GENETICS OF RESISTANCE TO ASCOCHYTA BLIGHT IN LENTIL

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctorate of Philosophy
In the Department of Plant Sciences
University of Saskatchewan
Saskatoon

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ABSTRACT

The aim of this study was to gain insight into the nature of resistance genes and mechanisms of resistance present in different ascochyta blight (AB) resistant genotypes of lentil to efficiently select non-allelic AB resistance genes mediating different mechanisms of resistance for gene pyramiding. Recombinant inbred lines (RILs) from all possible crosses among AB resistant *Lens culinaris* genotypes CDC Robin, 964a-46, ILL 7537 and ILL 1704 were subjected to allelism tests. Efforts were also made to understand the genetics of resistance in the *L. ervoides* accession L-01-827A. LR-18, a RIL population from the cross CDC Robin × 964a-46 was subjected to quantitative trait loci (QTL) mapping using a comprehensive genetic linkage map previously developed from polymorphic SNPs, SSRs and phenotypic markers. Results of allelism tests suggested that genes conditioning resistance to ascochyta blight in all lentil genotypes were non-allelic. Two complementary recessive resistance genes in L-01-827A were detected. QTL analysis indicated that CDC Robin and 964a-46 were different at two AB resistance QTLs. Histological tests suggested that cell death inhibition in CDC Robin, and reduced colonization of epidermal cells in 964a-46 might be the mechanisms of resistance in these genotypes. Comparing the expression of key genes in the salicylic acid (SA) and jasmonic acid (JA) signaling pathways of CDC Robin and 964a-46 suggested that the SA pathway was strongly triggered in 964a-46. However, the JA pathway was triggered in both, but at a lower expression level in 964a-46 than in CDC Robin. RNA-seq analysis revealed a number of candidate defense genes differentially expressed among genotypes with hypothetical actions in different layers of the plant defense machinery. The expression levels of the six candidate defense genes measured by quantitative real-time PCR analysis was correlated with those of RNA-seq. In conclusion, 964a-46 and CDC Robin mediated resistance to ascochyta blight through different resistant mechanisms, making them ideal candidates for resistance gene pyramiding. Gene pyramiding can be accelerated using closely linked markers to CDC Robin and 964a-46 resistance genes identified through QTL analysis.

Acknowledgements

I would like to express my heartfelt thanks to my supervisors, Drs. Albert Vandenberg and Sabine Banniza for their supports and encouragements. The successfulness of this thesis is also indebted to the restless efforts of my committee members, Drs. Yuguang Bai, Bruce Coulman, Aaron Beattie, Kirstin Bett, Jose Andres and Peta Bonham-Smith. I would like to specially thank Drs. Bett, Beattie and Andres for their helps with data analysis. I would also like to express my immense indebtedness to Drs. Hossein Borhan, Judith Lichtenzveig, Rebecca Ford, Vijai Bhadauria and Ravindra Chibbar for their assistance with qRT-PCR and RNA-seq experiments. I would also like to thank Dr. Paul Goodwin for agreeing to be my external examiner.

I would like to appreciate the technical assistance of Pulse Pathology Lab members Cheryl Armstrong-Cho, Candace Knihniski, Stephanie Boechler, Pulse Molecular Breeding Lab members Parvaneh Hashemi, Carmen Breitkreutz and Robert Stonehouse. The technical assistance of Eldon Simons and members of Crop Science Field Lab Brent Barlow, Thiago Do Prado and Stacey Wagenhoffer with the greenhouse experiments is greatly appreciated. I am also gratefull to graduate students Rajib Podder, Udhaya Kannan and post-doctoral fellow Dr. Rupam Sharma for their technical supports with my experments.

I would like to express my gratitude to the project sponsors, Natural Sciences and Engineering Research Council (NSERC) of Canada Industry Research Chair Program, Saskatchewan Pulse Growers (SPG) and University of Saskatchewan.

In the end, I would like to extend my heartfelt thanks to my wife Shirin for her patience, supports and sacrifices for completion of this degree. I also appreciate the patience and supports of my parents and wish health and wellness for them.

Dedication

To my wife who supported me with unconditioned love.

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LIST OF ABBREVIATIONS

% DS	Percentage of disease severity
AB	Ascochyta blight
ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
BHNs	Broad host-range necrotrophs
CDC	Crop Development Centre
CIM	Composite interval mapping
CLSM	Confocal laser scanning microscopy
cM	Centimorgan
CT	Threshold cycle
CV	Coefficient of variation
DAMPs	Damage-associated molecular patterns
DEGs	Differentially expressed genes
ESTs	Expressed sequence tags
ET	Ethylene
ETI	Effector-triggered immunity
FAO	Food and Agriculture Organization of United Nations
FPKM	Fragments per kb of exon per million mapped reads
GO	Gene ontology
GTL	Germ tube length
Hpi	Hours post inoculation
HR	Hypersensitive reaction
HSNs	Host specific necrotrophs
HST	Host specific toxin

JA	Jasmonic acid
LG	Linkage group
LOD	Logarithm of the odds
MAMPs	Microbe-associated molecular patterns
MAS	Marker-assisted selection
MMT	Million metric tones
NBS-LRR	Nucleotide binding site leucine rich repeat protein
NGS	Next generation sequencing
OGs	Oligogalacturonides
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCG	Percentage of conidia germination
Proc Mixed	Procedure Mixed
PTI	PAMP-triggered immunity
QRT-PCR	Quantitative reverse transcription real-time PCR
QTL	Quantitative trait loci
R-gene	Resistance gene
RAPD	Random Amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance gene analogue
RIL	Recombinant inbred lines
RLK	Receptor like kinase
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance

SD	Standard deviation
SE	Standard error
SIM	Simple interval mapping
SNP	Single Nucleotide Polymorphism
SSRs	Simple Sequence Repeats

CHAPTER 1

Introduction and research hypotheses

1.1. Introduction

Lentil is a self-pollinating annual cool-season legume crop that is produced throughout the world and is highly valued as a high protein food. It is the world's sixth largest pulse crop with annual production of about 4.5 MMT (FAOSTAT, 2012). Due to limited exploitation of available genetic resources, the biodiversity has dramatically declined (Erskine et al., 1998). The importance of preserving variability is accentuated by continuous demand for improving yield and quality. To keep pace, the introduction of new genetic resources to the breeding population and efficient application of available resources is required. To reach this goal, the lentil breeding program of the Crop Development Centre (CDC) at the University of Saskatchewan, Canada uses a system nicknamed SINGing or Systematic Introduction of New Germplasm. New traits of interest are introduced from non-adapted lentil germplasm and they are incorporated into high-yielding adapted lines using intra- and inter-species hybridization made possible by overcoming hybridization barriers present in the genus *Lens*. Exploitation of these valuable sources of genetic variability as well as integration of genomic and genetic resources, will lead to a sustainable and profitable lentil industry.

Lentil has been grown commercially in western Canada since 1970. Surveys of commercial lentil fields in the early to mid-1970s showed that the crop was relatively disease free, particularly with respect to foliar diseases (Morrall et al., 1972; McKenzie and Morrall, 1973; McKenzie and Morrall, 1975). In September 1978 a sample of severely discolored lentil seeds was received from Laird, Saskatchewan (Morrall and Sheppard, 1981). *Ascochyta lentis* was isolated from these lentil samples and was identified as the causal agent of ascochyta blight (AB), which is now established as one of the economically important diseases of lentil in Western Canada.

Keeping the lentil industry sustainable requires the application of methods of control with higher efficiency and durability. The environmentally most acceptable and economically most profitable method of control is developing cultivars with high levels of durable resistance. To reach this goal, the exploitation of resistance sources in different lentil gene pools as well as pyramiding available resistance genes in cultivated lentil has been the predominant strategy at the CDC.

The first identification of resistance to AB in lentil in Canada dates back to 1987 (Tay and Slinkard, 1989). Early research suggested that resistance to AB was conditioned by either a dominant or recessive gene (Tay and Slinkard, 1989; Andrahennadi, 1994; Ahmad et al., 1997; Andrahennadi, 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001). These findings led to the release in Canada of the first two AB-resistant lentil cultivars, CDC Matador and CDC Redwing, with recessive and dominant resistance genes, respectively. Andrahennadi (1994) reported that a recessive gene (resistance to *Ascochyta lentis* 2, *ral2*) conditioned resistance to *A. lentis* in the lentil cv. Indianhead. Tay and Slinkard (1989) and Ford et al. (1999) reported a major dominant gene (Ascochyta blight resistance gene1, *AbR1*) that controlled resistance to AB in ILL 5588. Recently, Tullu et al. (2006) inferred a quantitative trait locus (QTL) for resistance to AB from a study of recombinant inbred lines (RIL) derived from a cross between lentil cv. Eston and PI 320937.

Resistance genes for AB have been reported in wild lentil species, and *Lens ervoides* (Brign.) accessions have a relatively high frequency of resistance to AB compared to the other five wild lentil species (Bayaa et al., 1994; Tullu et al., 2010). Two complementary resistance genes were characterized for resistance to AB in both *L. ervoides* and *L. odemensis* (Ladiz.) (Ahmad et al., 1997). Limited genetic gain in yield of lentil caused by low genetic diversity can be compensated for by the introduction of new alleles from exotic germplasm (Tullu et al., 2010). As an example, there was no resistance to lentil anthracnose pathogen race 0 in the cultivated lentil before interspecific transfer of resistance from *L. ervoides* L-01-827A (Fiala et al., 2009).

To increase the efficiency of gene pyramiding, it is highly desirable to obtain insight into the quality and number of unique resistance genes available in resistant sources. In classical genetics, the allelism test is a means of identification of distinct resistance genes. By developing populations from resistant parent × resistant parent (R × R) crosses, the genetic resources were developed to test through allelism the hypothesis that AB resistance genes reported in lentil over the past 20 years are non-allelic. RIL populations of all possible combinations of crosses had been made among four AB resistant lentil genotypes: CDC Robin, 964a-46, ILL 1704 and ILL 7537. Segregation for disease severity was considered proof for the existence of different resistance genes in the parental lines. Microscopy of different genotypes of lentil infected with *A. lentis* also contributed to understanding differences between resistant genotypes in the cellular interaction with *A. lentis*. In addition, differences among resistant lentil genotypes in the phytohormone

signaling pathways was investigated by quantifying the expression of four genes that are markers for the salicylic acid (SA) and jasmonic acid (JA) signaling pathways. The differences among resistance mechanisms of resistant genotypes were also investigated using global transcriptome by RNA-seq analysis.

1.2. Research Hypotheses:

1- Known sources of resistance to AB in lentil carry non-allelic resistance genes.

2- Resistant genotypes carrying non-allelic AB resistance genes are different in cellular defense responses and activation of the SA and JA signaling pathways

3- Non-allelic AB resistance genes mediate resistance through divergent mechanisms.

CHAPTER 2

Literature Review

2.1. The genus *Lens*

Three different gene pools have been characterized according to hybridization barriers present in the genus *Lens* (Cubero et al., 2009). The primary gene pool includes the cultivated lentil *L. culinaris* (Medik.) subsp. *culinaris* and *L.* c. subsp. *orientalis* (Boiss.) Ponert, and the secondary gene pool includes *L. odemensis* (Ladiz.) *L. tomentosus* (Ladiz.). The tertiary gene pool includes *L. nigricans* (M. Bieb.) Godr. and *L. ervoides* (Brign.) Grande and *L. lamottei* Czefr. Hybrid lines can be developed by overcoming hybridization barriers using embryo rescue techniques which enable transfer of genes across the *Lens* gene pools.

2.1.1. The cultivated lentil

Lentil is a self-pollinating diploid plant which is grown in many temperate areas such as the entire Mediterranean basin, Central Asia, Western Asia, South Asia, Ethiopia, temperate regions of North and South America and regions with Mediterranean climate in Australia (Durán et al., 2004). Lentil has a thin taproot system with many lateral roots. *Lens* species vary remarkably in the taproot length and number of lateral roots (Sarker et al., 2005). The lentil stems are thin, with a square to angular shape with defined ribs. Leaves are alternate, compound and pinnate with elliptical and lanceolate leaflets. Small pairs of stipules are formed at the base of each leaf. The rachis may terminate in a bristle or simple tendril that forms on leaves unfurled at the onset of flowering, and may have the same length as the rachis. Lentil comes to flower after a juvenile period of vegetative growth which varies among genotypes. Lentil has an indeterminate growth which means flowering can continue until the end of the season. Maturity also varies among genotypes and environments, and usually ranges between 90-100 days.

2.2. Lentil production in Canada

The area planted to lentil in Canada in 2012 was recorded to be about 1.018 million ha, about 95 % of which was in Saskatchewan (Saskatchewan Agriculture Trade Update, 2012). Canada is the world's leading lentil producer and exporter (FAOSTAT, 2012). Besides direct economic benefits, the inclusion of lentil in cropping systems benefits the succeeding crop by improving the physical, chemical and biological properties of the soil as a result of biological nitrogen fixation and other rotational effects (Wisal, 2003). Internationally, the most common crop rotations are

wheat-lentil, rice-lentil, maize-lentil, cotton-lentil, pearl millet-lentil, sorghum-lentil, and groundnut-lentil. Previous studies proved rotation with lentil and some other legumes improved yield and protein levels of succeeding cereal crops (Miller et al., 2003).

Large green lentil (yellow cotyledons with green seed coats) and small red lentil (red cotyledons typically with gray seed coat) represent about 90 % of Canadian lentil production. Additional market classes include medium green, small green, French green, Spanish brown and various sizes of red cotyledon lentils. Main importers of lentil from Canada are India, Turkey, Bangladesh, UAE, Algeria and Sri Lanka (Agriculture and Agri-Food Canada, 2010). Canada's lentil exports had a value of $CDN 673 million in 2012 (Saskatchewan Agriculture Trade Update, 2012).

2.3. The lentil genome and genetic mapping

Lentil is a diploid crop with a haploid genome size of 4,063 Mbp (Arumuganathan and Earle, 1991). It has a symmetrical karyotype with 2n=14 chromosomes, with both metacentric and sub-metacentric chromosomes (Sharma, 2009). The average chromosome and chromatin lengths, chromatin volume, arm ratio and position of centromeres vary across varieties (Sharma, 2009). For example, the average chromosome length is 39.31 and 31.77 µm in varieties Pant L639 and Type 36, respectively (Gupta and Singh, 1981; Dixit and Dubey, 1986).

Isozymes, restriction fragment length polymorphisms (RFLPs) and some morphological markers were initially used to develop the first genetic maps of lentil (Havey and Muehlbauer, 1989; Weeden et al., 1992; Tahir et al., 1993). Subsequent efforts to map the lentil genome were made by Eujayl et al. (1997) based on 89 random amplified polymorphic DNA (RAPD) markers, 79 amplified fragment length polymorphism (AFLP) markers and six co-dominant, mostly restriction fragment length polymorphism (RFLP) markers. In another effort, a linkage map was developed using a F_2 population and 100 RAPDs, 11 inter-simple sequence repeats (ISSRs) and three resistance gene analog (RGA) markers (Rubeena et al., 2003). Duran et al. (2004) applied 62 RAPDs, 29 ISSRs, 65 AFLPs, four morphological and one microsatellite markers to construct a genetic linkage map of lentil. Hamwieh et al. (2005) focused on microsatellite markers and reported a map which consisted of 41 microsatellite and 45 AFLPs. Tullu et al. (2008) used 30 microsatellite markers to construct an intraspecific linkage map for QTL analysis of earliness and plant height in lentil. The number of linkage groups detected in these studies varied between 9 and 14 (Rubeena et al., 2003; Duran et al., 2004; Tullu et al., 2008; Hamwieh et al., 2005). Recently, Gupta et al. (2012) used 196 EST-SSR sequences from the model legume species *Medicago*

truncatula to enrich an existing intraspecific lentil linkage map. The most recent genetic map of lentil was developed from a set of Single Nucleotide Polymorphism (SNP) markers developed from a pool of transcriptome sequences obtained from multiple wild and cultivated lentil genotypes (Sharpe et al., 2013).

2.4. Ascochyta blight of lentil

AB of lentil caused by *A. lentis* Vassilievsky (teleomorph: *Didymella lentis* W.J. Kaiser, B.C. Wang, and J.D. Rogers) is prevalent throughout many lentil production regions of the world and has been reported to cause yield losses of up to 70 %, 30-50 % and 50 % in Canada, USA and Australia, respectively (Gossen and Morrall, 1983; Kaiser, 1992; Brouwer et al., 1995). Morrall and Sheppard (1981) described the Saskatchewan isolates of *A. lentis* as generally producing 2-celled conidia of 10-20 × 4-8 µm (Mean: 15.8 × 5.7 µm) with a few multi-septate conidia among a majority of 2-celled forms. The taxonomy of *A. lentis* was a topic of discussion in the 1980s when it was initially considered to be a *forma specialis* of *A. fabae* because of high levels of microscopic and cultural resemblance (Gossen and Morrall, 1984). In 1993, *Didymella lentis*, the teleomorph of *A. lentis*, was found on overwintered lentil debris in the Palouse region of eastern Washington and northern Idaho (Kaiser and Hellier, 1993), and Kaiser et al. (1997) differentiated the teleomorphs of *A. fabae* and *A. lentis* on the basis of pathogenicity tests, morphology and molecular markers. Recent phylogenetic studies suggest that pathogenic isolates of *Ascochyta* spp. on each host species form a cluster, in line with the morphological and biological species definition (Peever, 2007).

The life-cycle of *A. lentis* has two stages. The asexual stage is characterized by the production of pycnidia in lesions that have minute round ostioles (reviewed by Morrall, 1997). These pycnidia release conidia which move short distances via rain splash and wind. Conidia infect lentil plants following germination at the optimum temperature of 20-25 °C and in the presence of free moisture (Bondartzeva-Monteverde and Vassilievsky, 1940). The pathogen survives between lentil crops on or inside the seeds, on crop residue and volunteer lentil plants. Taxonomically, the teleomorph belongs to *Ascomycota, Pleosporales*, *Didymellaceae* and was observed for the first time in the USA (Kaiser and Hellier, 1993). Recently, Galloway et al. (2004) reported the teleomorph on lentil stubble in Victoria and Western Australia. *A. lentis* is heterothallic with two typical mating types (MAT1-1 and MAT1-2) (Kaiser et al., 1997).

2.5. Infection process of *Ascochyta lentis*

The initial infection process of *A. lentis* was studied by Roundhill et al. (1995). Conidia of *A. lentis* inoculated onto detached leaflets germinated within six h of inoculation, and germ tubes and appressoria developed after 10 h. A penetration peg then pierced the cuticle, often near the junction of two epidermal cells and cytoplasm aggregated adjacent to the infection hyphae. Within 40 h, the plasmalemma was disrupted and by 52 h, the cytoplasm and nucleus broke down and the entire cell became largely occupied by the fungus with only remnants of the protoplast present. Once the epidermis was fully colonized, the pathogen invaded the mesophyll with microscopic symptoms being evident by nine days after inoculation.

2.6. Population structure of *Ascochyta lentis* in Canada

No definitive structure of pathogenic races for *A. lentis* has been established. Population structure of *A. lentis* was first investigated using 39 isolates of *A. lentis* and 22 lentil lines, suggesting the presence of 6 pathovars (Nasir and Bretag, 1997). By contrast, Ahmed et al. (1996) suggested a lack of pathovar differentiation using 100 isolates of *A. lentis* collected from Canada and 13 other countries. They also suggested that the aggressiveness of *A. lentis* isolates had increased over time. A typical example is the Canadian cultivar 'Laird' which was moderately resistant at the time of release in the 1980s, and was reported to be susceptible by Pedersen and Morrall (1994). Banniza and Vandenberg (2006) reported that the host reaction of 16 lentil genotypes to 65 isolates of *A. lentis* collected in Canada also resulted in a continuum of severity of infection, without any distinct pathotypes.

2.7. Prevalence and management of ascochyta blight

AB of lentil is prevalent in regions that produce lentil in temperate summers or Mediterranean winters, causing yield and quality loss as reported in Canada, USA and Australia (Gossen and Morrall, 1983; Kaiser, 1992; Brouwer et al., 1995). Recommendations for disease control for western Canadian lentil growers are at present based only on integrated disease management (Tivoli et al., 2006), and includes crop rotation, use of disease-free seed, and use of resistant varieties. Growers may include other grain legumes in rotation as *A. lentis* appears to be host-specific and will not be transmitted by native legumes, weeds, forage crops or other pulses. Since AB is seed borne, seed treatments may help to control the disease.

Gossen and Morrall (1983) used frequent applications of a foliar-applied fungicide to achieve control and demonstrated yield increase of 30-40 % compared to unsprayed controls in susceptible

common Chilean and 'Eston' (Slinkard, 1981) and 10-15 % in 'Laird' cultivars (Slinkard and Bhatty, 1979).

2.8. Genetic control of resistance to *Ascochyta lentis*

Resistance breeding is important for developing a durable and sustainable strategy for managing AB. Several germplasm sources with AB resistance genes have been identified (Tay and Slinkard, 1989; Andrahennadi, 1994; Ahmad et al., 1997; Andrahennadi, 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001). Cultivars with improved levels of resistance to AB have also been developed (Vandenberg et al., 2001; Vandenberg et al., 2002).

Previous research suggested that resistance to AB was conditioned by either a dominant or recessive gene (Tay and Slinkard, 1989; Ahmad et al., 1997; Andrahennadi, 1994; 1997; Ford et al., 1999; Nguyen et al., 2001; Ye et al., 2000). A recessive resistance gene (*ral2*) reported by Andrahennadi (1994) in the lentil cultivar 'Indianhead' was later confirmed by Chowdhury et al. (2001). A major dominant gene controlling resistance to AB (*AbR1*) was also identified in ILL 5588 (Tay and Slinkard, 1989; Ford et al., 1999).

Ye et al. (2001) suggested that an additive effect of minor genes also contributed to resistance of lentil to AB, although to a much lower extent than the major genes. Quantitative trait loci (QTL) analysis conducted by Tullu et al. (2006) using a RIL population developed from cv. Eston (susceptible) × PI 320937 (resistant) resulted in the identification of a QTL for resistance to AB in PI 320937. The existence of a continuum in the reaction to AB in the RIL population led the authors to conclude a polygenic inheritance of resistance to AB in PI 320937. The major QTL explained 41 % of the phenotypic variation in the reaction to AB. Capturing resistance from those minor genes within populations with the same major resistance gene requires a novel phenotyping system that distinguishes intermediate phenotypes. Evidence for quantitative inheritance of resistance to AB of lentil is increasing. Recently, Gupta et al. (2012) developed a map including 196 EST-SSR markers from *M. truncatula* and a RIL mapping population from a cross between ILL 5588 and ILL 5722. Three QTLs for AB resistance were detected in lentil seedlings and three additional QTLs for resistance to pod infection (Gupta et al., 2012).

2.9. Developing durable resistance by gene pyramiding

The history of gene pyramiding dates back to 1978 when Nelson advised " Go young man and gather up your weary and defeated genes of the past, take your currently successful genes, find some new ones if you can and build yourself a pyramid" (Pedersen and Leath, 1988). The theory

of gene pyramiding arose from the development of pathogen resistance to fungicide after applications of single-mode of action fungicides. There is evidence that single resistance genes are also ephemeral and will be broken down soon after release, nevertheless, it has not yet been proven that pyramiding will increase the durability of resistance. There is little knowledge available on the performance of lines carrying multiple resistance genes under field conditions. It is not yet clearly known whether the co-occurrence of resistance genes causes a fitness penalty for plants. It is still a question whether genes accumulated in a genetic background act in the same way as when employed on their own in a different background. A very important fact in this context is that the construction of a pyramid of genes is costly, labour-intensive and time-consuming. This emphasizes even more the need for the ability to predict the durability of resistance genes prior to being subjected to gene pyramiding. In theory, durability of resistance is a function of stabilizing selection (Van der Plank, 1963). This suggests that the selection pressure is much lower when compatible reactions exist for all the corresponding host-pathogen loci (Pedersen and Leath, 1988). Therefore, if the resistance in a line is achieved through the accumulation of partial effects of many resistance genes, it should exert less selection pressure on the pathogen. This is what Nelson believed was the simulation of a natural ecosystem (Nelson, 1972). Kolmer (1995) studied the diversity in virulence of a heterogeneous population of *Puccinia recondita* f.sp. *tritici* after 12 generations of selections on three different multi-lines of dusty meadow rue (*Thalictrum speciosissimum)* carrying different proportions of susceptible and isogenic resistant lines. He suggested that application of multi-lines increased the ratio of pathogen genotypes with an intermediate amount of virulence. In this way, the release of multi-lines, and lines with multiple resistance genes, may decrease the fitness of highly virulent pathogen genotypes.

Whereas limited experimental evidence exists on the effectiveness of pyramiding resistance genes for increasing the durability of resistance, several studies have been conducted on the application of multi-lines and cultivar mixtures for managing resistance break-down occurring in mono-cultures (Mundt, 2002). For example, Cox et al. (2004) used a cultivar mixture of wheat for management of leaf rust and tan spot diseases in a multi-year experiment. The results showed that disease severity decreased on the susceptible high-yielding cultivar, although yield was equal or higher than in monoculture fields, suggesting the effectiveness of cultivar mixture for management of these diseases (Cox et al., 2004).

Pyramiding resistance genes could be an approach to achieving durable resistance; however it is difficult to separate progeny carrying multiple resistance genes; since the effect of one major resistance gene can mask the effects of other major or minor genes, resulting in progeny with similar phenotypes but different genotypes. With an increase in the availability of large numbers of dominant and co-dominant molecular markers closely linked to resistance genes or QTLs, it may be easier to establish pyramids of resistance genes. The only remaining issue is what type of genes should be selected for pyramiding. Few studies have been published on the development of gene pyramiding, let alone on the evaluation of efficiency of this method under field conditions. Selection of specific genes for pyramids requires availability of methods that will assist in the prediction of the durability of resistance genes. Biological studies on the nature of resistance mechanisms in host plant and virulence genes in pathogens can help to predict the durability of resistance genes. This can be achieved by molecular studies of plant resistance mechanisms that identify key genes with a role in resistance. It is critical to increase the efficiency of resistance gene pyramiding by investigating the distinctiveness of resistance genes targeted for gene pyramiding in a breeding program. To infer this, classical genetics offer the allelism test, a method for identifying the allelic relationship of resistance genes by crossing the resistance sources and examining the segregation patterns in the progeny.

2.10. Allelism tests

The allelism test has been applied in classical genetics to understand the allelic relationship among resistance genes (Hibberd et al., 1987; Potts, 1990; Ye et al., 2001). The test of allelism is a genetic test that predicts the allelic relationship of genes through phenotyping of the progeny. In such a test, segregation of progeny is an indicative of the presence of non-allelic genes in the parents (Griffiths et al., 2000). This test was initially used for determining the allelic relationship among mutants lacking a trait of interest through a complementation test (Griffiths et al., 2000). In this type of allelism test, the recessive mutants are crossed and progeny are examined for the expression of the wild type phenotype. If none of the progeny show the wild type phenotype, it is suggested that mutation occurred at different genes; therefore non-allelic genes condition the trait. Ye et al. (2001) studied the allelic relationship of AB resistance genes in lentil genotypes ILL 5588, ILL 5684, Indianhead and Laird for the first time. For ILL 5588 and ILL 5684, the resistance genes proved to be allelic whereas non-allelic resistance genes were identified in Indianhead and Laird.

2.11. Marker-assisted selection (MAS) for ascochyta blight resistance in lentil

Marker-assisted selection (MAS) improves the resolution of selection for progeny carrying different resistance genes. The availability of molecular markers for screening for AB resistance would allow selection in early generations in the absence of the pathogen, decreasing the cost and time required for establishing disease nurseries. Little published research exists on the application of molecular markers in resistance gene pyramiding. Previous efforts are published on the discovery of tightly linked marker for genes conferring AB resistance in lentil. Ford et al. (1999) identified RAPD markers RV01 and RB18, approximately 6 and 14 cM, respectively, away from and flanking the foliar AB resistance locus Ral1 (*AbR1*) in ILL 5588. Molecular markers were developed that were linked to the complementary dominant resistance genes in ILL 7537 (Muehlbauer et al., 2006). QTL analysis of AB resistance in ILL 7537 was also conducted using a population comprising 153 F_2 individuals (ILL 7537×ILL 6002) and a linkage map comprising 72 markers spanning 412.5 cM (Rubeena et al., 2003). Three QTL were observed. QTL1 and 2 were observed on Linkage Group (LG) 1 in close proximity and accounted for approximately 47 % of the variance, whereas QTL3 on LG2 accounted for approximately 10 % of the variance of the trait (Rubeena et al., 2003). The AFLP marker C-Ttalm-AC_{285} was found to be 3.4 cM away from QTL2 (Rubeena et al., 2003). The RAPD marker $M20_{700}$ was located at the same position as QTL3. Tar'an et al. (2003) pyramided AB resistance genes in lentil using RAPD markers tightly linked to *ral1* and *AbR1* genes including $UBC227_{1290}$ and $RB18_{680,}$ respectively. According to Tara'an et al. (2003), 82 % of the lines which had either or both of the markers were resistant and 80 % of the lines that had none of the RAPD markers were susceptible.

Until recently, a major limitation to lentil mapping was the unavailability of locus-specific and co-dominant markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). Highly informative SSR markers are preferred over dominant markers, however, size homoplasty impedes the application of SSRs for association mapping. Size homoplasty is defined as the occurrence of SSR alleles of identical size but different evolutionary origin (Estoup et al., 1995; Viard et al., 1998). Size homoplasty can be caused by insertion/deletion in SSR flanking regions (Curtu and Finkeldey, 2004). SNP assays avoid problems with size homoplasty and can be automated in an assay-plate format or on microchips, making SNPs easier to locate in most single copy regions of the genome compared to SSRs. SNPs are biallelic and their expected heterozygosity is low, e.g. 0.263 in maize germplasm compared to 0.77 for SSRs

(Taramino and Tingey, 1996). SNPs are essentially an inexhaustible source of polymorphic markers for use in the high-resolution genetic mapping of traits, and for association studies that are based on candidate genes or possibly the whole genome.

As indicated in 2.3., the first comprehensive genetic map of lentil was developed by deep transcriptome sequencing of multiple cultivated and wild lentil genotypes followed by the discovery of gene-based SNPs (Sharpe et al., 2013). Among these, 536 SNP markers were polymorphic between the parents of a RIL population (LR-18). This genetic map was successfully used in lentil for QTL mapping of seed size shape and colour (Fedoruk et al., 2013). Kaur et al. (2011) used lentil transcriptome sequences for the discovery of EST-SSR markers which yielded 166 applicable markers. These new generation lentil genetic maps have not yet been used for QTL analysis of AB resistance.

2.12. Increasing the efficiency of gene pyramiding by molecular studies of resistance mechanisms

To increase efficiency of breeding programs, especially for gene pyramiding, it is desirable to have access to resistance genes with different modes of action. Durable resistance can be governed by single or multiple genes, however most types of durable resistance developed to this day have been of polygenic nature. Understanding mechanisms of resistance can help increase the efficiency of selecting resistance genes with different modes of action and higher durability for a gene pyramiding program. Different types of plant resistance and components of plant defense are explained below.

2.13. Types of plant resistance

2.13.1. Non-host resistance

Pathogens differ in their ability to infect plant species, hence have different host ranges. Whereas some pathogens have narrow host ranges, others are able to infect more than one plant species and are called broad host range pathogens. Plant species are immune to the majority of microbes with pathogenic potential, and this immunity is called “non-host resistance” (Schulze-Lefert and Panstruga, 2011). This type of resistance is of great interest to plant breeders as it is thought to be durable and effective against all races/strains of a pathogen species (Schulze-Lefert and Panstruga, 2011).

2.13.2. Host resistance

2.13.2.1. Preformed defense

Irrespective of specific defense mechanisms activated by and / or effective against particular groups of plant pathogens, plant defense mechanisms are categorized into two main groups: passive and active defenses (Guest and Brown, 1997). Plant cells, as a source of food for plant pathogens, are protected by natural barriers. Parasites need to breach these barriers prior to establishing the parasitic relationship. Barriers can be physical, like cuticles, cell walls, stomatal apertures or lenticels, or can be chemical inhibitors, or the absence of chemical components required for pathogenesis.

2.13.2.2. Pattern triggered immunity (PTI)

A large number of gene expression modifications induced by resistance genes (R-genes) in plants occur during compatible interactions (absence of R-gene), although at a lower speed and reduced magnitude (Lamb et al., 1992; Tao et al., 2003). Resistance mechanisms that counteract pathogen growth during compatible interactions and do not require specific interaction are known as “PAMP triggered immunity (PTI)”. PTI is induced upon recognition of structural components of the pathogen, pathogen associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs). Examples of PAMPs are the bacterial flagellins and chitin fragments from fungal cell walls (Boller and Felix, 2009). The typical examples of PRRs perceiving PAMP are receptor like kinases (RLKs) such as the putative chitin receptor LysM/CERK1 (Wan et al., 2008), peptide receptors (Yamaguchi et al., 2010). As PAMPs usually have vital roles in survival of pathogens, evolution of pathogen mutants that escape PTI resistance is not frequent. PTI can also be triggered by components of host cells released after degradation by pathogen effectors called damage-associated molecular patterns (DAMPs). A typical example of a DAMP is a plant cell wall fragment released by the activity of cell wall degrading enzymes (CWDEs

Multiple genes condition PTI resistance, which is effective against all types of pathogens including biotrophs, hemibiotrophs and necrotrophs (Mengiste, 2012). Plant mutations in PTI components have been shown to reduce resistance, which contradicts the idea of resistance as binary alternatives alternating from complete resistance to complete susceptible postulated in the gene-for-gene model. In fact, a continuum of reactions to a pathogen exists, ranging from complete resistance to extreme susceptibility.

2.13.2.3. Effector triggered immunity (ETI)

Plants are armed with biological systems that recognize and respond to pathogen infection in a timely manner. Before the advent of molecular genetics, this recognition was thought to be based on the interaction of the protein product (effector) of a single dominant gene in a pathogen (avirulence gene) with a matching product of a dominant R-gene in a host plant. This model is recognized as "gene-for-gene" resistance (Flor, 1955). When the matching products interact, resistance mechanisms are activated, the pathogen is not able to induce disease (avirulent pathogen) and the host will be resistant. Susceptibility in this interaction model occurs when gene-for-gene recognition is absent due to the absence of either the R-gene or the avirulence gene, a situation where the pathogen is virulent and the host is susceptible (Glazebrook, 2005). This type of defense is important in the context of breeding for resistance to pathogens. With the recent advances in the molecular biology of plant-pathogen interactions, many cases have been identified where an indirect interaction of R-gene and avirulence gene products has been postulated, leading to a modified gene-for-gene model called the guard hypothesis. In this model, the product of the R-gene monitors the alteration of a pathogen effector target in the host cell (van der Biezen and Jones, 1998). The defense response induced upon specific recognition of avirulence gene products by R-gene products is called effector-triggered immunity (ETI).

Many R-genes and avirulence genes have been identified and classified. The most common R-gene class is the nucleotide binding site-leucine rich repeat (NBS-LRR) (Belkhadir et al., 2004). For bacterial plant pathogens, virulence genes encode type III effectors which are also avirulence factors in the presence of the matching R-gene products (Abramovitch and Martin, 2004).

2.14. Signal transduction pathways in plant defense

Both ETI and PTI use overlapping signaling networks which transfer the resistance signal initiated at the signal receptor to the cell nuclei to express defense related genes. This process is known as signal transduction and is mediated mainly by the plant hormones SA, JA, ethylene (ET) and abscisic acid (ABA) (Glazebrook, 2005).

The main difference between the ETI and PTI is the nature of pathogen recognition and the strength and intensity of defense responses. Otherwise, PTI and ETI share many components of the downstream pathways. Both ETI and PTI induce systemic resistances in tissues distant from the infection site (Mishina and Zeier, 2007). The high level of resemblance between defense responses triggered by PTI and ETI suggests that a continuum of defense responses triggered by

PTI and ETI conditions plant resistance to pathogens (Thomma et al., 2011). It is challenging to theoretically distinguish between R-genes and PRRs, as well as PAMPs and avirulence gene products (Tiedemann, 1997). The resemblance is more obvious in the downstream pathways when the signaling cascades trigger almost a similar set of defense genes (De Lorenzo et al., 2011). For example flagellin, a PAMP from bacteria also triggers defense against *B. cinerea* (Laluk et al., 2011).

2.14.1 Salicylic acid-dependent signaling

SA signaling pathway and its roles in plant resistance to biotrophs and hemibiotrophs and in systemic acquired resistance (SAR - discussed below) have been fully described (reviewed by Glazebrook, 2005). A rise in SA induces various defense components mainly pathogenesis related protein-1 (PR-1) (Figure 2.1). Mutation screening in *Arabidopsis thaliana* suggested that *PAD4* and *EDS1* are required for SA signaling activated by some, but not all, SA stimuli (Zhou et al., 1998; Falk et al., 1999). *PAD4* has an additional role, as in the infection of *A. thaliana* by *Pseudomonas syringae*; SA-dependent defense gene activation requires *PAD4* but not *SID2* and *EDS5* which are SA-biosynthesis genes (Glazebrook et al., 2003).

The product of *SID2* is an isochorismate synthase. A mutation in this gene (*sid2*) caused a significant decrease in SA levels, refuting the hypothesis that SA is only produced from the phenylalanine pathway (Dewdney et al., 2000; Wildermuth et al., 2001). The low concentration of SA in *sid2* mutants suggested the existence of parallel pathways of SA synthesis in *A. thaliana.* *EDS5* is also required for SA synthesis in SA signaling (Nawrath and Métraux, 1999). It synthesizes a multidrug and toxin extrusion protein (MATE) family transporter which seems to have a role in the transportation of SA biosynthesis components (Nawrath et al., 2002). *Eds5* mutants showed impairment of SA-related genes as observed for *sid2*, supporting the idea that *EDS5* is required for SA biosynthesis (Glazebrook et al., 2003). *EDS1* (enhanced disease susceptibility 1) is another important component of SA signaling involved both in basal defense and ETI mediated by TIR-NBS-LRR (toll-interleukin receptor domain–nucleotide binding domain–leucine rich repeat) resistance genes (Falk et al., 1999). There appears to be a requirement of an EDS1-PAD interaction for SA-dependent activation of defense genes (Rietz et al., 2011). An *A. thaliana* plant harboring SA-degrading bacterial gene *NahG* has been used to demonstrate the role of SA in defense signaling and induction of SAR (Gaffney et al., 1993; Delaney et al., 1994). NPR1 is the central component of the SA signaling pathway. At low SA concentrations, NPR1 is

present in an oligomeric form in the cytoplasm. Increases in SA levels cause the disassociation of NPR1 monomers by reduction of disulfide bonds between the monomers. Monomers are able to enter the nucleus and interact with TAG transcription factors which activates the expression of response genes such as PR-1 (Zhang et al., 1999; Després et al., 2003; Johnson et al., 2003). NPR3 and NPR4, the other members of the NPR protein family have also been shown to directly interact with SA (Fu et al., 2012). These proteins are involved in the ubiquitination of NPR1 through using CUL3 E3 ligase, and are key regulatory components of the SA signaling pathway (Dharmasiri et al., 2013). The activities of NPR3 and NPR4 in regulating NPR1 degradation is very dependent on the cytosolic concentration of SA and are associated with different functions of the SA pathway in PTI and ETI (Moreau et al., 2012). It has been proven that TAGs 2, 5 and 6 transcription factors then activate *PR-1* expression (Zhang et al., 2003). The transcription factor WRKY70 is also required for *PR-1* expression and is activated by NPR1, however no direct interaction between NPR1 and WRKY70 has been reported (Li et al., 2004).

Induction of some SA-induced genes is independent of NPR1, suggesting existence of some other parallel mechanisms of SA perception in plants. For example, *AtWhy1* is induced by *Peronospora parasitica* and an exogenous treatment with SA in *A. thaliana*, and is independent of NPR1 (Desveaux et al., 2014). According to Desveaux et al. (2014), there appeared to be two branches of the SA signaling pathway, both contributing to induction of *PR-1* and defense activation.

The order of events in the SA pathway is complex. For example, the hypersensitive reaction (HR) HR activates the SA pathway while SA accumulation induces the HR. Research has also shown that *PAD4* and *EDS1* are not required for SA production in all plant species, and in some plants their expression is enhanced by SA accumulation (Falk et al., 1999; Jirage et al., 1999). On the other hand, *A. thaliana npr1* mutants had higher SA levels than wild type plants suggesting a role for *NPR1* in controlling SA levels (Shao et al., 2003).

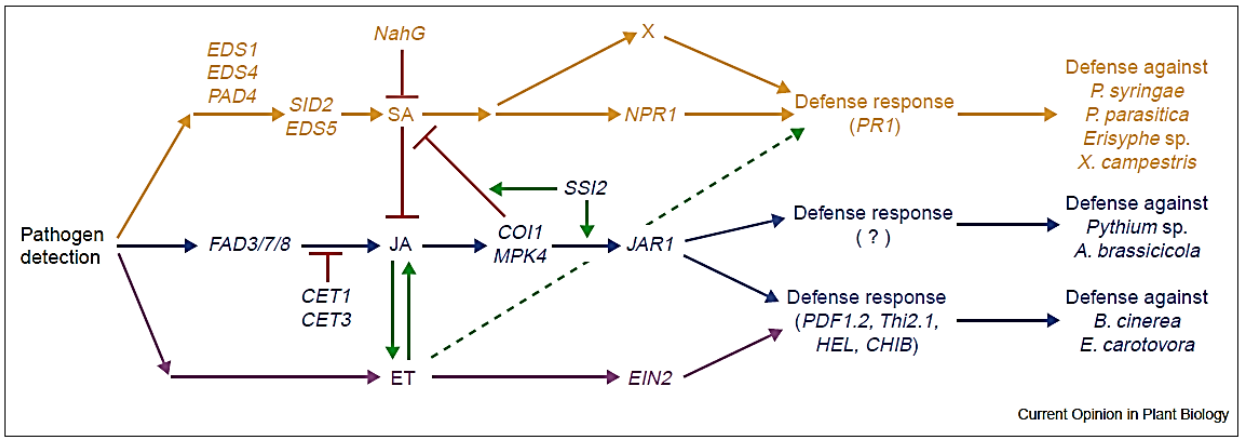


Figure 2.1. Predicted model for the role of SA and JA-ET pathways in activation of defense genes in *Arabidopsis thaliana* (Kunkel and Brooks, 2002; reproduced with permission). Green and red arrows indicate positive and negative interactions between signaling pathways. Dashed line represents infrequent observations. *P. syringae*= *Pseudomonas syringae*; *P. parasitica*= *Pronospora parasitica*; *X. campestris*= *Xanthomonas campestris*; *A. brassicicola*= *Alternaria brassicicola*; *B. cinerea*= *Botrytis cinerea*; *E. caratovera*= *Erwinia caratovera*.

When the HR occurs, the SA signaling is activated throughout the plant. Therefore, a plant challenged with an avirulent pathogen will trigger the SA pathway and induce resistance against all pathogens sensitive to SA-induced defense responses. This phenomenon is called systemic acquire resistance (SAR) (Durrant and Dong, 2004).

PR-1 is widely accepted as a hallmark of the SA signaling pathway in *A. thaliana* (Rogers and Ausubel, 1997) and some other crop plants (Niderman et al., 1995; Tornero et al., 1997). The involvement of SA signaling in a defense reaction against biotrophs has been confirmed by using gain- and loss-of-function mutants and exogenous applications of SA analogues. For instance, two SA-deficient mutants *sid*2 and *eds*5 of *A. thaliana* had increased susceptibility to many virulent pathogens (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999; Dewdney et al., 2000). By contrast, the SA signaling pathway is ineffective against pathogens with a necrotrophic life-style and JA plays a crucial role in plant defense instead.

2.14.2. Jasmonic acid- and ethylene-dependent signaling

JA signaling pathway is involved in the induction of plants resistance to necrotrophic plant pathogens and herbivores (Figure 2.1). JA levels are heightened in response to the attack of some necrotrophic pathogens and result in an increase in the expression of defense genes like *PDF1.2*.

Some cyclopentenone compounds related to JA show signaling activities and induce the expression of defense genes that are sometimes different from conventional JA-induced genes (Glazebrook et al., 2005).

JA is synthesized through a release of α-linoleic acid (α-LeA) from galactolipids by the activity of a phospholipase1 enzyme (Wasternack and Hause, 2013). Lipoxygenase (*LOX*) genes are also important components of the JA pathway through oxygenation of α-LeA. *A. thaliana* has six *LOX* genes, among which *LOX2* has been extensively studied. *LOX2* is induced upon wounding stress and was involved in the biosynthesis of oxylipins during senescence (Seltmann et al., 2010). The activity of *LOX2* was shown to be regulated both by coronatin-insensitive 1 (COI1) and a Ca^{2+}-dependent pathway (Bonaventure et al., 2007). A role for allene oxide cyclases (AOCs) in JA biosynthesis has also been suggested (Stenzel et al., 2012). AOCs were shown to be involved in the biosynthesis of JA in chloroplasts (Farmaki et al., 2007). The role of AOCs in tolerance to drought and oxidative stress was shown using constitutive expression of soybean AOCs in tobacco (Wu et al., 2011).

Some defense response genes are regulated by both the JA and ET signaling pathways, which is why they are considered to be integrated. For example, *PDF1.2* is induced by both the JA and ET (Thomma et al., 1998). By contrast, JA-inducible *VSP1* was not expressed upon ET signaling (Norman-Setterblad et al., 2000). The involvement of cell-wall cellulose synthases in the regulation of JA levels is documented. The cellulose synthase mutant *cev1* displayed high levels of JA and high expression of the JA-dependent genes (Ellis et al., 2002). Another important component in this pathway is *jasmonic acid responsive 1* (*JAR1*) gene which encodes a JA-amino synthase which has a role in the conjugation of JA and isoleucine (Ile, Staswick et al., 2002; Staswick and Tiryaki, 2004). It has been shown that JA-Ile is the active form of JA (Staswick and Tiryaki, 2004).

COI1 is also an important component of the JA signaling pathway. Mutations in *COI1* in *A. thaliana* cancelled all JA activity (Xie et al., 1998). This gene encodes an F-box protein which has a role in proteolysis (Xie et al., 1998). The *COI1* product interacts with cullin, Skp1-like proteins and AtRbx1 to produce SCF_{COI1} complexes that have a predicted function in E3 ubiquitin ligase (Xu et al., 2002). Recent studies suggested that regulation of the JA signaling pathway through a positive feedback loop associated with SCF_{COI1}-jasmonate zim domain (JAZ) protein-protein interaction (Wasternack and Hause, 2013). The accumulation of JA and its active form JA-Ile

causes targeting of JAZ, a negative regulator of JA to the proteasome through ubiquitination. Degradation of JAZ results in activation of MYC2 transcription factor and induction of the JA responsive defense genes (Mosquera et al., 2009). Response to JA is also related to mitogen-activated protein (MAP) kinase activity encoded by *MPK4*, as a *mpk4* mutant fails to express *PDF1.2* and *THI2.1,* the key JA-responsive genes (Petersen et al., 2000).

Three types of transcription factor, ethylene-responsive 1 (ERF1), ethylene-resposive RAP2.6 (AP2 family), and jasmonate-insesitive 1 (JIN1, AtMYC2) are induced by JA (Lorenzo et al., 2003; He et al., 2004; Lorenzo et al., 2004). ERF1 is at the convergent point between the JA and ET pathways and it needs expression of both *COI1* and ethylene-insesitive 2 (*EIN2*) for its complete function (Lorenzo et al., 2003). Overexpression of *ERF1* is accompanied by the expression of many defense genes as well as compensating for the mutants *ein2* and *coi1* that are normally required for expression of *PDF1.2* (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). This suggests the involvement of *ERF1* downstream of *COI1*. *JIN1* and *ERF1* each regulate sets of genes in opposite ways. Genes induced by ERF1 are repressed by JIN1 and *vice versa* (Glazebrook et al., 2005). The genes induced by ERF1 seem to be only important for resistance development as *ERF1* overexpression in *jin1* mutants resumed resistance (Lorenzo et al., 2004).

Fatty-acid derived signals can also induce *PDF1.2*. Mutation of *SSi2,* a stearoyl-acyl carrier protein desaturase caused lower levels of oleic acid and resulted in suppression of *PDF1.2*. This suggested that fatty acid signaling worked mutually with JA in the activation of JA-depended defense (Kachroo et al., 2003). Details of the JA signaling pathway have been comprehensively reviewed by Wasternack and Hause (2013). A study of loss of function mutants in the JA pathways showed enhanced susceptibility to necrotrophic pathogens (Thomma et al., 1998).

2.14.3. Cross-talk between salicylic acid and jasmonic acid/ethylene signaling

Extensive cross-talk exists between SA and JA/ET signaling pathways (Figure 2.1). The cross-talk is dominated by mutual suppression, although exceptions exist for some genes that are expressed by both SA and JA exogenous treatments (Schenk et al., 2000; Kunkel and Brooks, 2002; Glazebrook et al., 2003). Enhanced SA levels achieved by genetic alteration can lead to suppression of JA synthesis, as is shown by *mitogen activated protein kinase 4* (*mpk4*) mutants and when *Arabidopsis thaliana chlorophyllase 1* (*AtCLH1*) is silenced (Kariola et al., 2005). In *suppressor of npr1-5* allele *ssi2* mutants that have impaired fatty acid signaling, SA is

constitutively expressed, suggesting that the fatty acid signaling in JA pathway also regulate SA accumulation (Kachroo et al., 2001).

Cross-talk between SA and JA signals happens at multiple points in the pathways. In *A. thaliana* plants transformed with *NahG,* which fails to accumulate SA after *Pseudomonas syringae* infection, higher expression of JA-dependent defense genes was observed (Spoel et al., 2003). The inhibition of the JA pathway by SA required NPR1 function in the cytoplasmic oligomer form but not as nuclear localized monomers, confirming the cytosolic suppression of JA by SA (Spoel et al., 2003). The WRKY70 transcription factor has a role in the cross-talk as its overexpression caused constitutive expression of SA (Li et al., 2004). However, antisense suppression of this transcription factor induced COI1-dependent genes. These results suggest that this transcription factor affects both the SA and JA pathways downstream of their biosynthesis cycle (Li et al., 2004).

Recent evidence indicated a drastic difference between SA and JA crosstalk in monocots and dicots (Tamaoki et al., 2013). In monocots like rice (*Oryzae sativa* L.), SA and JA induced similar sets of defense genes (Tamaoki et al., 2013). This might suggest the absence of the well-characterized antagonism between the SA and JA signaling pathways in monocots

2.14.4. Abscisic acid signaling

The role of ABA in plant defense is controversial. Both, augmented resistance and susceptibility to pathogens have been reported in ABA-deficient mutants. The ABA pathway triggers multifaceted defense responses in plants which vary with the type of plant tissues, the infection stage and strategy of pathogens (Ton et al., 2009). As an example, mutation in *ABI1* and *ABI2* (*abscisic insensitive 1 and 2*) which are negative regulators of ABA showed a greater magnitude of the JA/ET responsive gene expression and higher levels of resistance to necrotrophic pathogens in *A. thaliana* (Anderson et al., 2004; Hernández-Blanco et al., 2007). Sanchez-Vallet et al. (2012) suggested a significant impact of ABA signaling on other signaling pathways as 65 and 30 % of up and down-regulated genes, respectively, in the ABA deficient mutants *aba1-6* were associated with the JA, ET and SA pathway. A role in regulation of R-gene products by ABA had been recently suggested (Mang et al., 2012). Exogenous application of ABA reduced the nuclear localization of resistant to Pseudomonas syringae 4 (RPS4) and resulted in suppressed resistance to *P. syringae* (Mang et al., 2012).

Defense responses triggered by ABA are generally divided into two main groups, pre-invasive penetration defense and post-invasive defense, based on the timing of defense response activation

(Ton et al., 2009). Defense responses in the pre-invasive penetration phase include closing of stomata early after perception of PAMPs and are mostly effective against bacterial and fungal pathogens which infect plants exclusively through stomata (Melotto et al., 2006; Sun et al., 2014). However, virulent pathogens are equipped with effectors to subvert ABA induced stomata closure, e.g. secretion of coronatin by *P. syringae* pv. *tomato* (Melotto et al., 2006) and fusicoccin and oxalate by *Fusicoccum amygdali* and *Sclerotinia sclerotiorum*, respectively (Turner and Graniti, 1969; Guimara and Stotz, 2004). Post-invasive defense responses activated by ABA include deposition of callose at the penetration site and accumulation of reactive oxygen species (ROS). At this step, multifaceted responses are reported depending on the type of plant tissues and the infection stage and strategy of the pathogens (Ton et al., 2009). For example in tomato (*Solanum lycopersicon* L.) a mutation in the ABA pathway called sitiens increased resistance to *B. cinerea* as a result of increased ROS accumulation (Audenaert et al., 2002; Asselbergh et al., 2007). In contrast, callose deposition mediated by ABA signaling had a positive effect on the resistance of *A. thaliana* to *Leptosphaeria maculans* (Kaliff et al., 2007). However, this positive effect was restricted to only a few components of ABA signaling as in contrast to *Abi1-1,* a mutation in *Abi2-1*did not compromise resistance to *L. maculans* (Kaliff et al., 2007).

ABA is also synthesized by fungal pathogens such as *B. cinerea* (Inomata et al., 2004). A few studies have proposed pathogen-produced ABA as a mechanism of virulence in fungal pathogens through suppression of host immune responses (Sánchez-Vallet et al., 2012), but the real mechanism of ABA in the induction of disease remains to be elucidated.

2.15. Mechanisms of resistance against different groups of pathogens

Plant pathogens are classified into three groups based on their mode of obtaining nutrition: necrotrophs, hemibiotrophs and biotrophs. A necrotrophic plant pathogen kills plant cells before starting parasitism and cellular death is a prerequisite for the parasitic relationship. Necrotrophs are dependent on toxins to kill plant cells in order to colonize plant tissue. If these toxins are not produced at the right time at adequate concentrations, and/or host cells lack the susceptibility factors for these toxins, the pathogen fails to parasitize plants (Glazebrook, 2005). Necrotrophs are generally divided into two types: (i) broad host-range necrotrophs (BHNs) or (ii) host-specific necrotrophs (HSNs) (Glazebrook, 2005). The main difference between them is the specificity of the toxins. BHNs produce toxins that act on metabolic pathways common to all plant species whereas HSNs produce host-specific toxins which are products of a single gene acting on the

product of a single susceptibility gene in the host plant. HSNs usually have physiological races or pathovars which attack only some of the genotypes of the host plant species.

Biotrophic plant pathogens are obligate parasites only able to obtain nutrients from living host cells. Biotrophs are forced to establish a compatible relationship with the living host cell. These pathogens must establish feeding structures without eliciting defense mechanisms, or must parasitize during a short time-frame in which the plant defenses may be elicited but are not yet activated. These intricate levels of pathogenesis result in a very restricted host range leading to existence of physiological races in biotrophs. In contrast to necrotrophs, resistance against biotrophs is mediated through rapid programmed cell death known as a HR (Glazebrook, 2005).

Hemibiotrophic pathogens like the fungi *Colletotrichum graminicola*, *Phytophthora infestanse*, and *Magnaporthe grisea* and the bacterium *P. syringae* experience both biotrophic and necrotrophic phases at different stages of their life-cycle. An early asymptomatic phase is achieved through the secretion of biotrophic effectors which suppress cell death and defense responses. Later stages of hemibiotrophic infection are recognized by a switch from the biotrophic to the necrotrophic phase, which is recognized by the development of disease symptoms. The ability to produce both biotrophic and necrotrophic effectors enable hemibiotrophs to switch between the initial biotrophy and later necrotrophy life-cycles (Lee and Rose, 2010).

2.15.1. Mechanisms of resistance against necrotrophs

Roundhill et al. (1995) suggested that *A. lentis* hyphae are not capable of colonizing normal living cells, suggesting that *A. lentis* is either a necrotroph or a hemibiotroph with a very short phase of biotrophy. Host-specific necrotrophs (HSNs) secrete host-specific toxins (HST) which may determine the host range or the range of genotypes of a host plant species that it can infect (Wolpert et al., 2002). An example is the HC-toxin produced from *Cochliobolus carbonum*, which induces corn leaf spot disease but is only toxic on some host genotypes (Williams et al., 2011). Examples of broad host range necrotrophs (BHNs) are the fungi *B. cinerea*, *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and *Sclerotinia sclerotiorum*, and the bacterial pathogen *Erwinia carotovora.*

Necrotrophy is mainly dependent on host cell-death prior to infection and therefore requires a different mode of defense from biotrophy. As the mechanisms of cell death induction vary among necrotrophs, defense responses also vary based on the pathogen's mechanisms of cell death. Cell

death in necrotrophs is usually accompanied by production of secondary metabolites, antimicrobial peptides, and phytohormones such as SA, JA/ET, ABA and ROS as well as callose deposition and other modification of cell walls (Mengiste, 2012). The kinetic and relative accumulation of these compounds may vary for each plant-necrotrophic pathogen interaction. The contribution of each of these components also varies depending on the pathogen's virulence factors (Mengiste, 2012). Some of these defense responses are exploited by necrotrophs for inducing cell death and infection. Cell death promotes colonization of plants by necrotrophs like *B. cinerea* (Govrin and Levine, 2000). Enhanced cell death mutants showed comparably higher susceptibility to necrotrophs but lower susceptibility to biotrophs (Veronese et al., 2004).

Disease associated cell death similar to HR-associated cell death is dependent on host components like hormones and ROS (Desmond et al., 2008; Rossi et al., 2011). Although it is generally accepted that cell death promotes necrotrophic infection, it is not clear if this occurs with all necrotrophs. In addition to biotrophs, the HR plays a role in resistance to hemibiotrophs like *Phytophthora infestans* (Vleeshouwers et al., 2000) and *M. oryzae* (Jia et al., 2000), although they also have a necrotrophic phase in their life cycles. In a screen for *A. thaliana* mutants impaired for resistance to *B. cinerea*, the gene *BOS1* (*Botrytis Susceptible* 1) was shown to restrict necrosis induced by necrotrophs and stresses. The *BOI* (*Botrytis Susceptible Interactor*) also inhibited cell death and resulted in resistance to *B. cinerea* (Veronese et al., 2004). However, reduction in cell death by *BOI* did not impair HR cell death mediated by disease resistance genes. Transformation of plants with an inhibitor of apoptosis proteins (IAPs) for some viruses also caused higher levels of resistance to necrotrophs by reducing the occurrence of cell death (Dickman et al., 2001).

MPK3 and MPK6 are at the core of convergent signaling pathways triggered by PTI and ETI (Tsuda and Katagiri, 2010). These signaling components mediate resistance against BHNs through triggering ET and camalexin synthesis (Brodersen et al., 2006; Qiu et al., 2008; Ren et al., 2008). *PAD3* and *UPS1* which are required for camalexin synthesis were shown to be important for initiating PTI against *B. cinerea* triggered by chitin, OGs and flg22 (Ferrari et al., 2007). MPK3 and MPK6 induced camalexin synthesis by activation of the WRKY33 transcription factor (Mao et al., 2011) and phosphorylation of 1-amino cyclopropane-1-carboxylic acid synthases (ACS) 2 and ACS6 (Han et al., 2010). Loss of function mutations in MPK3 and MPK6 mediated decrease in resistance to *B. cinerea* through a decline in the camalexin biosynthesis (Ren et al., 2008). MKS1 and WRKY33 transcription factors, which have roles in defense against BHNs are physically

bound to MPK3 and MPK6 in normal living cell. After triggering PTI by PAMPs, MKS1and WRKY33 transcription factors are released, leading to the induction of downstream defense gene expression (Qiu et al., 08). Similar binding is shown between ERF104, a component of the ET pathway in PTI-induced defense, and MPK6 (Bethke et al., 2009).

Except for a toll/interleukin 1 receptor domain (TIR)- R-protein which plays a role in resistance to *L. maculans*, ETI is ineffective as a resistance mechanism against necrotrophs. ETI is also hijacked by some of the HSNs to suppress plant defense by specific recognition between an HST and a plant receptor (Oliver and Solomon, 2010). For example, the semi-dominant NBS-LRR Pc protein interacts with an HST called PC toxin, and is required for a compatibility reaction to *Periconia circinata* (Nagy et al., 2007). Another example is the NBS-LRR protein LOV1, which specifically interacts with an HST secreted from *Cochliobolus victoriae* (Victorin), and is required for successful colonization of *A. thaliana* (Lorang et al., 2007).

The inhibition of cell death is thought to be mediated by autophagy, defined as "a cellular process for degradation and recycling of cytoplasmic contents in response to stress and senescence as well as during normal conditions" (Mengiste, 2012). In *A. thaliana*, autophagy is mediated through the formation of autophagosomes after infection by *B. cinerea* (Lai et al., 2011). The WRKY33 transcription factor is capable of interacting with *ATG18a*, a gene required for induction of autophagy in *A. thaliana* (Lai et al., 2011). Mutations in autophagy-inducing genes including *atg2-2*, *atg5*, *atg7* and *atg10* increased levels of resistance to biotrophs (Veronese et al., 2004).

ROS are also produced during plant infection by necrotrophs. This is a common response to pathogen infection, recorded for both necrotrophs and biotrophs (Mengiste, 2012). ROS have an adverse effect on resistance to necrotrophs, as shown in resistance to *B. cinerea* and *S. sclerotiorum* infection (Tiedemann, 1997; Temme and Tudzynski, 2009; Williams et al., 2011). Besides their direct roles, ROS contribute to signaling activity in plant defense, leading to induction of defense genes and cell-death (Kotchoni and Gachomo, 2006). Induction of cell-death has been suggested to be the main action of ROS in defense against hemibiotrophs and biotrophs (Mengiste, 2012). A linear relationship was found between H_2O_2 and superoxide (-OH) radical concentrations and the intensity of colonization by necrotrophs (Govrin and Levine, 2000). *B. cinerea* is able to tolerate high levels of H_2O_2, and a positive correlation was found between the aggressiveness of *B. cinerea* and the concentration of –OH radicals (Tiedemann, 1997; Temme and Tudzynski, 2009). Exceptions were reported where ROS played a role in resistance against necrotrophs when it was

produced at the very early phase of infection. For example, a mutation in ABA biosynthesis caused an increase in ROS levels and levels of resistance to *B. cinerea* (Asselbergh et al., 2007). Wound-induced resistance to *B. cinerea* was mediated by a quick increase in the level of ROS at an early phase of infection (L'Haridon et al., 2011). More supportive evidence for the role of ROS in resistance against necrotrophs is provided by the increase in resistance to *B. cinerea* and *Plectosphaerella cucumerina* in *ocp3,* an *A. thaliana* mutant with higher H_2O_2 levels (Coego et al., 2005). Early stages of ROS production also have a role in resistance against *S. sclerotiorum* (Williams et al., 2011).

2.16. RNA-seq transcriptome analysis

Interactions between plants and pathogens are complex and dynamic, and are controlled by interwoven downstream signaling pathways. Traditional genetic tools are insufficient for understanding these complex host-pathogen interactions, but novel high-performance transcriptome technology, such as RNA-seq analysis allow for large scale monitoring of gene expression.

In the RNA-seq platform, a population of RNAs (total or fractionated as poly(A)+) is converted to a library of cDNA fragments with adaptors linked to one or both ends (Figure 2.2). The cDNA library will then be sequenced by NGS in a way that short reads are produced from one or both ends of the cDNA. The reads usually span between 30-400 base pairs based on the DNA sequencing technology used for RNA-seq. Generally, RNA-seq can be conducted using any high-throughput sequencing technology like Illumina IG18 (Lister et al., 2008; Marioni et al., 2008; Morin et al., 2008), Applied Biosystems SOLiD (Cloonan et al., 2008) or Roche 454 Life Sciences (Vera et al., 2008). After sequencing, the reads will be usually aligned to a reference genome if it is available. In the absence of a reference genome, reads will be assembled *de novo* to develop a genome-wide transcription map which consists of transcriptional structure and/or level of expression of each gene (Wang et al., 2009).

RNA-seq has many advantages over the traditional transcriptome analysis methods, although it is a new technology and is under active development. In contrast to hybridization-based methods, RNA-seq does not require a genome sequence, making it an ideal strategy for the study of large numbers of organisms with no genome sequence data. RNA-seq has the adaptability to provide sequence of different lengths. Shorter reads like 30 bp can help to reveal how the neighboring exons are connected, while longer reads or pair-end short reads are useful for tracking the

connectivity of multiple exons. This adaptability is useful for the study of complex transcriptomes (Wang et al., 2009). It is feasible in RNA-seq platforms to call for sequence variation like SNPs in the expressed genes which are valuable for developing genetic maps. RNA-seq has little background noise compared to hybridization methods like microarrays, because reads are mapped against a reference genome with minimum errors. There is no saturation level for highly expressed genes, and there is a large dynamic range in which the gene expression can be measured whereas microarray technology is unable to deal with genes with both very high and very low expression levels. Finally, RNA-seq does not require cloning and it can be set up with samples with very low amounts of RNA (Wang et al., 2009).

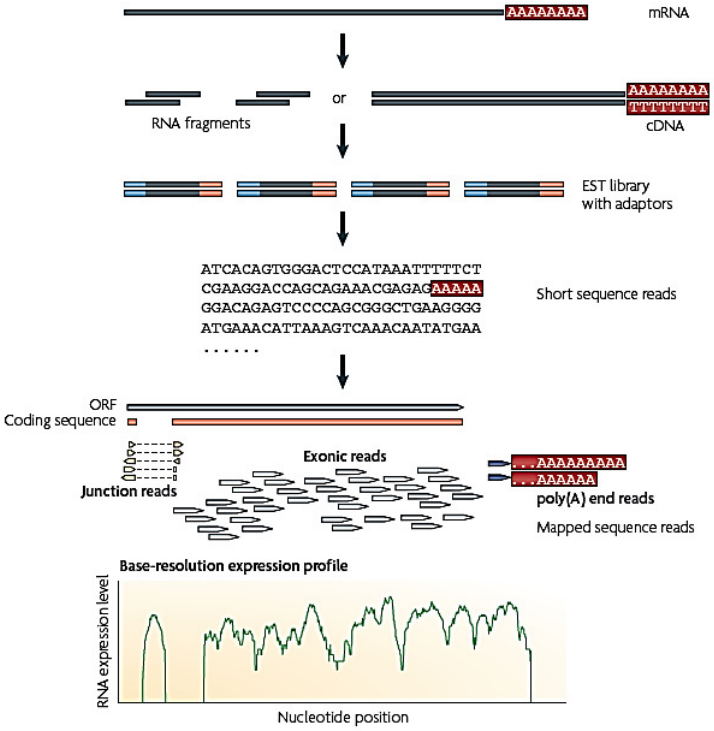


Figure 2.2. Common steps involved in sequencing mRNA by NGS in RNA-seq platforms. In the first step, a cDNA library is constructed from fragmented mRNA. The sequencing adaptors are then linked to the cDNA fragments. Short sequence reads are then obtained through sequencing the cDNA adaptor complex by NGS technology. Sequencing data are then either aligned to a reference genome or assembled *de novo* using the assembly algorithms. Aligned or assembled reads are then categorized to three main types: exonic reads, junction reads and poly(A) end-reads. The expression pattern of genes is finally inferred from these three types of data (Wang et al., 2009, reproduced with permission).

The statistics analyses required for RNA-seq fall into three categories (i) read mapping, (ii) transcriptome reconstruction and (iii) expression quantification (Garber et al., 2011). The first step in all NGS data analysis is mapping the sequenced reads to a reference genome, and if not available, developing a transcription map from the *de novo* assembly of reads (Figure 2.3). In advance of mapping or assembling reads, a preliminary step is quality filtering in which reads with poor sequence qualities are omitted from the data files. This significantly reduces the computational time required for the alignment or assembly. In the case of organisms with no preliminary genome sequence, *de novo* assembly leads the way toward construction of a transcription map. There is potential for using closely related organisms genome or expressed sequence tags (ESTs), however, some data will be neglected due to sequence difference or splicing events (Garber et al., 2011).

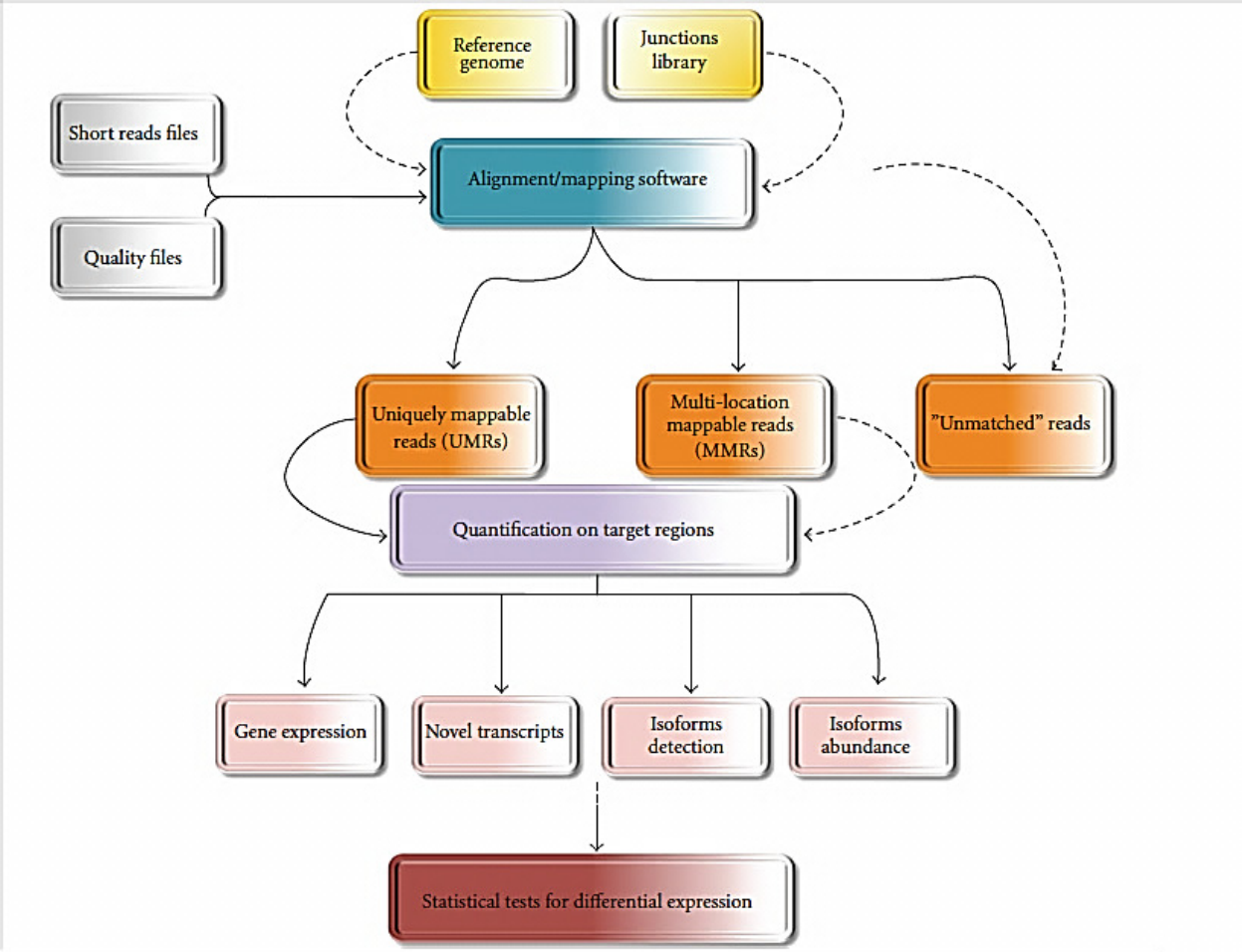


Figure 2.3. RNA-Seq computational pipeline (Costa et al., 2010; reproduced with permission).

The next step in analysis of RNA-seq data is quantifying gene expression, which is of great importance for assessing the quality of data and for judging events based on the available annotations. Only a qualitative image is provided of the analysis, and the large amount of data hinders a higher focus on the most relevant data at this step. Therefore, the second step in the analysis of RNA-seq is the automatic quantification of transcripts for the entire transcriptome. The final objective is quantifying the identified well-annotated genes and newly expressed transcripts which are referred to as "transcribed segments of DNA but not annotated" as an exon. Analysis of data for eukaryotes is more compounded by alternative splicing events which give a single gene the ability to produce multiple products. Exon skipping, alternative 5' or 3' splicing, mutually exclusive exons, intron retention and cryptic splice sites are the most frequent splicing events (Costa et al., 2010). The algorithm used in this step solves two common problems induced by splicing events, finding different isoforms and determining their ratio of expression (Garber et al., 2011).

The final step is to compare the expression levels among different treatments. Following the analysis procedure suggested for microarrays, the first generation of analysis is built upon tests combined with multiple comparisons of error of the observed count at the gene or isoform levels. Because RNA-seq data are not normally distributed, it is more appropriate to use a Poisson distribution model to aggregate read counts with binomial distributions (Marioni et al., 2008). More recently, Robinson et al. (2010) suggested an empirical Bayesian approach to differentiate among genes expression in different treatments. More details about this can be found in the review by Costa et al. (2010).

2.17. Conclusion

CDC breeding program for AB resistance has been successful and many resistant variety have been released during last three decades. The current strategy at CDC is to assure the durability of resistance genes through introduction of resistance genes from non-adapted germplasm and pyramiding of previously introduced resistance genes. As pyramiding of all the available genes is costly and time-consuming, an efficient gene pyramiding program requires prior knowledge on the allelic relationship among resistance genes and availability of closely linked markers for marker-assisted gene pyramiding. In addition, it is advantageous to know if non-allelic resistance genes mediate resistance through different mechanisms to avoid pyramiding of resistance genes with

identical actions. This study was conducted to gather required information for an efficient AB resistance gene pyramiding in the lentil breeding program.

Prologue to Chapter 3

As suggested in the literature review, the allelism test is a genetic tool capable of determining the allelic relationship of resistance genes. Allelism tests were used to determine allelic relationship among AB resistance genes reported in lentil. Recombinant inbred lines (RILs) from all the possible crosses among AB resistant *L. culinaris* genotypes CDC Robin, 964a-46, ILL 7537 and ILL 1704 were subjected to pathogenicity tests. Efforts were also made to understand the genetics of resistance in the *L. ervoides* accession L-01-827A. For this purpose, segregation analysis was performed for an interspecific RIL population derived from the *L. culinaris* cv. Eston × *L. ervoides* L-01-827A. The LR-18 RIL population developed from the cross CDC Robin × 964a-46 was subjected to QTL mapping using a comprehensive genetic linkage map previously developed from 563 polymorphic markers (SNPs, SSRs and morphological markers). The allelic relationships among resistant *L. culinaris* genotypes and the genetic inheritance of resistance from *L. ervoides* L-01-827A are discussed in this chapter.

CHAPTER 3

Allelism tests decipher the allelic relationships among resistance genes for ascochyta blight in lentil

3.1. Introduction and objectives

Lentil (*Lens culinaris* Medik.) is a self-pollinating annual cool season legume crop that is produced throughout the world. Lentil is highly valued as a high protein food and is the world's sixth largest pulse crop with annual production of about 4 Mt (FAOSTAT, 2013). Cultivated crop species, including lentil, often experience genetic 'bottlenecks' (Erskine et al., 1998; Tester and Langridge, 2010). The importance of preserving variability is integral to future efforts in genetic improvement. To keep pace with the demands for continuous genetic improvement, introduction of new genetic resources to breeding programs and efficient application of available resources is required. A system of Systematic Introduction of New Germplasm named SINGing has been developed at the CDC, University of Saskatchewan, Canada. In lentil, this involves the following aspects: i) Potential new genes of interest are systematically introduced from non-adapted lentil germplasm through intraspecific and interspecific hybridizations made possible by overcoming hybridization barriers present in the genus *Lens*; ii) new traits from cultivated and wild species are eventually incorporated into high-yielding adapted lines, and iii) genomic technologies are applied to decrease the cost and time required for genetic improvement (Tullu et al., 2011).

AB of lentil caused by *Ascochyta lentis* Vassilievsky (teleomorph: *Didymella lentis* W.J. Kaiser, B.C. Wang, and J.D. Rogers) is prevalent throughout many temperate lentil production regions of the world. Yield losses have been reported up to 70 % in Canada, 30-50 % in the USA, and 50 % in Australia (Gossen and Morrall, 1983; Kaiser, 1992; Brouwer et al., 1995). The most environmentally acceptable and economically profitable method of control is to develop varieties with high levels of durable resistance. A few major AB resistance genes have been characterized in different lentil genotypes (Tay and Slinkard, 1989; Andrahennadi, 1994; Ahmad et al., 1997; Andrahennadi, 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001), and AB resistant varieties have been released (Ali, 1995; Vandenberg et al., 2001; Vandenberg et al., 2002). The exploitation of resistance sources in different species of the genus *Lens* and pyramiding available

resistance genes in cultivated lentil genotypes are the predominant new strategies for developing resistant cultivars.

First identification of resistance to AB of lentil in Canada was in 1987 (Tay and Slinkard, 1989). This and other research suggested that resistance to AB was conditioned by either a dominant or a recessive gene in different AB resistant genotypes (Tay and Slinkard, 1989; Andrahennadi, 1994; Ahmad et al., 1997; Andrahennadi, 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001). Andrahennadi (1994) reported that a recessive gene (*ral2*) conditioned the resistance to AB in the lentil cv. Indianhead. Tay and Slinkard (1989) and Ford et al. (1999) reported a major dominant gene (*AbR1*) that controlled resistance to AB in ILL 5588. The first two AB resistant lentil cultivars in Canada CDC Matador and CDC Redwing, with recessive and dominant resistance genes, respectively were released in 1994. Tullu et al. (2008) inferred a major QTL for resistance to AB from a study of a RIL population derived from a cross between PI 320937 and cv. Eston. They suggested that a single major QTL explained 41 % of variation of resistance to AB in PI 320937. QTL analysis of AB resistance in ILL 7537 was conducted using a population comprising 153 F_2 progeny (ILL 7537 × ILL 6002) and a linkage map comprising 72 markers spanning 412.5 cM (Rubeena et al., 2003). Three QTLs were detected. QTL1 and QTL2 were in close proximity to each other on LG1 and accounted for approximately 47 % of the variance, whereas QTL3 on LG2 accounted for approximately 10 % of the variance of the trait (Rubeena et al., 2003). Recently, Gupta et al. (2012) developed a genetic map by integrating 196 EST-SSR markers from *Medicago truncatula* with an intraspecific linkage map from a mapping RIL population from a cross between ILL 5588 and ILL 5722. They detected three QTLs for seedling and a further three for pod resistance to AB (Gupta et al., 2012). The range of results suggests that resistance to AB in lentil is conditioned by a few major QTLs.

Resistance genes for AB have also been reported in wild lentil species (Bayaa et al., 1994; Tullu et al., 2010). *L. ervoides* (Brign.) accessions have a relatively high frequency of resistance to AB compared to the other five wild lentil species (Bayaa et al., 1994; Tullu et al., 2010). Two complementary resistance genes for AB were characterized in both *L. ervoides* and *L. odemensis* (Ladiz) (Ahmad et al., 1997). Genetic gain in the development of AB resistance in cultivated lentil can be improved by introduction of new alleles from exotic germplasm (Tullu et al., 2010). As an example, there was no resistance to anthracnose pathogen race 0 in the cultivated lentil before

interspecific transfer of resistance from *L. ervoides* accessions L-01-827A (Fiala et al., 2009) and IG 72815 (Tullu et al., 2013).

Ahmed et al. (1996) described significant levels of variability of aggressiveness in the *A. lentis* population, and suggested that aggressiveness of *A. lentis* isolates had increased over time as cultivar 'Laird' which was moderately resistant at the time of release in the 1980s had become susceptible later (Pedersen and Morrall, 1994). Banniza and Vandenberg (2006) reported that the host reaction of 16 lentil genotypes to 65 isolates of *A. lentis* collected in Canada resulted in a continuum of severity of infection, indicating natural variation of aggressiveness in the population without any distinct pathotypes. The available evidence suggests that large scale and long-term cultivation of lentil cultivars with single resistance genes may enable the pathogen to overcome this resistance (Nasir and Bretag, 1997). To avoid this, the strategy to breed for durable resistance relies on combining multiple resistance genes into elite cultivars, or gene pyramiding.

Gene pyramiding may be difficult if conventional breeding approaches are used due to possible epistatic effects of the genes governing resistance. Selection of progeny carrying multiple resistance genes can be difficult because the effect of one major resistance gene may obscure the effects of other major or minor genes, resulting in progeny with similar phenotypes but different genotypes (Crute, 1998). Marker-assisted selection (MAS) can improve the resolution of selection by addressing this issue. The availability of molecular markers used to select at early generations in the absence of the pathogen can decrease the cost and time required. An example is the application of the RAPD markers linked to the AB resistance genes *AbR*1 and *ral*1 and a dominant gene conditioning resistance to anthracnose for gene pyramiding in lentil (Tar'an et al., 2003). Reliable co-dominant markers closely linked to lentil AB resistance genes are preferred to track different resistance genes for gene pyramiding.

The first comprehensive genetic map of *L. culinaris* was developed by deep transcriptome sequencing of multiple cultivated and wild lentil genotypes followed by the discovery of gene-based SNPs (Sharpe et al., 2013). For the LR-18 RIL population developed from CDC Robin × 964a-46, 563 polymorphic markers including SNPs, SSRs and some morphological markers were mapped. The markers were used for developing the first SNP-based linkage map in lentil used for QTL mapping of seed size, shape and colour (Fedoruk et al., 2013). In a separate effort, Kaur et al. (2011) used lentil transcriptome sequences for the discovery of EST-SSR markers which

yielded 166 applicable markers. This new generation of lentil genetic maps has not yet been used for QTL analysis of AB resistance.

To increase the efficiency of gene pyramiding, it is desirable to obtain insight into the nature and number of available resistance genes from various resistant sources. In classical genetics, the allelism test is a means of identification for distinct resistance genes. Using allelism tests, the hypothesis was tested that AB resistance genes reported in lentil over the past 20 years are non-allelic. RIL populations from all possible combinations of crosses among four AB resistant lentil genotypes CDC Robin, 964a-46, ILL 1704 and ILL 7537 were developed previously. Transgressive segregation for disease severity was considered proof for the existence of non-allelic resistance genes in the parental lines.

This study was conducted to (1) determine if the known AB resistance genes in lentil are allelic, (2) find closely linked genetic markers to be used for marker-assisted selection in AB resistance gene pyramiding and (3) determine the genetic control of AB resistance genes in *L. ervoides* L-01-827A.

3.2. Materials and Methods

3.2.1. Plant materials and fungal isolate

The lentil genotypes CDC Robin, 964a-46, ILL 1704 and ILL 7537 with known resistance to AB were chosen for this study. CDC Robin is resistant to both AB and race 1 of the anthracnose pathogen (Andrahennadi, 1994; Vail et al., 2012) and was released in Canada in 2001 (Vandenberg et al., 2001). Resistance to both AB (*ral2*) and anthracnose in CDC Robin was derived from the cv. Indianhead. Breeding line 964a-46 has a dominant AB resistance gene (*AbR1*) from ILL 5588, which was released as the cultivar Northfield in Australia (Ali, 1995). ILL 7537 is a land race from Jordan with a resistance gene different from ILL 5588 (Nguyen et al., 2001). The genetic control of AB resistance in ILL 1704, a land race from Ethiopia with moderate resistance (Tullu et al., 2010) has not yet been determined.

The AB resistance reactions of these lentil genotypes were compared with other genotypes including PI 320937, CDC Redberry, ILL 6976, *L. ervoides* L-01-827A, *L. ervoides* IG 72815 and LR-59-81 in the parental test. PI 320937 is a germplasm accession from Germany with resistance to both AB and anthracnose race 1 (Tullu et al., 2003; Tullu et al., 2006). Tullu et al. (2006) suggested that resistance to AB in PI 320937 was conditioned by a major QTL. CDC Redberry is a lentil cultivar with resistance to both AB and anthracnose race 1 (Vandenberg et al., 2006). ILL

6976 is an ICARDA accession which produced high numbers of pods in each peduncle in a field trial and is used in the CDC lentil breeding program as a potentially high yielding line (Abebe Tullu, Dpt. of Plant Sciences, University of Saskatchewan, pers. com.). Reaction of ILL 6976 to AB has not yet been determined. *Lens ervoides* L-01-827A was a single plant selection from accession IG 72847 in the ICARDA collection and was shown to be resistant to race 0 and 1 of *Colletotrichum lentis* Damm (Fiala et al., 2009). *L. ervoides* IG 72815 is an accession from the ICARDA collection and has resistance genes to anthracnose pathogen race 0 (Tullu et al., 2013). Hybrid progeny from both L-01-827A and IG 72815 derived from crosses with cv. Eston were developed previously for investigation of interspecific transfer of anthracnose resistance (Fiala et al., 2009; Tullu et al., 2013). The reaction of these wild genotypes to AB has not been reported. LR-59-81 is a RIL from $F_{7:10}$ RIL population LR-59 derived from the interspecific cross *L. culinaris* Eston × *L. ervoides* L-01-827A. It has resistance to race 0 and 1 of *C. lentis* (Vail et al., 2012).

CDC Robin, 964a-46 and Eston were developed at the CDC. All genotypes with ILL and IG prefixes are germplasm from the ICARDA collection. PI 320937 was obtained from the USDA germplasm collection.

RIL populations were previously developed by single seed descent for all cross combinations among four lentil genotypes (Table 3.1). *L. ervoides* L-01-827A was previously crossed with the AB susceptible *L. culinaris* cv. Eston. The F_1 hybrid was obtained by Fiala et al. (2009) using embryo rescue and a RIL population was advanced to F_7 by single-seed descent before bulking and selfing for several more generations. Lentil cvs. Eston and CDC Robin were included in all the tests as susceptible and resistant checks, respectively.

A monoconidial culture of *A. lentis* isolate AL57, an aggressive isolate from Landis, Saskatchewan (Banniza and Vandenberg, 2006), was used for the pathogenicity test. To confirm virulence, Eston plants were sprayed with 5×10^5 conidia mL^{-1} before the initiation of the test, the fungus was re-isolated from infected symptomatic tissues, and the fungal conidia suspension aliquots were stored in a cryopreservation solution containing 10 % skim milk and 20 % glycerol at -80 C°.

3.2.2. Plant growth condition and fungal inoculation

To prepare fungal inoculum, conidia were revitalized on 50 % oatmeal agar plates (30 g oatmeal [Quick Oats, Quaker Oats Co., Chicago, IL, U.S.A.], 8.8 g agar [Difco, BD®, Sparks Glencoe,

MD, U.S.A.], 1 L H_2O) and incubated for 7 days at room temperature. Plates were then flooded with sterile deionized water and conidia were harvested by scraping the colonies with the edge of a sterile glass microscope slide. The suspension was collected and filtered through one layer of Miracloth into a clean Erlenmeyer flask. The concentration of the conidia suspension was adjusted to 5×10^5 conidia mL^{-1} using a hemocytometer.

Four seeds from each line were sown in 10 cm square pots containing soilless mixture of Sunshine Mix no. 4 (Sun Grow Horticulture® Ltd.,Vancouver, BC, Canada) and Perlite™ (3/1 V/V). Seedlings with 10 - 15 expanded leaves (21 days after sowing) were inoculated with the conidia suspension at 2 mL per seedling using an airbrush, and then incubated in a humidity chamber for 48 h. Plants were subsequently incubated on a mist bench with mist applied for 30 s every 90 min during the day for the remainder of the test. Disease severity (DS) data were collected two to three weeks after inoculation using a 1-10 scale, where the DS score increased incrementally by 10 %. Data were converted to percentage DS (% DS) using the class midpoints for data analysis.

3.2.3. Experimental design for phenotyping lentil germplasm and data analysis

All data analyses were performed using Statistical Analysis System (SAS) version 9.3 (SAS Institute Inc., Cary, NC, USA). Homogeneity of variance was tested using Levene's test and, in the case of heterogeneity, the variance was modeled using the mixed model procedure.

The reaction to AB infection was first evaluated for the parental lines of RIL populations and compared with other genotypes including ILL 6976, CDC Redberry, LR-59-81, IG 72815 and PI 320937. This experiment was arranged in a randomized complete block design with four replicates. DS ratings were conducted for each of four plants in each pot, converted to percentage before being averaged for each pot representing one of four biological replicates. This parental test was conducted twice. Homogeneity of variances between the two repeats was determined with the Levene's test. The homogenous variance between the two repeats of parental test allowed pooling of the data for two repeats. The Mixed procedure (Proc Mixed) of SAS was used for variance analysis of parents with the replicate considered as a random effect and coefficient of variance (CV) was generated using generalized linear model (Proc GLM) of SAS.

RIL populations were subjected to pathogenicity testing using the same procedure as for the parental tests except for the rating strategy. Here, one estimate of DS was recorded for every pot containing four seedlings, which was regarded as one biological replicate. All experiments for RIL populations were arranged in a randomized complete block design with four replicates. Due to

limited space in the greenhouse, experiments for populations LR-04 and LR-20 were split into two sub-experiments each with two replicates. Non-significant block effects observed for both LR-04 and LR-20 indicated uniform test conditions over time. To assure the repeatability of pathogenicity tests for the RIL populations, data of the check lines (CDC Robin and Eston) measured across pathogenicity tests of RILs were subjected to analysis of variance by the mixed model procedure of SAS. Check line reactions were uniform across pathogenicity tests, supporting the uniformity of pathogenicity tests over time. For each RIL population, pathogenicity testing was conducted once. Homogeneity of variances was tested with the Levene's test. The Proc Mixed of SAS was used for variance analysis with block as a random factor. To determine the proportion of transgressive segregant lines in each population, means of % DS were compared with that of the parents using the LSMEANS option of Proc Mixed. RILs with significantly higher and lower % DS than both parents were regarded as transgressive segregants.

3.2.4. QTL analysis of the LR-18 population

QTL analysis was conducted using a lentil linkage map already developed from 550 SNPs, nine SSRs and four morphological markers, in a mapping population developed from two resistant parents, CDC Robin and 964a-46 (Fedoruk et al., 2013). Phenotypic data used for QTL mapping were obtained from the pathogenicity tests conducted by the pulse pathology group of the CDC following protocols described above, but with DS ratings for each of four plants in each pot, similar to the parental test. Data were converted to percent values before being averaged for each pot (replicate). The mean of three biological replicates was used as phenotypic data for QTL analysis.

QTL analyses conducted using composite interval mapping (CIM) by assigning markers with the highest LOD (logarithm of the odds) scores detected in simple interval mapping (SIM) as cofactors. QTLs were considered significant if the LOD scores exceeded a significant threshold level at $P = 0.05$, determined by a 1000 permutation test (Churchill and Doerge, 1994). The percentage of phenotypic variation explained by each QTL was estimated from the difference between the percentage of the total variance and the residual variance. QTL analysis was conducted with MapQTL® 5 (van Ooijen, 2004). The QTL graph was prepared with MapChart® 2.2 (Voorrips, 2002) using LOD scores and confidence levels calculated by CIM. To understand the origin of QTLs detected for LR-18, a single marker with the highest LOD score was selected from each QTL and mean phenotypic data were grouped based on their genotype-combinations at these loci. RILs with missing data at either one of the loci were removed from this test.

3.3. Results

3.3.1. Reaction of lentil germplasm to *Ascochyta lentis* inoculation

Significant differences in % DS ($P < 0.0001$) were observed among lines and they showed a continuum of reactions to *A. lentis* infection (Figure 3.1). Eston and ILL 6976 had similar % DS above 70 %. All other lines had significantly lower % DS than the susceptible check Eston, suggesting the existence of some level of resistance in all the tested genotypes except for ILL 6976. *L. ervoides* L-01-827A, CDC Robin and ILL 7537 had % DS ranging from 0-20 %. *L. ervoides* L-01-827A showed no macroscopic symptoms. To exclude the possibility of disease escape, the inoculation for this genotype was repeated two weeks after the first inoculation using the same plants. Plants of L-01-827A remained disease free, indicating that resistance was neither a case of escape nor age-dependency. The lines with significantly lower % DS than Eston included CDC Redberry, ILL 1704, 964a-46, PI 320937, LR-59-81 and *L. ervoides* IG 72815 with DS ranging from 30-50 %.

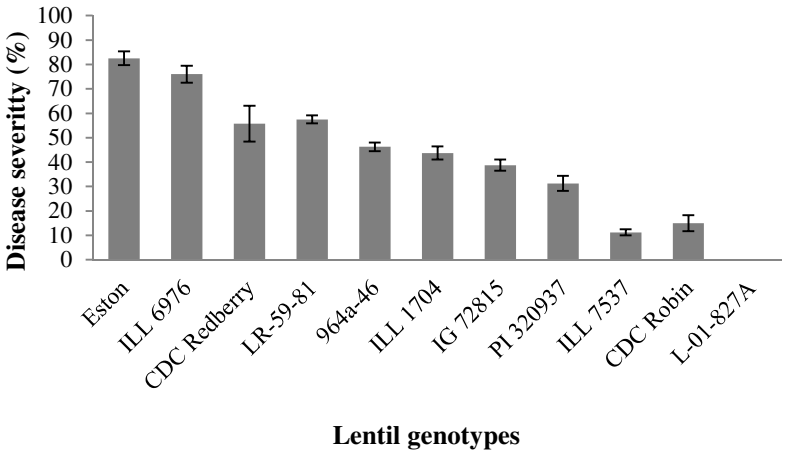


Figure 3.1. Percent ascochyta blight severity of eight *Lens culinaris* genotype, two *L. ervoides* genotypes (L-01-827A and IG 72815) and one interspecific genotype (LR-59-81 derived from *L. culinaris* cv. Eston × *L. ervoides* L-01-827A). Values are averages of four replicates. Error bars indicate ± SE. Disease was rated using a 1-10 scale with 10 % incremental increases in disease severity. Data were converted to % DS using the class midpoints.

3.3.2. Segregation pattern of reactions to *Ascochyta lentis* in RIL populations

Significant differences in % DS among the lines were observed for all RIL populations (Table 3.1). The CV for all seven experiments was less than 30 %, ranging from 14.0 for LR-04 to 27.5 for LR-18.

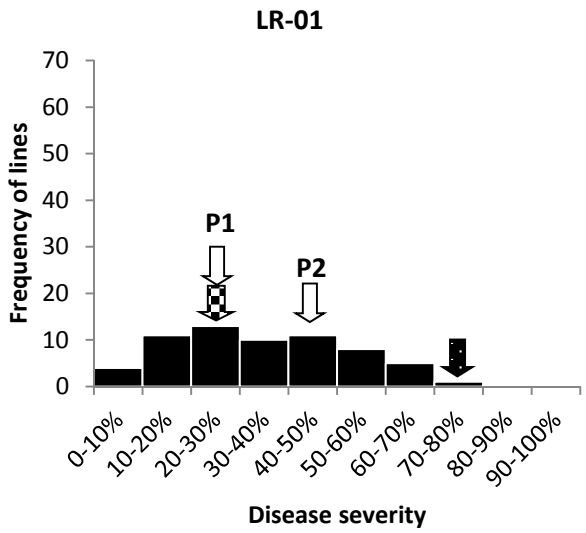
Table 3.1. ANOVA results for pathogenicity tests conducted for recombinant inbred line populations from crosses between lentil genotypes with resistance to ascochyta blight.

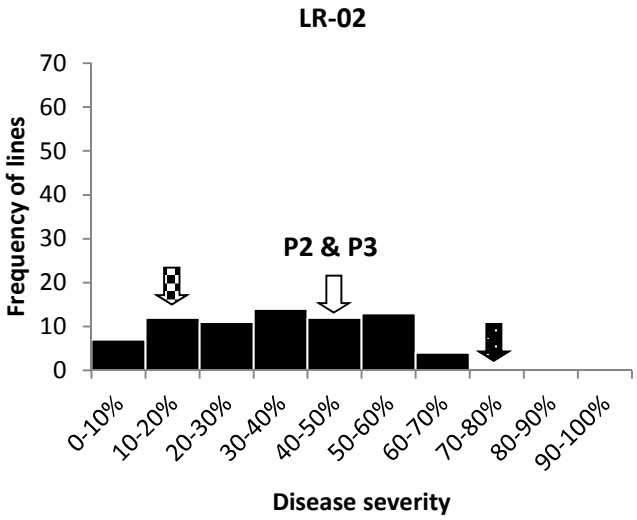
Tests	Number of lines	Cross	F	CV
Parents	--	--	112.9**[a]	14.2
LR-01	63	ILL1704 × CDC Robin	41.6**	17.3
LR-02	74	ILL1704 × 964a-46	49.9**	15.4
LR-03	107	ILL1704 × ILL7537	27.1**	18.4
LR-04	132	964a-46 × ILL7537	61.2**	14.0
LR-18	145	CDC Robin × 964a-46	53.7**	27.5
LR-20	164	ILL7537 × CDC Robin	38.1**	16.7
LR-59	74	Eston × L-01-827A	24.1**	13.8

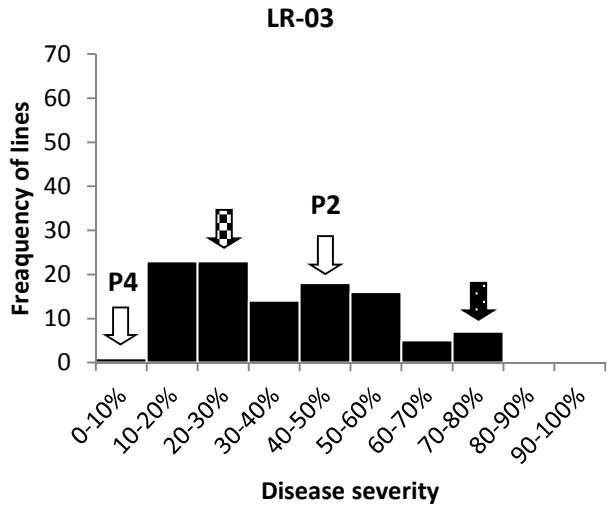
[a] significant with 99.99% confidence.

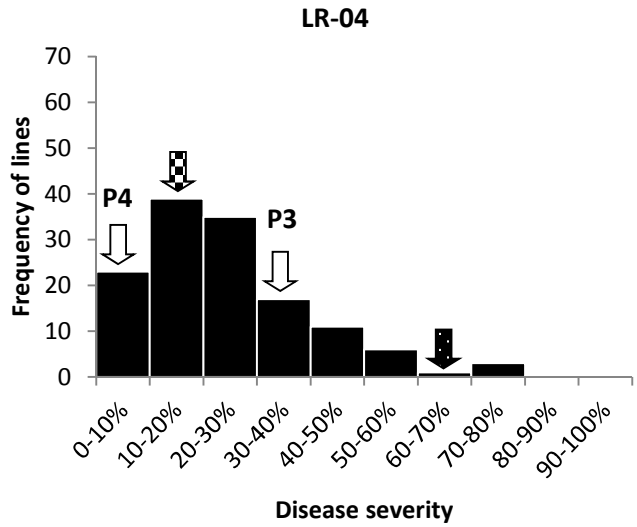
The mean % DS was 20 % and 45 % for LR-01 parents CDC Robin and ILL 1704 and they were significantly different from each other ($P < 0.0001$). RILs were distributed within and outside the parental DS score range, resulting in a monomodal distribution (Figure 3.2). Mean comparisons showed that 65 % RILs had similar AB reactions compared to at least one of the parents (Table 3.2). Almost equal numbers of RILs showed transgressive susceptible and resistant % DS. The proportion of transgressive lines was 35 % of the population. All RILs scattered between the parents had % DS either similar to both or one of the parents.

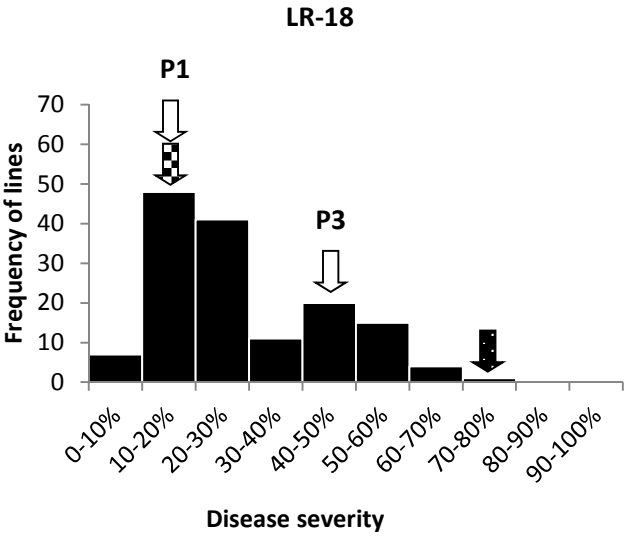
For LR-02 parents 964a-46 and ILL 1704, the mean % DS was 45 % and there was no significant difference between them ($P = 0.1366$). The frequency distribution for LR-02 population was monomodal and a continuum of % DS was observed (Figure 3.2). The majority of lines had significantly lower % DS than both parents and 54 % of the RILs were in the transgressive resistant group (Table 3.2). The proportion of transgressive susceptible lines was smaller than transgressive resistant with 18 % of the lines. Only 22 lines had similar % DS to one or both of the parents.


LR-01
Frequency of lines
70
60
50
40
30
20
10
0
P1
P2
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity


LR-02
Frequency of lines
70
60
50
40
30
20
10
0
P2 & P3
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity


LR-03
Freaquency of lines
70
60
50
40
30
20
10
0
P4
P2
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity


LR-04
Frequency of lines
70
60
50
40
30
20
10
0
P4
P3
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity


LR-18
Frequency of lines
70
60
50
40
30
20
10
0
P1
P3
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity

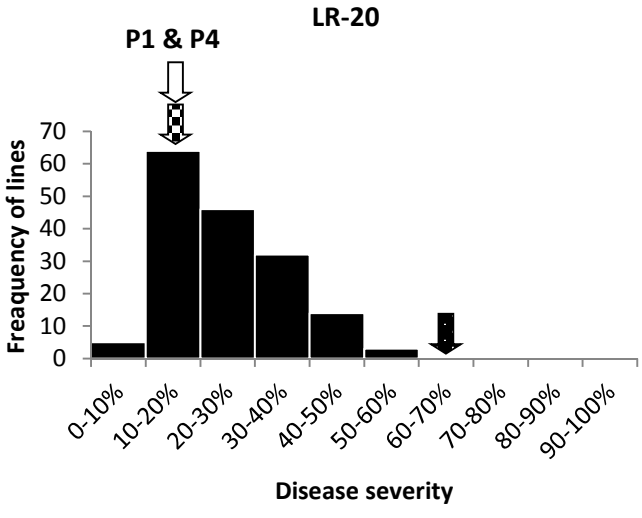

LR-20
Freaquency of lines
70
60
50
40
30
20
10
0
P1 & P4
0-10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Disease severity

Figure 3.2. Distribution pattern of percent ascochyta blight disease severity (DS) scores in RIL populations from six resistant × resistant *Lens culinaris* crosses. Values are means of four replicates. LR-01: CDC Robin (P1) × ILL 1704 (P2); LR-02: ILL 1704 (P2) × 964a-46 (P3); LR-03: ILL 7537 (P4) × ILL 1704 (P2); LR-04: ILL 7537 (P4) × 964a-46 (P3); LR-18: CDC Robin (P1) × 964a-46 (P3) ; LR-20: ILL 7537 (P4) × CDC Robin (P1). CDC Robin ( ) and Eston ( ) were included as resistant and susceptible checks, respectively. Blank arrows show the position of parents in the distribution. DS was rated using a 1-10 scale, where the DS score increased incrementally by 10 %. Data were converted to % DS using the class midpoints for data analysis.

Table 3.2. Frequency of recombinant inbred lines (RIL) (%) statistically similar to at least one of the parents (Parent), frequency of RILs (%) with disease severity scattered between, but significantly different from, both parents (Mid-parent), and frequency of RILs (%) with transgressive susceptible or resistant reactions to ascochyta blight. LR-01: CDC Robin × ILL 1704; LR-02: ILL 1704 × 964a-46; LR-03: ILL 7537 × ILL 1704; LR-04: ILL 7537 × 964a-46; LR-18: CDC Robin × 964a-46; LR-20: ILL 7537 × CDC Robin. Mean comparison was conducted with Fisher's Least Significant Difference at $p \leq 0.05$.

Population	Frequencies of RILs (%)			
	Parent	Mid-parent	Transgressive susceptible	Transgressive resistant
LR-01	65	0	19	16
LR-02	28	0	18	54
LR-03	36	52	12	0
LR-04	58	26	16	0
LR-18	59	39	2	0
LR-20	45	0	37	18
LR-59	16	84	0	0

The mean % DS was 10 % and 45 % for LR-03 parents, ILL 7537 and ILL 1704, respectively, and there was a significant difference between them ($P < 0.0001$). The distribution of % DS ratings in this population was bimodal (Figure 3.2). The majority of RILs had % DS scattered between the parents and the distribution was skewed toward lower % DS scores. Mean comparisons showed that 36 % of lines had similar % DS to at least one of the LR-03 parents (Table 3.2). The proportion of RILs with transgressive % DS was 10 % and all transgressive RILS were susceptible.

For LR-04, the parents ILL 7537 and 964a-46 had mean % DS of 10 % and 40 % respectively, with significant difference between them ($P < 0.0001$). Data distribution was monomodal and skewed toward lower % DS scores (Figure 3.2). Most of the RILs (58 %) were not significantly different from one or both of the parents (Table 3.2). Susceptible transgressive segregation was observed and 16 % of the RILs had significantly lower DS than both parents. No transgressive segregation was observed for resistance.

For the LR-18 parents CDC Robin and 964a-46, the mean % DS was 15 % and 45 % and they were significantly different ($P < 0.0001$). The distribution of frequency data was bimodal (Figure 3.2). Most RILs (59 %) had % DS not significantly different from either one or both of the parents (Table 3.2). Compared to the other crosses, the proportion of transgressive susceptible was the lowest in this population and only 2 % of lines were more susceptible than both parents.

For the LR-20 RIL population, the mean % DS was 15 % for both parents CDC Robin and ILL 7537 with no significant difference between them ($P = 0.99$). The distribution of frequency data was monomodal and skewed toward low % DS scores (Figure 3.2). Transgressive segregation was observed for LR-20 with the second highest proportion of transgressive progeny (Table 3.2). In addition, it had the highest proportion of transgressive susceptible lines (37 % of the RILs). The proportion of RILs similar to both parents was lower than transgressive lines in the LR-20 population.

For the interspecific RIL population LR-59, the % DS of the parents L-01-827A and Eston were significantly different ($P < 0.0001$). In this experiment, L-01-827A remained disease-free, similar to the results observed in the lentil germplasm test, whereas Eston was severely diseased in the end of the experiment. The proportion of RILs with % DS not significantly different from that of L-01-827A was 16 %, confirming that resistance was successfully transferred from L-01-827A to the LR-59 hybrid RILs (Figure 3.3). RILs were mostly distributed over the range from 0-40 % DS. Mean comparisons showed that the majority of RILs had significantly lower % DS than the susceptible check Eston (Table 3.2). A Mendelian genetic analysis based on the phenotypic data, where lines with % DS significantly higher than resistant parent L-01-827A were regarded as susceptible, revealed best fit with an expected ratio for two complementary genes in $F_{7:10}$ of RILs (1R:3S) ($P = 0.081$).

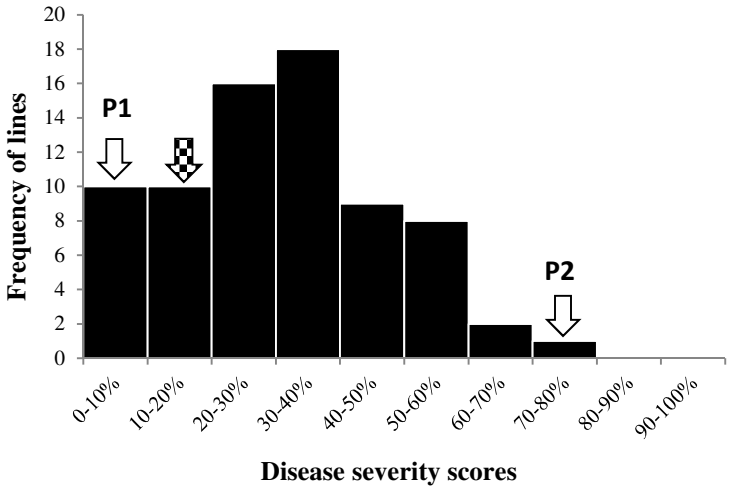


Figure 3.3. Frequency distribution of mean percent ascochyta blight severity for recombinant inbred line population LR-59 derived from interspecies cross *Lens culinaris* cv. Eston (P2) × *L. ervoides* L-01-827A (P1). Values are average of four replicates. CDC Robin (⚄) was included as a resistant check. Disease severity (DS) was rated using a 1-10 scale, where the DS score increased incrementally by 10 %. Data were converted to % DS using the class midpoints for data analysis.

3.3.3. Ascochyta blight resistance QTLs in the LR-18 population

SIM was conducted first to find markers with the highest LOD score to be used as co-factors for CIM analysis. Using SIM, two significant QTLs were detected, one on linkage group 1 (QTL1), the other on linkage group 2 (QTL2) explaining 25.8 % and 13.3 % of the phenotypic variation, respectively. Significant threshold levels of LOD scores at P = 0.05 were determined to be 2.8 and 2.9 for QTL1 and QTL2, respectively. CIM analysis confirmed the SIM results, and both QTL1 and QTL2 were detected again. When assigning the two most significant SNP markers from QTL1 and QTL2, LcC12416p463 and LcC03040p469, as co-factors in the CIM analysis of QTL1 and QTL2, respectively, the amount of phenotypic variation explained by QTL1 increased to 28.7, whereas that of QTL2 remained unchanged (Figure 3.4).The additive effects of markers calculated by CIM were 2.7 for QTL1 and -1.8 for QTL2 with reference to CDC Robin.

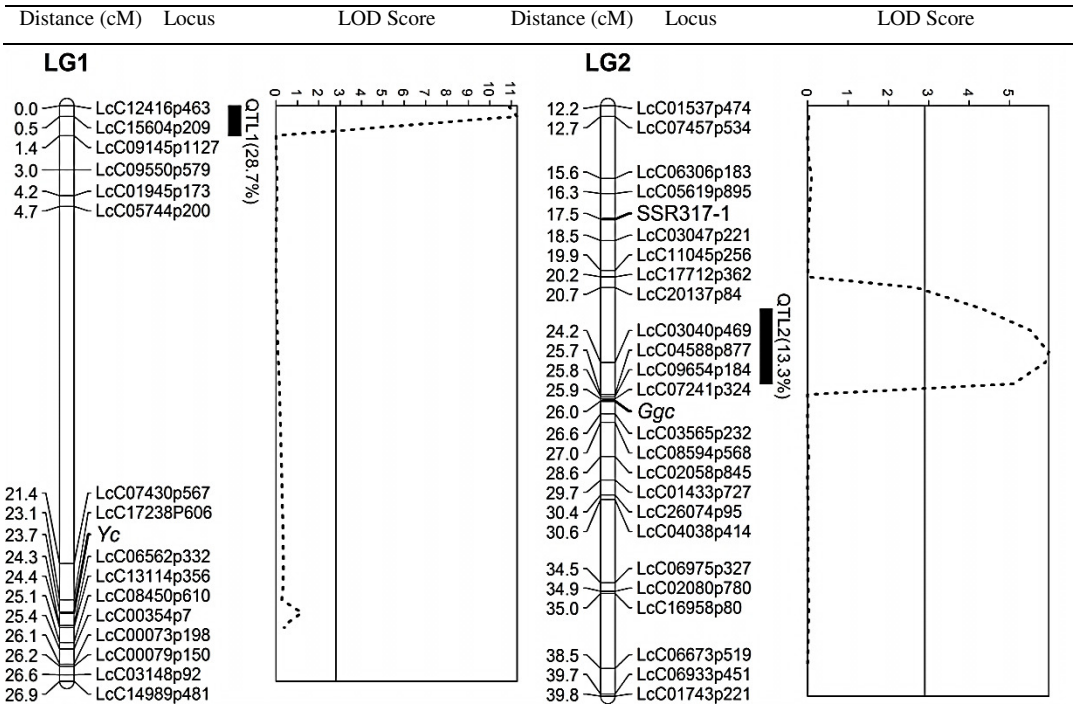


Figure 3.4. Ascochyta blight (AB) resistance QTLs obtained from QTL analysis of LR-18 population, a RIL population developed from cross between lentil AB resistant genotypes CDC Robin and 964a-46. Only LR-18 linkage groups LG1 and LG2 with the location of significant AB resistance QTLs are depicted. Markers on the linkage groups are SNPs except for *Yc* (yellow cotyledon color) and *Ggc* (grey ground color, seed coat) which are phenotypic markers, and SSR317-1. QTLs are highlighted with solid bars with the assigned name and percentage of phenotypic variation explained by each QTL (determined by simple interval mapping) noted on the bars. cM = centimorgan. LOD = logarithm of the odds.

When the allelic states at LcC12416p463 (L1) and LcC03040p469 (L2) were combined, four genotypes were identified among the LR-18 RILs. The genotypic groups included i) L1(a)L2(a), with the CDC Robin allele (a) at both loci ii) L1(b)L2(b) with the 964a-46 allele (b) at both loci, iii) L1(a)L2(b), with the CDC Robin and 964a-46 alleles at QTL1 and QTL2 loci, respectively, and iv) L1(b)L2(a), with the 964a-46 and CDC Robin alleles at QTL1 and QTL2 loci, respectively. The assumption for this test was that heterozygosity at these loci is close to 0, due to multiple generations of self-pollination during the development of the RIL population. Mean comparison tests suggested that only the L1(a)L2(b) genotypic group had significantly higher % DS than all

the other groups (Figure 3.5). This suggested that RILs with L1(a)L2(b) genotype may lack the effect of both QTLs and that QTL1 might be from 964a-46 and QTL2 from CDC Robin.

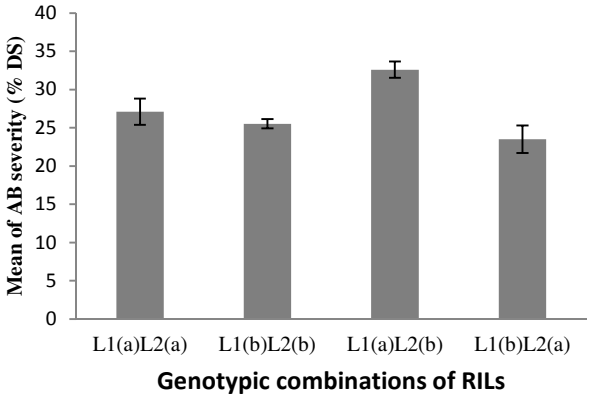


Figure 3.5. Prediction of the parental source of ascochyta blight resistance QTLs detected in QTL analysis of LR-18 population developed from a cross between AB resistant genotypes CDC Robin and 964-46. Error bars indicate ±SE. L1 and L2 represent two highest LOD scores SNP markers from QTL1 (LcC12416p463) and QTL2 (LcC03040p469) loci, respectively. The polymorphism in these loci was shown with (a) and (b), representing allele which belonged to CDC Robin and 964a-46, respectively. The parental source of detected QTLs was predicted as explained in the text. Mean of each genotypic group was the average of mean of % DS used for QTL analysis.

3.4. Discussion

This study was conducted to infer for the first time the allelic relationship of AB resistance genes in four genotypes that have been frequently used for the development of AB resistance in lentil breeding programs in the last 20 years. Transgressive segregation was observed for all RIL populations, suggesting that resistance genes may differ in all resistant parents tested in this study. The frequency distributions of % DS observed for RIL populations were monomodal except for LR-18 and LR-03 which had bimodal distribution. QTL analysis of the LR-18 population suggested that each of the parents, CDC Robin and 964a-46 had a major AB resistance QTL and confirmed the differences between resistant genes in the parents.

Transfer of immunity to AB from *L. ervoides* accession L-01-827A to LR-59 interspecific hybrid RILs was confirmed. The distribution of RILs for the LR-59 population was fit to a two-complementary-Mendelian gene model. The nature of AB resistance in L-01-827A was characterized for the first time in this study. There is a possibility that the resistance genes from

L-01-827A are mediating non-host resistance to AB which usually provide durable resistance. Future research on the molecular mechanisms of resistance in L-01-827A can answer this hypothesis. If this is the case, the introduction of resistance genes from L-01-827A would by itself provide durable resistance and there would be no need for gene pyramiding.

All experiments were conducted with isolate AL57, a local isolate collected from Saskatchewan, Canada, that was selected based on consistently high % DS on a range of lentil lines in a previous *A. lentis* population study (Banniza and Vandenberg, 2006). Testing of lines currently in use in the CDC lentil breeding program in the parental test showed that the susceptible and resistant checks, Eston and CDC Robin, could be differentiated based on reactions to AL57, confirming that it is an appropriate *A. lentis* isolate for resistance screening. Consistent reactions of these check lines to AB infection in different tests also confirmed the reliability of the inoculation procedure for pathogenicity testing.

The results of the parental tests showed a continuum of % DS rather than distinct groups of resistant and susceptible reactions of genotypes. ILL 7537, L-01-827A and the resistant check CDC Robin had % DS scores less than 20 %. The relatively high levels of resistance to AB in ILL 7537 appeared to be effective against a few *A. lentis* isolates, as Nguyen et al. (2001) also observed high levels of resistance using Australian isolates. In contrast, the resistance of 964a-46 was reported to vary depending on the fungal isolate used for the pathogenicity test (Tar'an et al., 2003). It seems that *A. lentis* AL57 was similar to the A1 isolate used by Tar'an et al. (2003) to which 964a-46 had shown moderate levels of resistance. Similarly, Nguyen et al. (2001) suggested that the resistance of ILL 5588, from which the resistance in 964a-46 is derived, is isolate-specific. There is no previous evidence for resistance to AB in ILL 1704 in the literature except for one study conducted by Tullu et al. (2010) in which ILL 1704 was used as a check. Moderate levels of resistance to AB observed here in this line agree with results of Tullu et al. (2010). LR-59-81, a RIL derived from the interspecific cross Eston × L-01-827A also showed moderate levels of resistance. This line had previously shown high resistance to lentil anthracnose pathogen race 0 (Fiala et al., 2009; Vail et al., 2012), the reason why it was selected for screening in this study.

The allelic relationship among resistance genes in the resistant parents was tested by segregation analysis using RIL populations developed from all crosses between resistant parents, and all combinations showed transgressive segregation. The greatest number of transgressive segregates was recorded for the LR-02 and LR-20. For the LR-02, this might be due to additive effects of

moderate resistance genes in both ILL 1704 and 964a-46 which gave rise to highly resistant lines in this population. In contrast, combination of resistance genes from ILL 7537 and CDC Robin gave rise to the occurrence of a high number of RILs with higher % DS than the parents suggesting the a possible effect of the genetic background on the performance of AB resistance genes (Quenouille et al., 2013).

In two (LR-03 and LR-18) of three RIL populations derived from crosses between highly and moderately resistant parents, progeny with % DS between, but significantly different from, both parents (mid-parent reaction) were detected. The occurrence of RILs with mid-parent reactions could indicate the presence of either minor genes with quantitative effects, epistasis effects of major genes or environmental effect, elucidation of which warrants research. Allelism tests were used by Ye et al. (2001) to study the allelic relationship of AB resistance genes in lentil genotypes ILL 5588, ILL 5684, Indianhead and Laird. For the first pair (ILL 5588 and ILL 5684), the resistance genes proved to be allelic, but there were different resistance genes in Indianhead and Laird.

Efforts to fit the distribution of phenotypic scores of RIL populations into Mendelian models failed in this study as none of the obtained ratios would fit to a Mendelian one and two gene model ratio. The segregation of minor genes in the crosses from two resistant parents might change the proportions of susceptible lines expected in the RIL populations. Alternatively, there might be more than one resistance gene in one or both parents which could obscure the prediction of parent genotypes in RIL populations from R × R crosses.

Previous results suggested that genes with major effects conditioned resistance to AB in lentil (Ford et al., 1999; Nguyen et al., 2001; Ye et al., 2001; 2002). Dominant, recessive and complementary dominant resistance genes were reported for different genotypes of lentil (Andrahennadi, 1994; Ahmad et al., 1997; Ford et al., 1999; Chowdhury et al., 2001; Nguyen et al., 2001; Ye et al., 2001). However, polygenic models have also emerged in more recent genetic studies, with the help of QTL analysis (Rubeena et al., 2003; Gupta et al., 2012). Nevertheless, most of the AB resistance QTLs detected so far had major effects, explaining simple inheritance and the easy transfer of AB resistance genes as reported in the lentil breeding programs (Albert Vandenberg, pers. com.). Ye et al. (2002) suggested the existence of minor AB resistance genes in lentil which could not be captured using their phenotyping systems. The occurrence of a continuum of disease severity in most RIL populations may also be due to the existence of both

major and minor genes in the resistant parents. Conclusive determination of the cause of the continuum of disease severity in the lentil germplasm, despite the frequent reports of major AB resistance genes, requires further genetic dissection of populations from resistant and susceptible crosses.

The QTL analysis of the LR-18 population showed that parents were different in two major AB resistance QTLs. The five most susceptible RILs in the phenotypic data used for segregation analysis had an L1(a)L2(b) genotype, confirming the correlation between two types of ratings (pot vs. single plants). The two QTLs detected in this study explained 42 % of the total phenotypic variation. The unexplained amount of phenotypic variation could be caused by unidentified loci due to incomplete genome coverage or weakness of phenotyping, either due to small population size and/or environmental effects (Gupta et al., 2012). QTL analysis with phenotypic data from multiple locations and years may clarify environmental effects and will allow an estimate of heritability of AB resistance. Previous results suggested that there are three QTLs for seedling resistance to AB in ILL 7537 (Rubeena et al., 2006) and three QTLs in ILL 5588 (Gupta et al., 2012), the donor of AB resistance to 964a-46, accounting for 50 and 34 % of phenotypic variation. Detection of one QTL from each LR-18 parent indicates that they are different at two QTLs, but does not exclude the presence of further QTLs common to both parents. Rubeena et al. (2006) used two mapping populations, one from ILL 7537 (resistant) × ILL 6002 (susceptible) and the other from ILL 7537 × ILL 5588 (moderately resistant). They concluded that the contribution of moderate levels of resistance from ILL 5588 in the latter population decreased the sensitivity of the QTL analysis for detection of major QTLs contributed by the resistant parent ILL 7537. This might also be the reason for the detection of lower numbers of QTLs in the present study compared to previous studies. QTL analysis of populations from each of the resistant parents with a common susceptible parent like Eston may better estimate the number of QTLs in the resistant parents.

Tara'an et al. (2003) used the same population (LR-18) and RAPD markers linked to a dominant resistance gene in 964a-46 and a recessive resistance gene in CDC Robin to assess the effectiveness of these markers for gene pyramiding. It seems that two QTLs found in this study are associated with the resistance genes *AbR1* and *ral2* previously identified in the parents and the RAPD markers might map with QTLs found in the current study. To test this, efforts were made to tag the RAPD markers used by Tara'an et al. (2003) to the LR-18 map. These RAPD markers

did not show polymorphism and hence could not be successfully mapped. Solving this discrepancy requires additional study in future.

The partial mRNA sequence of LcC12416p463 (marker with highest LOD score in QTL1) showed high homology to *M. truncatula* receptor like protein-like kinases gene (accession no. MTR_5g096530). Receptor like kinases form a family of more than 600 receptor proteins in *A. thaliana*, which have roles in responses to environmental stimuli such as pathogen attack (Morris and Walker, 2003). In addition, these receptors can be the product of resistance genes such as *Xa21*, which mediates resistance to *Xanthomonas oryzae* pv. *oryzae* (Wang et al., 1996). A BLASTN search showed that LcC03040p469 (marker with highest LOD in QTL2) showed homology to an ethylene-responsive transcription factors gene of *M. truncatula* (gene bank accession no. MTR_2g014300), which also have potentially important roles in plant defense. Co-localization of LR-18 QTL1 and QTL2 with genes with potential roles in plant defense might suggest the involvement of these genes in resistance to AB. However, introducing these as candidate resistance genes requires further studies, because SNP markers were also identified from ESTs from mock-inoculated plants, and the population size is not large enough for reliable physical mapping of these genes.

The region of LG1 next to QTL1 (4.7 to 21.4 cM) had no marker coverage, possibly because markers used to develop the LR-18 map were developed from expressed genes. Integration of markers from non-transcribed regions of the genome would increase the resolution of this map. QTL2 was tightly linked to the *Ggc* in coupling phase. *Ggc* encodes gray seed colour in lentil. This supports the previously observed linkage of AB resistance with gray seed colour in the germplasms used in the lentil breeding program at CDC (A. Vandenberg, pers. comm.)

L. ervoides L-01-827A showed complete resistance to *A. lentis* AL57. Erskine and Bayaa (1991) and Tullu et al. (2010) also found that some wild lentil accessions of *L. ervoides* and *L. nigricans* had high levels of resistance as no macroscopic symptoms appeared on the plants. The rationale for using the LR-59 population in this study was based on the hypothesis that L-01-827A had resistance genes different from those in cultivated lentil. This could be true if we assume that there is no complete resistance to AB in *L. culinaris.* Some RILs of LR-59 were found with immunity to AB, confirming the successful transfer of AB resistance from L-01-827A to hybrid RILs. Marker-assisted backcrossing could be used in future to accelerate the transfer of this complete resistance gene to the lentil varieties once reliable markers are identified. The frequency

distribution data for LR-59 was similar to the patterns observed for a two complementary gene phenotypic distribution. Ahmad et al. (1997) also found complementary AB resistance gene in other accessions of *L. ervoides* and *L. odemensis*. QTL analysis could unlock the complexity in the nature of resistance to both AB and anthracnose in L-01-827A.

Erosion of AB resistance and increases in the aggressiveness of pathogen population over time have already been reported (Pedersen and Morrall, 1994; Banniza and Vandenberg, 2006). The erosion of resistance may be related to the major resistance genes, possibly specific receptor genes, forcing a directional selection pressure on the pathogen. Pyramiding of resistance genes could be a strategy for decreasing the risk involved in resistance break-down. Accumulating evidence supports the hypothesis that different genes have different mechanisms of resistance, suggesting that it may be possible to develop more durable resistance through pyramiding of genes with different mechanisms of resistance. The possibility of different resistance mechanisms in genotypes with different resistance genes has been revealed in the wheat - fusarium head blight pathosystem in which different resistance genes were shown to regulate local and systemic resistance, respectively (Foroud et al., 2012). It would be more complicated, hence less likely, for a pathogen to break down different resistance mechanisms (Crute, 1998).

QTL analysis of populations developed from the crosses between resistant and susceptible parents and finally integrating the results with current data could simplify the prediction of genetic factors in the resistant parents. Allelism tests only allow determining whether resistance genes are different but provide no information on resistance mechanisms. Future studies on the mechanisms of resistance to AB in lentil using molecular tools might shed light on the extent of differences among these resistance genes. Recently, a genomic study was initiated to unravel the host-pathogen interaction of lentil based on the hypothesis that a complex toxin model is involved in the reaction of lentil to *A. lentis* (Lichtenzveig et al., 2012). In this hypothesis, the continuum of reactions to *A. lentis* in lentil germplasm is attributed to the specific interaction of numerous toxins produced by the pathogen with their hypothetical corresponding susceptibility receptors in the plant. The authors suggested that *A. lentis* toxin effectors are homologous to those of *Stagonospora nodorum* (E. Mull.) Hedjar. Friesen et al. (2007) suggested that genotype reactions to *S. nodorum* in wheat are determined by SnTox1 and SnToxA toxins. Similar to results here, pathogenicity tests of *S. nodorum* in a segregating wheat population also revealed a continuum of disease severity scores, despite the fact that only two genes were interacting with SnTox1 and SnToxA toxins.

Although resistance to most of HSNs like *S. nodorum* is mediated by recessive genes (lack of a dominant receptor), dominant resistance genes have also been reported e,g. *HM* which mediates resistance to the maize pathogen *Cochliobolus carbonum* is a dominant gene that encodes an enzyme involved in detoxification of HC-toxin (Meeley and Walton, 1991). Similarly both dominant and recessive resistance genes have been reported for AB resistance in lentil (Tay and Slinkard, 1989; Andrahennadi, 1994; Ahmad et al., 1997; Andrahennadi, 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001). A final conclusion about the *A. lentis*-lentil interaction model can only be drawn after supplementary tests for the corresponding plant resistance mechanisms have been conducted.

In conclusion, evidence from the allelism tests indicated that genes controlling AB resistance in lentil genotypes CDC Robin, ILL 7537, 964a-46 and ILL 1704 were non-allelic. The immunity to AB observed in *L. ervoides* L-01-827A was shown to be successfully transferred to hybrid lines. Evidence from QTL analysis suggested that CDC Robin and 964a-46 carry two distinct AB resistance QTLs. SNP markers tightly linked to AB resistance genes can be used for marker-assisted gene pyramiding in future lentil breeding programs. Further fundamental research on the plant-pathogen interactions using advanced genomic tools will help decipher details of genotype-dependent interaction of lentil with *A. lentis*.

Prologue to Chapter 4

Results of allelism tests suggested that genes controlling AB resistance are different among the tested genotypes. However, it is not known how these differences may manifest themselves at the cellular and molecular levels through the intricate plant defense machinery. To investigate further, quantitative microscopy was conducted using all the tested genotypes in Chapter 3, to develop a more accurate understanding of the quantitative differences among the parents observed in the parental tests. This was followed by an analysis of some of the cellular and molecular responses of CDC Robin, 964a-46 (the parents of the LR-18 population) and the susceptible genotype Eston to *A. lentis* infection. Cellular responses of these lentil genotypes to infection were described using light and confocal laser scanning microscopy. The involvement of the main defense signaling pathways, the SA and JA, and the putative differences among the genotypes in invoking these pathways upon infection were studied by measuring gene expressions of pathogenesis related (PR) proteins exclusively induced by these pathways, PR-1, PR-5 and PR-4, and allene oxide cyclase (AOC), an enzyme involved in the biosynthesis of JA. The extent, to which these genotypes differed in the type of resistance responses, is discussed in Chapter 4.

CHAPTER 4

Genotype-dependent responses of lentil lines to *Ascochyta lentis* infection

4.1. Introduction and objectives

AB of lentil caused by *Ascochyta lentis* Vassilievsky (teleomorph: *Didymella lentis* W.J. Kaiser, B.C. Wang, and J.D. Rogers) is prevalent throughout many temperate lentil (*Lens culinaris* Medik) production regions of the world. It has been reported that AB can cause yield losses of up to 70 %, 30-50 % and 50 % in Canada, the USA and Australia, respectively (Kaiser, 1992; Brouwer et al., 1995; Gossen & Morrall, 1983). The environmentally most acceptable and economically profitable method of control is to develop varieties with high levels of durable resistance. Previous research suggested the polygenic nature of lentil resistance to AB (Banniza and Vandenberg, 2006). A few QTLs have been detected in different lentil genotypes, explaining the presence of a continuum of reaction from partial resistance to susceptibility in genotypes (Rubeena et al., 2003; Tullu et al., 2008; Gupta et al., 2012). An investigation into the allelic relationship among AB resistance genes in lentil genotypes frequently used in lentil breeding programs indicated the presence of non-allelic resistance genes (Chapter 3).

Due to the constant exposure to insects and pathogens, plants are armed with a sophisticated immune system which recognizes various types of stimuli and responds accordingly by activating intricate and effective defense pathways (Jones and Dangl, 2006; Howe and Jander, 2008). Conclusive evidence exists for the involvement of the phytohormones SA, JA, ET and ABA as primary signals in fine-tuning the plant immune system (Pieterse et al., 2009; Verhage et al., 2010). The accumulation of individual or blends of phytohormones upon pathogen challenge can generally be linked to the infection strategy of pathogens. The activation of these "signal signatures" causes the downstream activation of distinct groups of defense genes (De Vos et al., 2005). By balancing the biosynthesis of these signaling compounds through an intricate network of cross-talk, plants are able to spatially and temporarily adjust their defense responses (Pieterse et al., 2009). However, compatible pathogens can harness these pathways to their own benefit by secreting effectors which directly or indirectly antagonize the host immune responses (Pieterse and Dicke, 2007; Grant and Jones, 2009). Recent evidence suggests that some necrotrophs even hijack

resistance mechanisms that are effective against biotrophs to induce cell-death and promote host cell colonization (Hammond-Kosack and Rudd, 2008).

Defense signal transduction pathways are classically categorized into three main groups. One is SA-dependent and induces resistance against biotrophic pathogens, and is also activated upon invasion by hemibiotrophs, whereas the other is SA-independent and involves the JA and ET signaling pathways acting primarily against necrotrophs and hemibiotrophs (Kunkel and Brooks, 2002). These two main groups of signaling pathways act antagonistically in the initiation of an appropriate defense response to various invaders (Glazebrook, 2005). The defense responses induced by the ABA signaling pathway are more complicated, and both, augmented resistance and susceptibility to pathogens have been reported in ABA-defective mutants. In fact, the ABA pathway triggers multifaceted responses in plants that can vary with the type of plant tissue, and the infection stage and the strategy of pathogens (Ton et al., 2009). A number of PR proteins are expressed upon systemic and local accumulation of SA. For some PR proteins, direct antimicrobial activity against biotrophic pathogens has been identified (Spoel and Dong, 2012). Accumulation of JA activates a set of PR proteins different from those induced by SA (e.g. Penninckx et al., 1996; Lorenzo et al., 2003). Loss of function mutants in the JA pathways have shown enhanced susceptibility to necrotrophic pathogens (Thomma et al., 1998).

PR proteins have been frequently used to monitor the activation of the SA and JA/ET signaling pathways in plant-microbe interaction studies. This is feasible because of clear separation of PR protein groups induced by the SA and JA/ET pathways. Previous results suggested the requirement of the SA signaling for induction of *PR-1*, *PR-2* and *PR-5*, and the JA for *PDF1.2*, *HEL*, *CHI-B*, *PR-3* and *PR-4* in various plant species (Thomma et al., 1998). *PR-1* has been widely accepted as a hallmark of the SA signaling in *A. thaliana* (Rogers and Ausubel, 1997) and other crop plants such as tomato (*Solanum lycopersicum* L.) (Niderman et al., 1995; Tornero et al., 1997). Evidence exists for anti-microbial activity of PR-1 proteins. For example, PR-1a increased tobacco (*Nicotiana tabacum* L.) resistance to the pathogens *Phytophthora parasitica* Dastur and *Peronospora hyoscyami* f.sp. *tabacina* (Adam) Skalicky (Alexander et al., 1993). The function of PR-1 proteins in the SA-mediated signaling pathway is unknown (Tornero et al., 1997). Members of the PR-2 (β-1, 3-glucanases) and PR-3 (chitinases) protein groups directly degrade the cell wall of fungal pathogens through hydrolytic activity (Mauch et al., 1988). Proteins of the PR-5 family

are homologous to thaumatin and osmotin-like proteins and show destructive effects on the permeability of fungal plasma membranes (Abad et al., 1996).

The only PR proteins studied in lentil are those of the PR-4 group. Transcriptome analysis of lentil genotypes resistant to AB suggested up-regulation of *PR-4a* after pathogen challenge in a resistant but not in a susceptible genotype (Mustafa et al., 2009). The antifungal activity of PR-4 proteins has been described in other plant-pathogen systems (Caruso et al., 2001). Some members of the PR-4 group inhibit fungal growth by their chitinase activity and possess a chitin-binding domain, while others possess ribonuclease and DNAse activities (Lu et al., 2012). Vaghefi et al. (2013) demonstrated the antifungal activity *in vitro* of a recombinant lentil PR-4a protein (LcPR4a) on *A. lentis.*

Allene oxide cyclase (AOC) is a key enzyme in the JA pathway, involved in JA biosynthesis from α-linolenic acid (Vick and Zimmerman, 1983). The *AOC* gene has been cloned from *A. thaliana* (Stenzel et al., 2003), *Lycopersicon esculantum* Mill (Ziegler et al., 2000) and *M. truncatula* Gaertn and is of primary importance in the JA signaling for legume mycorrhization (Isayenkov et al., 2005). *AOC* expression can be used as a marker for monitoring the JA signaling pathway (Leon-Reyes et al., 2010).

Microscopic examination of cellular reactions to a plant pathogen is frequently used in the study of plant-fungal interactions (Hood and Shew, 1996). Success of microscopic studies depends on the application of staining techniques that differentiate plant and pathogen tissues, and the detection of cytological cascades of events happening after infection. Understanding the timing of stages in the development of a pathogenic relationship is a prerequisite for the determination of sampling intervals required for gene expression analysis in plant-pathogen interactions. Microscopic studies can help to determine the appropriate number of sampling times required for gene expression analysis. Little is known about the histology of lentil infected by *A. lentis*. The initial infection process of *A. lentis* was studied by Roundhill et al. (1995), who showed that colonization of epidermal cells by *A. lentis* happened after the disruption of the cytoplasm, suggesting that *A. lentis* is either a necrotroph or hemibiotroph with a short biotrophic phase.

Necrotrophic plant pathogens are mainly dependent on cell-death prior to infection. The mechanisms of cell death induction and mode of virulence vary among necrotrophs, and defense responses vary among plant species (Mengiste, 2012). Cell death promotes colonization of plants by necrotrophs like *Botrytis cinerea* Pers.: Fr (Govrin and Levine, 2000). Enhanced cell death

mutants showed comparably higher susceptibility to necrotrophs but lower susceptibility to biotrophs (Veronese et al., 2004).

Allelism tests conducted on four lentil genotypes, assumed to have different resistance genes, supported the hypothesis that the genotypes represent different sources of AB resistance (Chapter 3). Little is known about how these differences are reflected at the cellular and molecular level of host defense responses. The present study was conducted to i) examine whether lentil genotypes carrying different resistance genes differ in activating the SA and JA signal transduction pathways by monitoring the expression of key genes involved in the hormonal signaling pathways, and ii) determine cellular reactions of these genotypes at the microscopic level to *A. lentis* infection.

4.2. Materials and methods

4.2.1. Plant materials

The lentil cultivars and genotypes CDC Robin, 964a-46, ILL 1704 and ILL 7537, used frequently in various lentil breeding programs for improving resistance to AB, were chosen for this study. *L. ervoides* L-01-827A was a single plant selection from accession IG 72847 in the ICARDA germplasm collection (Fiala et al., 2009), and is highly resistant to AB (Chapter 3). Lentil cv. Eston was also included in this study as a susceptible line. CDC Robin is resistant to both AB and race 1 of *C. lentis* causing anthracnose, another economically important disease of lentil. AB resistance in the breeding line 964a-46 is derived from ILL 5588 which is the source of resistance for cultivar Northfield (Ali, 1995). ILL 7537 and ILL 1704 are landraces from Jordan and Ethiopia, respectively, with resistance to AB reported in previous studies (Rubeena et al., 2003; Tullu et al., 2010). ILL 7537 carries an AB resistance gene different from ILL 5588 (Nguyen et al., 2001). CDC Robin, 964a-46 and Eston were developed at the Crop Development Centre (CDC), University of Saskatchewan, Canada. Allelism tests using RILs developed from R × R crosses among these lines suggested that non-allelic resistance genes condition resistance to AB in ILL 7537, CDC Robin, 964a-46 and ILL 1704 (Chapter 3).

4.2.2. Fungal isolate and inoculation procedure

A. lentis isolate AL57 is an aggressive isolate from Landis, Saskatchewan (Banniza and Vandenberg, 2006). A conidial suspension prepared from a monoconidial culture of this isolate was stored in a cryopreservation solution containing 10 % skim milk and 20 % glycerol at -80 °C. Inoculum was prepared by revitalizing conidia on 50 % oatmeal agar plates (30 g oatmeal [Quick Oats, Quaker Oats Co., Chicago, IL, USA], 8.8 g agar [Difco, BD®, Sparks Glencoe, MD, USA],

1 L H_2O) and incubating them for 7 days at room temperature. Plates were then flooded with sterile deionized water and conidia were harvested by scraping the colonies with the edge of a glass microscope slide. The suspension was collected and filtered through one layer of Miracloth into a clean Erlenmeyer flask. The concentration of the conidia suspension was adjusted to 5×10^5 conidia mL^{-1} using a hemocytometer.

Four seeds of each genotype were sown in 10 cm square plastic pots containing a soilless mixture of Sunshine Mix No. 4 (Sun Grow Horticulture® Ltd., Vancouver, BC, Canada) and Perlite™ (3/1 V/V). Seedlings with 10 to 15 expanded leaves (21 days after sowing) were inoculated with the conidia suspension at a rate of 2 mL per seedling using an airbrush, and were incubated in a humidity chamber for 48 h. Plants then were incubated on a mist bench where they were misted for 30 s every 90 min during the day for the remainder of the test.

4.2.3. Microscopy of cellular reaction of lentil genotypes to *A. lentis* infection

4.2.3.1. Quantitative measurements by epifluorescent microscopy

Quantitative microscopy was conducted to investigate how defense mechanisms counteracted the growth and development of *A. lentis* on the different lentil genotypes. The experiment was conducted as a randomized complete block design with three replicates. All inoculated leaflets, pooled from four lentil seedlings per genotype grown in one pot and representing one biological replicate were collected at 10, 12, 24, 30 and 48 h post inoculation (hpi). Fungal structures were stained with Uvitex-2b (Polyscience Inc., Warrington, USA) following the protocol of Moldenhauer et al. (2006). The procedure involved clearing tissue in ethanol-chloroform (3:1, v/v) containing 0·15 % (w/v) trichloroacetic acid for at least 18 h followed by washing leaflets in 50 % ethanol. Leaflets were then soaked in 0.1 M Tris-HCL buffer (pH = 5.8) for 30 min and stained in 0.1 % (w/v) Uvitex-2b in 0.1 M Tris-HCL buffer (pH = 5.8) for 5 min. Samples were then de-stained by washing four times for 10 min in water. Specimens were mounted in 50 % glycerol for slide preparation.

Three leaflets were randomly selected from the pool of leaflet specimens of each biological replicate and were subjected to quantitative measurements. Percentage of conidial germination (PCG) was determined for leaflet samples collected at 10 and 12 hpi by examining 100 conidia per three fields of vision. Conidia were considered germinated when they produced germ tubes equal to, or longer than, the conidial diameter. For germ tube length (GTL) determination, leaflets collected at 24, 30, 48 hpi were examined in 10 fields of vision (each containing more than 10

conidia) and images were recorded for each field of vision using an AxioCamICc1 digital camera installed on a Zeiss Axioplan fluorescent microscope (Carl Zeiss, Göttingen, Germany). The GTL was determined using the curve spline tool of the Axiovision 4.7 digital image processing software. All quantitative data were collected using the Zeiss Axioplan fluorescent microscope with BP excitation/emission cubes (546/FT580/LP590).

4.2.3.2. Description of epidermal cell response to *A. lentis* infection using confocal laser scanning microscopy (CLSM)

To determine the underlying cellular defense reactions and differences between resistant genotypes in cellular reaction to *A. lentis* infection, descriptive microscopy was conducted for CDC Robin and 964a-46, with Eston as a susceptible check. Ten infected leaflet samples randomly selected from the pool of leaflets collected from single plants of each genotype at 60 and 90 hpi were discolored and stained with Uvitex-2b following the protocol described above. The reaction of epidermal cells to pathogen penetration was studied using a two photon Carl Zeiss confocal laser scanning microscope as described by Moldenhauer et al. (2006). The specimens were excited with UV-laser beams at 351 and 364 nm, then scanned with filter settings at 400–500 nm for Uvitex 2b-stained fungal structures, and with argon-laser beams at 514 and 543 nm, and then scanned with filter settings at 560–680 nm for epidermal cells responses. Observations of pathogen and plant tissues located in different fields of vision were conducted by collecting images in a number of Z stacks with 0.5 µm intervals. The Z stacks were then compiled to a single micrograph using the Z projection tool in Image J 1.7 p (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012).

4.2.3.3. Test of cell viability by light microscopy

The viability of epidermal cells of CDC Robin, 964a-46 and Eston was investigated following the method of O'Connell et al. (1991) with the following modifications. Randomly selected samples of 10 infected leaflets collected at 48 and 72 hpi were subjected to viability staining as follows: Leaflets were cut in half, then vacuum-infiltrated in 0.85 M KNO_3 containing 0.01 % Neutral Red (Sigma-Aldrich, St. Louis, USA) for 5 h. Specimens were mounted in infiltration solution and fungal structures were stained with a drop of 0.1 % Aniline Blue solution (BDH Prolabo, U.K.) in lactic acid. Specimens were examined under a Zeiss light microscope (Carl Zeiss, Göttingen, Germany) and images were recorded using an AxioCamICc1 digital camera.

4.2.4. Analysis of the SA and JA signal transduction pathways by quantitative real-time PCR

The temporal pattern of the SA and JA signaling pathways upon *A. lentis* infection was indirectly assessed by expression analysis of *PR-1a* and *PR-5* as hallmarks of the SA pathway (Bhadauria, pers. comm.), and *PR-4a* (Vaghefi et al., 2013) and *AOC* (Leon-Reyes et al., 2010) as hallmarks of the JA pathway. Lentil genotypes CDC Robin and 94a-46 were selected for this test, with Eston as the susceptible check. Plants were inoculated in a similar manner to the microscopy test, except that a higher concentration of conidia (10^6 conidia mL^{-1}) was used. The experiment was arranged in a randomized complete block design with three replicates. All inoculated leaflets of seedlings were collected at 6, 12, 18, 24, 36, 48, 60 hpi and flash frozen in liquid nitrogen. Leaflets were also collected from mock-inoculated control plants sprayed only with water and sampled prior to inoculation. Leaflet samples were stored at -80 °C. Leaflets pooled for each biological replicate were ground in an RNAse-free mortar, pre-cooled by liquid nitrogen. Two subsamples were taken from the pool of ground tissue of each biological replicate and subjected to RNA extraction. RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Total RNA was then treated with DNAse I (Invitrogen, Carlsbad, USA) to remove any trace of genomic DNA according to the manufacturer's recommendation. The purity and quantity of RNA was determined using a NanoDrop ND8000 (Thermo Scientific, Wilmington, USA). Samples with an A260/280 ratio less than 2.0 were discarded. RNA integrity was determined by denaturing agarose gel electrophoresis (Barill and Nates, 2012).

Total RNA was used for reverse transcriptase-dependent first strand cDNA synthesis, primed by Oligo dt_{12-18} primer (Invitrogen, Carlsbad, USA) according to Klickstein et al. (2001). In brief, 1 µg total RNA was mixed with 0.5 µg Oligo dt_{12-18} and 5 mM dNTP mix. The mix was spin-collected and heated at 65 °C for 5 min. Transcriptase buffer solution containing 1X first strand buffer, 10 mM DTT and 40 U of RNAse-out was added to the first mix and heated at 40 °C for 2 min. Finally, 200 U of Superscript II® Reverse Transcriptase was added and first strand cDNA was synthesized by incubating at 42 °C for 50 min followed by reaction inactivation at 70 °C for 15 min. The template RNA strand was removed by treating the first strand cDNA-RNA complex with 2 U of RNAse H (Invitrogen, Carlsbad, USA) at 37° C for 20 min followed by the reaction inactivation at 95° C for 10 min.

Residual genomic DNA contamination of total RNA samples was detected by running a PCR using ubiquitous actin primers designed for an exon-exon junction (Table 4.1) and first strand cDNA following the protocol of Vaghefi et al. (2013). Each PCR was conducted in a 20 µl reaction mix containing 4 µl of 1:10 diluted cDNA, 1X PCR buffer, 0.13 µM of each primer, 0.25 mM dNTPs, 3 mM $MgCl_2$ and 1 U Taq polymerase (GenScript, Piscataway, USA). The PCR cycles were 3 min at 95 ºC, followed by 40 cycles of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C, followed by a final extension of 72 °C for 7 min. PCR products were visualized by staining with 1:1000 dilution of GelRed® (Invitrogen, Carlsbad, USA) added to the loading dye, on a 1.4 % agarose gel. Samples with genomic DNA contamination were discarded and cDNA synthesis was repeated after total RNA treatment with a doubled concentration of DNAse I.

Primer sequences were obtained from other researchers except for AOC-69 and Actin-257 (Table 4.1), which were designed based on lentil cv. CDC Redberry transcriptome sequencing data available on Knowpulse (contig No. LcRBContig10217 and LcRBContig09231, http://knowpulse2.usask.ca/portal/). Actin 257 was selected from a group of β-actin primers designed for lentil based on its higher amplification efficiency. Primers were designed using primer blast search tool provided by the National Centre for Biotechnology Information (NCBI), (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

The PCR amplification included 10 µl of Applied Biosystems® Power SYBR® (Applied Biosystems, Warrington, UK), 0.2 µM of each primer and 5 µl of 1:10 diluted cDNA. The cycling program was executed in an ABI StepOnePlus™ Real-Time PCR machine (Applied Biosystems, Foster City, USA) and included 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s followed by a melting curve from 60 °C to 95 °C with 0.3 °C intervals. Each PCR was conducted in duplicate. A PCR was repeated if the standard deviation of CT of two PCR replicates was higher than 0.3. The expression level was reported relative to the mock-inoculated control by calculating fold changes in expression levels following the method suggested by Salzman et al. (2005). Mean fold change induced by infection was equal to:

$2^{(\Delta\Delta CT)}$, with upper and lower SD levels of $2^{(\Delta\Delta CT+s)}$ and $2^{(\Delta\Delta CT-s)}$, respectively, where:

$\Delta\Delta CT = (\text{mean } \Delta CT_{\text{control cDNA}}) - (\text{mean } \Delta CT_{\text{treatment cDNA}})$

$\Delta CT = (CT \text{ cDNA}_{\text{test primer}}) - (CT \text{ cDNA}_{\text{actin primer}})$

$S = \sqrt{(\text{SD of CT}_{\text{test primer}}^2) + (\text{SD of CT}_{\text{actin primer}}^2)}$.

Table 4.1. Names and sequences of gene-specific primer pairs used for quantitative real-time PCR

Primer name	Sequence 5'->3'	Gene bank ACC	Signaling pathway	References
PR-1a	F: AGATCCGAGGTTGGTGTTTC R: CCCACAATTTCACAGCATCT	JG294109	SA	Vijai Bhadauria, CDC, University of Saskatchewan, personal communication
PR-5	F: CACTGTATGGCCAGGAACAC R: TACCAAAGTTGCTGGTGGAA	JG293995	SA	Vijai Bhadauria, CDC, University of Saskatchewan, personal communication
PR-4a	F: ACCTGGGATGCTAACCAGCCTTT R: TTTGCCGCAAGAATCTCTGCCTG	JX273653	JA	Vaghefi et al. (2013)
AOC-69	F: AGAGTAGGCATAACTGCAGGCT R:TGGTACGTCAGATAAGCTCCCTGT	AJ866733[a]	JA	E. Sari, this study[a]
Actin-257	F: CACTGTACTTCCTCTCCGGC R:TATGTTCCCCGGGATTGCTG	EU664318[a]	-	E. Sari, this study[a]
Ubiquitoous Actin	F: GTTCCACAATGTTCCCTGGT R: ATTCTGCCTTTGCAATCCAC	HQ247603[b]	-	Vaghefi et al. (2013)

[a] The *Actin-257* and *AOC* sequences of *M. trancatula* were used to look up the corresponding lentil sequence from ESTs of lentil cv. CDC Redberry using the blast tool available at http://knowpulse.usask.ca/portal/blast/nucleotide/nucleotide. [b] The primer was designed by Mustafa et al. (2009) using the pea actin (ACC HQ247603) and was then successfully used in lentil by Vaghefi et al. (2013). The successful application of this primer in pea and lentil was the reason for calling this primer "ubiquitous". SA= salicylic acid, JA= jasmonic acid.

Amplification efficiency was calculated for each primer pair using cDNA samples serially diluted 1:4 (V/V) five times for a total of six dilutions. Dilutions were used as template for PCR following the protocol described above. A linear equation was fitted to the CT values obtained for various cDNA dilutions. Percentile of amplification efficiency (PAE) was calculated from the slope of the regression line using the equation $PAE = 10^{(-1/slope)} - 1$.

4.2.5. Statistical analysis

All data analyses were performed using Statistical Analysis System (SAS) version 9.3 (SAS Institute Inc., Cary, NC). Homogeneity of variance was tested using Levene's test and in the case of heterogeneity, the variance was modeled with the mixed model procedure. Quantitative microscopy data were subjected to mixed model analysis with block assigned as random and

sampling time-points as repeated measure effects, respectively. No significant difference occurred among data collected from microscopic fields of visions for both, PCG and GTL, therefore the mean of data from all fields of vision per sample was used for statistical analyses. Means of GTL for each genotypes were compared at each individual time point using Fisher's least significant differences (LSD) ($\alpha = 0.05$). The experiment for GTL was conducted twice and repeatability was tested by including the factor repeat, repeat × genotype interaction and block (repeat) in the model.

Quantitative PCR data were subjected to analysis using a mixed model with replicates as random effects and sampling time-points as repeated measurements. The mean CT of two PCR replicates was used for data analysis. Analysis was conducted using the mean ΔCT of RNA samples extracted from two subsamples randomly selected from the pool of ground tissues of each biological replicate. The validity of the reference genes for normalizing the CT of target genes was tested using the Kruskal-Wallis test following the procedure suggested by Schmittgen and Zakrajsek (2000).

4.3. Results

4.3.1. Quantitative microscopy measurements

4.3.1.1. Conidial germination

PCG was determined on leaflets of lentil genotypes to investigate a potential association between germination inhibition and resistance to AB. Results suggested PCG to be a host genotype-independent trait as differences among genotypes were non-significant ($P = 0.47$). The PCG generally increased from 10 to 12 h (Table 4.2). In this study the maximum germination recorded was 93 % on leaflets of *L. ervoides* accession L-01-827A.

4.3.1.2. Germ tube length

To understand the stage at which *A. lentis* growth was inhibited in the resistant genotypes, GTL was measured at three time points spanning the period from penetration to colonization. No significant repeat × genotype effect was observed ($P = 0.1643$) and data of two repeats were pooled for analysis. Analysis of variance showed significant differences among genotypes ($P < 0.001$), sampling time points ($P < 0.001$) and their interaction ($P < 0.001$), suggesting that GTL was a genotype-dependent trait and the reaction of genotypes changed over time. No significant differences were detected among genotypes at 24 hpi (Table 4.3). Significant differences were observed between resistant genotypes and the susceptible check Eston, and among resistant genotypes at both 30 and 48 hpi. GTL for CDC Robin, ILL 7537 and L-01-827A were significantly

lower than those in the susceptible check Eston at 30 and 48 hpi. There was no significant difference between ILL 1704 and the susceptible check Eston at 48 hpi, although it had significantly longer germ tube at 30 hpi. There were no significant differences between 964a-46 and Eston at all the sampling times. At all sampling times, conidia on L-01-827A generated the shortest germ tubes; however GTL on this genotype was significantly lower compared to that on Eston only at 30 and 48 hpi.

Table 4.2. Mean percentage of conidial germination (PCG) of *Ascochyta lentis* on leaflets of *Lens* genotypes at 10 and 12 hours post-inoculation. Means and standard errors (± SE) were generated from three biological replicates using a mixed model analysis. Each biological replicate represents the mean of three microscopy fields of vision. All genotypes are accessions of *Lens culinaris,* except for L-01-827A which is an accession of *L. ervoides.*

Genotypes	Incubation time (h)	
	10	12
	% Conidia germination	
Eston	59.2±6.5	81.4±3.5
CDC Robin	79.2±6.5	73.5±3.5
964a-46	71.4±6.5	81.6±3.5
ILL 7537	60.2±6.5	75.5±3.5
ILL 1704	70.7±11.3	78.2±3.5
L-01-827A	59.9±6.5	93.0±3.5

Table 4.3. Mean length of germ tube (µm) extending from germinated *Ascochyta lentis* conidia measured at 24, 30 and 48 hours post-inoculation on the leaflet surface of lentil genotypes. Data are means of three biological replicates. Data from each biological replicate were obtained from the average of 10 microscopy fields of vision. Fisher's Least Significant Difference (LSD) was determined for each time point data. Means were estimated by the mixed model procedure of SAS.

Genotypes	Incubation time (h)		
	24	30	48
	Germ tube length (µm)		
Eston	54.2	60.3	80.3
CDC Robin	46.2	45.8	53.2
964a-46	47.6	51.2	80.0
ILL 7537	47.4	46.6	46.3
ILL 1704	61.4	79.2	80.4
L-01-827A	38.5	42.1	41.0
LSD (P= 0.05)	20.6	10.5	15.2

4.3.1.3. Cellular reaction of lentil genotypes to infection by *Ascochyta lentis*

Descriptive microscopy was conducted in search of cellular defense responses differentially activated by different lentil genotypes. CDC Robin and 964a-46 were selected for this purpose. The susceptible cultivar Eston was also included for comparison. Composite CLSM micrographs of fungal and plant epidermal cells were obtained for samples collected at 6, 12, 18, 24, 36, 48, 60 and 90 hpi. Reactions were similar for all genotypes up to 48 hpi, whereas at 60 and 90 hpi genotypic reactions were divergent. For Eston, penetration of epidermal cells was accompanied by the emission of fluorescent signals from the entire protoplast of epidermal cells at 60 hpi (Figure 4.1A). The fluorescent signal was strongest right beneath the penetration site, suggesting the reinforcement of the cell-wall and formation of papillae. Formation of thick and dense papillae was also confirmed by capturing the differential interference contrast (DIC) micrograph in the same field of vision (Figure 4.1a). The strength of the fluorescent signal emitted from the papillae was lower near the appressorium, probably due to the destruction of papillae by infection vesicles. The fungus had colonized epidermal cells by 90 hpi, resulting in massive disruptions of cytoplasm (Figure 4.1.D). At this time point, a dense network of mycelium was formed inside the necrotic epidermal cells with only cell walls and the remnants of protoplasts adhering to the cell walls (Figure 4.1.D, d). By contrast, an autoflourescent signal was just detected at the site of penetration attempts in CDC Robin at 60 hpi and the destruction of papillae was not observed in this genotype (Figure 4.1.B, b). In contrast to Eston, the majority of infection attempts failed in CDC Robin and massive colonization was not observed at 90 hpi (Figure 4.1.E, e). These results suggested that reactions of CDC Robin epidermal cells to *A. lentis* did not change from 60 to 90 hpi. Cellular events induced by *A. lentis* infection in 964a-46 were very similar to those of Eston (Figure 4.1. C, c, F, f). The entire cell protoplast responded to infection, and cell-wall reinforcement and papillae were observed. Destruction of papillae by infection vesicles was also detected in this genotype similar to Eston; however, 964a-46 developed thinner papillae than Eston. In addition, the colonization by fungal mycelium at 90 hpi was denser in 964a-46 than Eston.

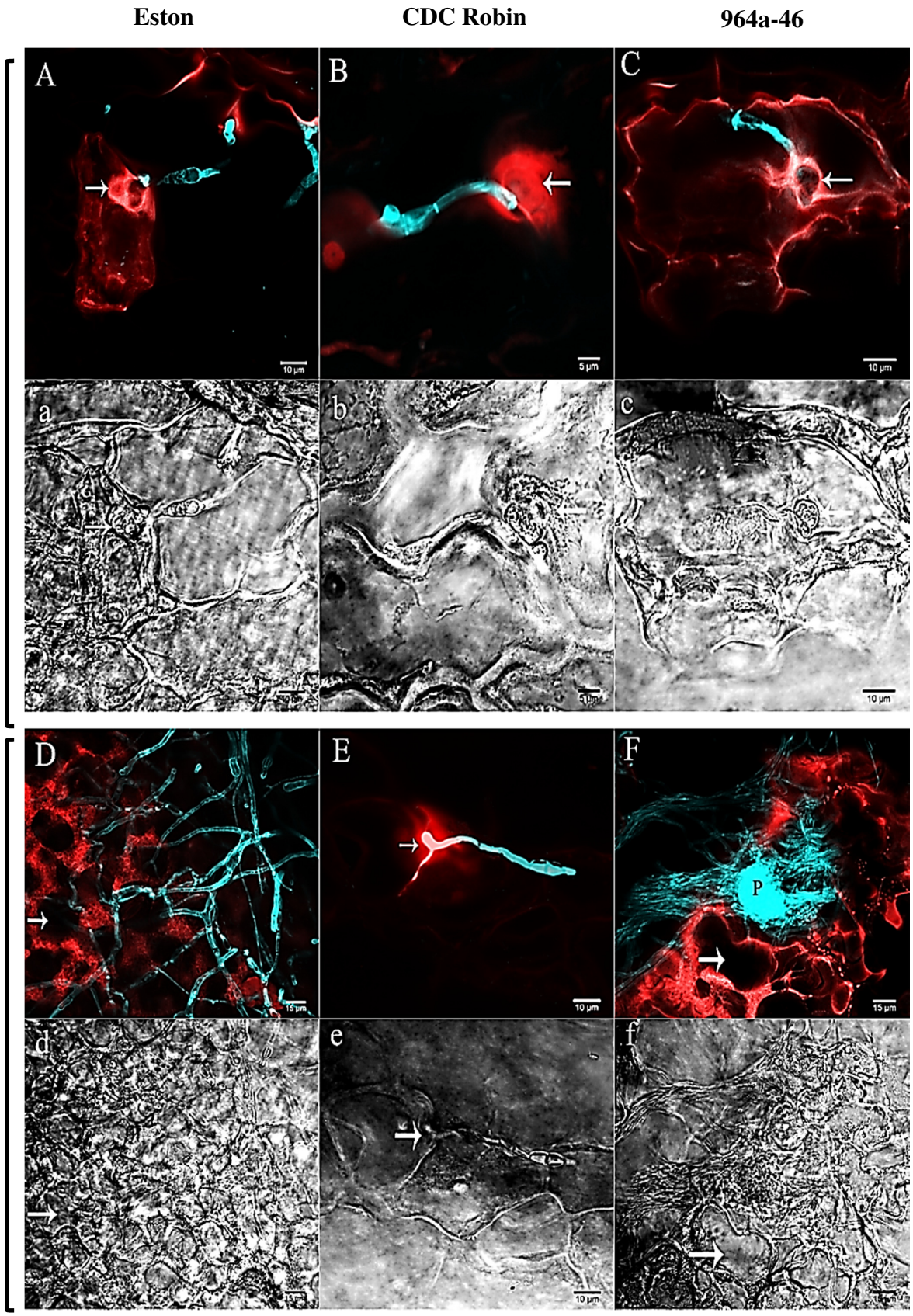
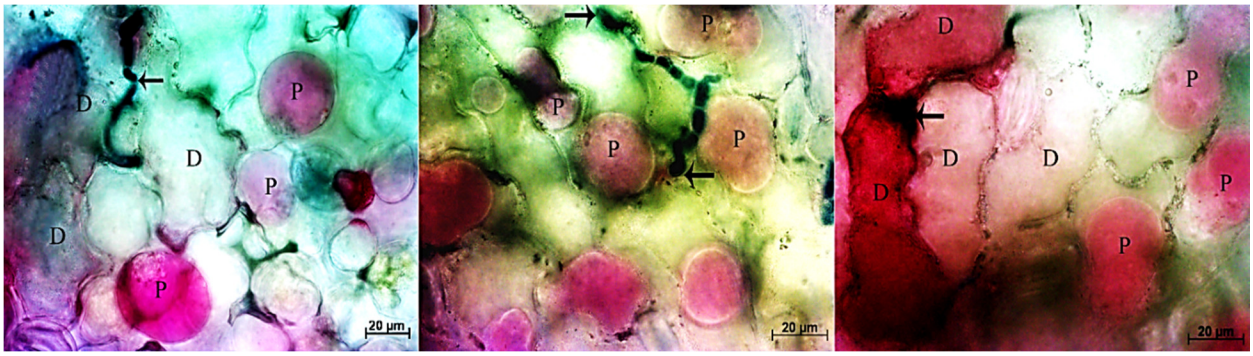

Eston
CDC Robin
964a-46
60 hpi
90 hpi
A
B
C
a
b
c
D
E
F
d
e
f
P
10 µm
5 µm
15 µm

Figure 4.1. Cellular reaction of three lentil genotypes to *Ascochyta lentis* infection captured by two photon confocal laser scanning microscopy (CLSM). Each image was recorded using fluorescence (capital letters) and differential interference contrast filter (small letters). Fungal structures (cyan) were stained with uvitex-2b. Auto-fluorescent signals developed in response to pathogen attack are in red. Arrows in images show the penetration site except for d) and f), where arrows indicate the cavity developed as a result of the destruction of cell contents by the fungus. P in image F shows a newly-developed pycnidium formed on the mass of mycelium. Scale bars are indicated at the bottom right of each image.

4.3.1.4. Viability of cells in response to *Ascochyta lentis* infection

A cell viability test was applied to determine whether the fluorescent signal emitted from epidermal cell protoplasts observed in CLSM micrographs of Eston and 964a-46 was due to cell-death. Results showed that at 72 hpi, cell-death occurred in Eston and 964a-46 when epidermal cells were challenged with *A. lentis* infection, but not in CDC Robin (Figure 4.2). In Eston, most cells attacked by the pathogen lost their viability, whereas cell-death was detected in a few non-infected cells neighboring the infection site in 964a-46. Penetration into epidermal cells was observed in Eston and 964a-46 but not in CDC Robin.



Eston CDC Robin 964a-46

Figure 4.2. Viability of lentil epidermal cells at 72 hours post inoculation with *Ascochyta lentis*. Fungal tissues were stained with aniline blue-lactic acid solution (dark blue). Arrows indicate the penetration site. Viability was postulated when the host protoplast (P) contracted and absorbed red pigments after vacuum infiltration of leaflet tissues in 0.85 M KNO3/0.01 % Neutral Red solution. Dead cells (D) absorbed the red pigments but did not show contracted protoplast. Scale bars are indicated at the bottom right of each image.

4.3.1. Quantitative measurement of *PR-1a*, *PR-5*, *PR-4a* and *AOC* gene expression

To observe differences in molecular components of defense responses activated among lentil genotypes, the transient expression of two contrasting hormonal signaling pathways, SA and JA was indirectly investigated by measuring the expression of *PR-1a* and *PR-5*, and *PR-4* and *AOC* genes as hallmarks of the SA and JA pathways, respectively. All gene-specific primers had amplification efficiencies close to 100 % (data not presented). To confirm the validity of the reference gene (*β-actin*) for normalization, the CT values of mock-inoculated leaflets were compared with those of inoculated samples. No significant difference was observed between inoculated and mock-inoculated samples for all three tested genotypes (Eston: $P = 0.4414$; CDC Robin: $P = 0.4159$; 964-46: $P = 0.1037$, raw CT are presented in Appendix 2).

4.3.2.1. *PR-1a*

Variance analysis showed that genotype ($P = 0.0175$) and hpi ($P < 0.0001$) had significant effects on *PR-1a* expression, with a significant interaction between genotype and hpi ($P < 0.0001$) (Appendix 1). Different patterns of *PR-1a* expression were observed among genotypes (Figure 4.3.A). CDC Robin and Eston were similar in relative *PR-1a* expression over the tested time-points except for 12 hpi, when *PR-1a* expression in Eston was significantly higher than CDC Robin. *PR-1a* expression was exponentially increased at 18 hpi in 964a-46 and the expression of this gene was estimated to be 7084 times higher than in mock-inoculated samples at 24 hpi. The expression then declined and all three genotypes had similar levels of expression at 36 hpi. The expression of *PR-1a* was significantly higher in 964a-46 than CDC Robin and Eston at both 24 hpi and 48 hpi.

4.3.2.2. *PR-5*

Variance analysis suggested that the effect of genotype was not significant ($P = 0.0786$), for *PR-5* expression, however, both hpi ($P < 0.0001$) and the interaction between genotype and hpi ($P < 0.0001$) were very highly significant (Appendix 1). Significant differences in *PR-5* expression were detected for all three genotypes at all time-points except for 6, 12 and 60 hpi (Fig 4.3.B). For 964a-46, *PR-5* expression was not different from other genotypes at 6 and 12 hpi. However, its expression exponentially increased after 12 hpi and reached a 4910 fold-increase compared to the mock-inoculated plants at 24 hpi. Similar *PR-5* expression levels was also observed at 48 hpi in 964a-46 before declining at 60 hpi to an expression level that was equal to those at 6 and 12 hpi.For Eston and CDC Robin, *PR-5* expression remained nearly unchanged. *PR-5* expression increased

at 48 hpi in Eston and CDC Robin, however at a lower magnitude than 964a-46. Nevertheless, Eston had significantly higher expression than CDC Robin at this time point. In a similar way to 964a-46, *PR-5* expression declined to the levels similar to 6 and 12 hpi for both Eston and CDC Robin.

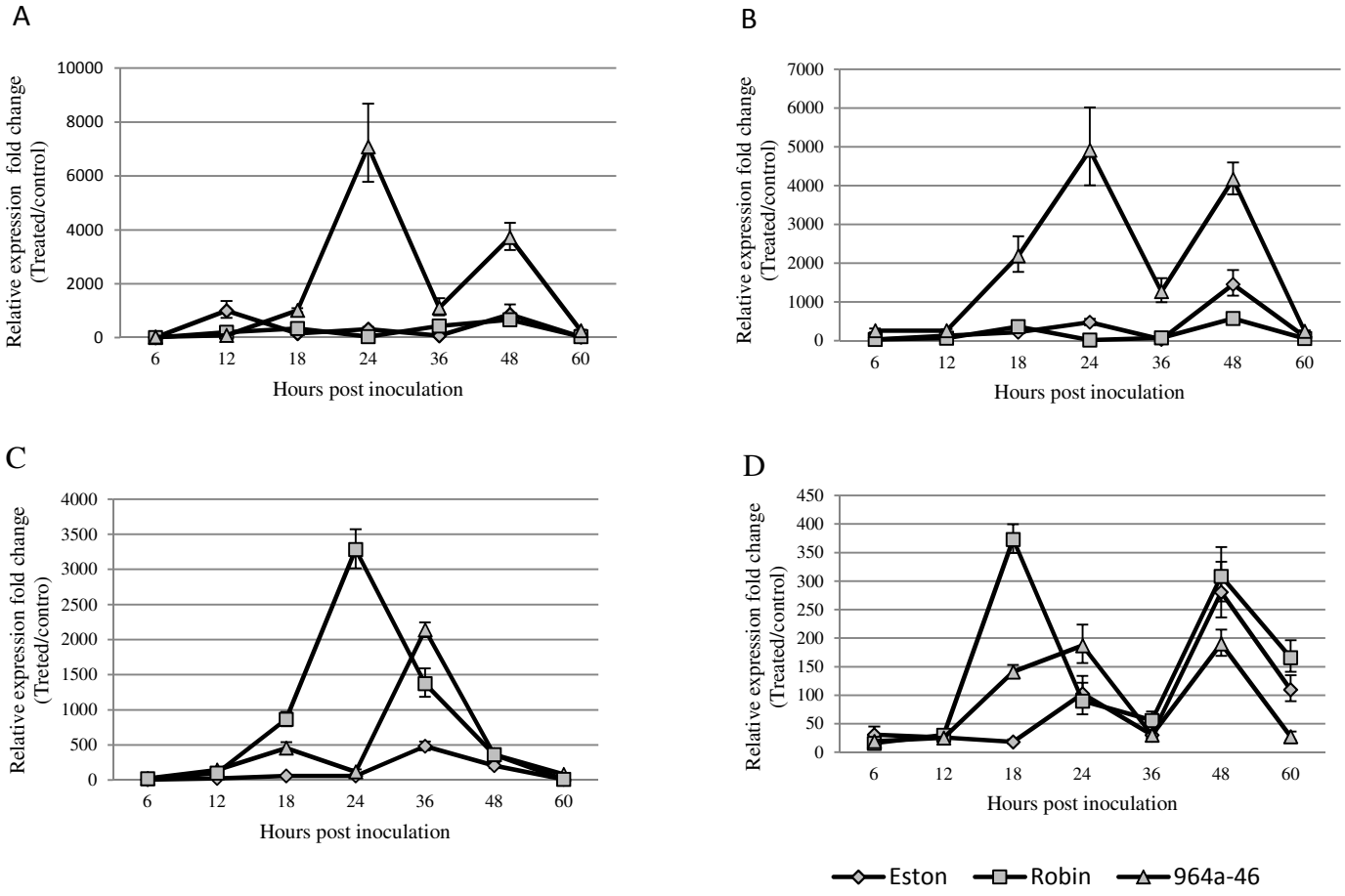


Figure 4.3. Quantitative assessment of *PR-1a* (A), *PR-5* (B), *PR-4a* (C) and *AOC* (D) expression by quantitative real-time PCR after inoculation with *Ascochyta lentis* in lentil genotypes Eston, CDC Robin and 964a-46. Data are means of three replicates. Error bars indicate ±SD. Gene expression was relative to mock-inoculated samples collected just prior to inoculation. Data were normalized using *β-actin* gene expression as a reference gene.

4.3.2.3. *PR-4a*

There was no significant effect of genotypes (P = 0.5530) on *PR-4a* expression, whereas the effects of hpi (P < 0.0001) and the interaction between genotype and hpi (P = 0.0027) was significant (Appendix 1). *PR-4a* expression was almost the same in the genotypes at 6, 12, 48 and 60 hpi (Fig 4.3.C). In Eston, it remained low until 24 hpi and then increased to a peak at 36 hpi, before gradually declining thereafter. For CDC Robin, *PR-4a* expression increased from 12 hpi, reached a peak level at 24 hpi (3281 times higher than mock-inoculated plants) and then declined. There were significant differences between CDC Robin and Eston at all time-points from 12 to 48 hpi. For 964a-46, the expression increased starting at 12 hpi but declined at 24 hpi. At this time-point, Eston and 964a-46 had almost identical levels of *PR-4a* expression. The expression increased again for 964a-46 at 36 hpi and was significantly higher than that for CDC Robin at this time.

4.3.2.4. *AOC*

Variance analysis indicated significant effects of genotypes (P < 0.0001), hpi (P < 0.0001) and the interaction between genotype and hpi (P = 0.0001) on expression levels of *AOC* (Appendix 1). Genotypes were significantly different in the pattern of *AOC* expression from 12 to 36 hpi (Fig 4.3.D). *AOC* expression in Eston remained at the baseline until 18 hpi, while it started increasing at 12 hpi for both CDC Robin and 964a-46. This increase was nearly double in CDC Robin compared to 964a-46 at 18 hpi. *AOC* expression then declined at 24 hpi in CDC Robin, but this decline was not observed for 964a-46 and this genotype had significantly higher *AOC* expression at 24 hpi compared to 18 hpi. Similar patterns of *AOC* expression were observed among genotypes from 36 to 60 hpi.

4.3. Discussion

Cellular and molecular defense responses to *A. lentis* infection were studied in resistant lentil genotypes and compared with a susceptible genotype. Microscopic examination of infected leaflets of these genotypes suggested that cell-death is of relative importance in resistance of lentil to AB. Genotypes showed different patterns in the expression of genes connected with the SA and JA signal transduction pathways. The involvement of both, the SA and JA pathways, in the reaction of lentil to AB was implicated here, however, only the expression of JA-related genes could explain differences among susceptible and resistant genotypes. The involvement of both pathways

in this interaction could indicate conformation with the "tunable dial" theory, suggesting that temporal changes of hormonal balances through a complex cross-talk network is the dominant strategy to adjust plant defense response (Reymond and Farmer, 1998). Based on this theory, plants use these signaling pathways to precisely adjust the expression of defense responses according to the types of aggressors. However, some types of aggressors are capable of exploiting these pathways for their pathogenesis. Only 964a-46 showed the augmented expression of SA-related genes *PR-1a* and *PR-5*. This suggested the possible existence of fundamentally different mechanisms of resistance in CDC Robin and 964a-46, which has not been reported previously.

In this series of studies, efforts were made to integrate quantitative and descriptive microscopic observations to provide evidence for determining the type of cellular resistance mechanisms in AB resistant lentil genotypes. The second objective for the microscopic studies was to provide relevant phenotypic data for a better understanding of molecular data. Quantitative microscopy suggested genotype-independent PCG of *A. lentis*, supported by non-significant difference of genotypic effects in the variance analysis. This was similar to results of previous microscopic studies of *A. rabiei* in chickpea (Höhl et al., 1990). Although it was not the case in this study, inhibition of spore germination has been suggested as a mechanism of resistance e.g. resistance in onion to *C. circinans* (Walker and Stahmann, 1995). After germination, conidia developed a short germination tube which penetrated into the epidermal cells by developing appressoria and penetration pegs. Production of germ-tubes and development of penetration structures was similar on all genotypes from 10 to 24 hpi. The differences among genotypes appeared after penetration, based on measurements of GTL at 24, 30 and 48 hpi. Significant differences in GTL between the susceptible check and CDC Robin, ILL 7537 and L-01-827A were observed at both 30 and 48 hpi, suggesting that germ tube growth was inhibited in these genotypes which are highly resistant (less than 30% of disease severity) to *A. lentis* infection (Chapter 3). These high levels of resistance can be attributed to the inhibition of fungal growth observed at 30 and 48 hpi in these genotypes. In ILL 1704 and 964a-46, germ tubes grew similarly to the susceptible check Eston. In the pathogenicity tests, ILL 1704 and 964a-46 were significantly more resistant than Eston (Chapter 3). This suggests the involvement of a different defense mechanism in these lines which was activated later, after penetration of epidermal cells by the fungus. Similarly, Sillero and Rubiales (2002) found differences in the number of epidermal cells colonized among faba bean *(Vicia faba* L.) genotypes that varied in their level of resistance to the rust pathogen (*Uromyces*

viciae-fabae). They measured quantitative traits from germination to colonization and suggested that the highest differentiation among genotypes occurred after penetration of the stomata by the fungus (Sillero and Rubiales, 2002). Similarly, Kema et al. (1996) found differences in the number of epidermal cells colonized among wheat *(Triticum aestivum* L.) genotypes that varied in their level of resistance to *Mycosphaerella graminicola* (Fückel) Schroeter.

AB resistant genotypes could be categorized into two groups based on GTL measurements. The first group (CDC Robin, ILL 7537 and L-01-827A) were those with shorter germ tubes at 30 and 48 hpi compared to the susceptible check Eston. The second group (ILL 1704 and 964a-46) had similar GTL at 30 and 48 hpi compared to Eston. Genotypes within the two groups had similar GTL. GTL in the second group increased over time, but remained almost unchanged in the first group, suggesting that germ tube development was inhibited for the first group.

Data from descriptive microscopy by CLSM and viability tests supported the observation of a difference in reaction to AB among genotypes. CLSM images revealed obvious differences among genotypes at 60 hpi. Observations starting at 60 hpi suggested that an accumulation of auto-fluorescent compounds at the attempted penetration site occurred in all lentil genotypes. The emission of fluorescent signals from cell protoplasts was only observed in the susceptible Eston and the moderately resistant genotype 964a-46. Complementary viability tests suggested that emission of fluorescent signals from cell-protoplasts was due to cell death and disruption of cell protoplast. This process was also suggested by previous microscopic studies of *A. lentis* on two lentil genotypes with different levels of resistance to AB (Roundhill et al., 1995). Differences between susceptible and resistant genotypes were reported to occur after the penetration stage, when susceptible cells became necrotic followed by growth of the penetration peg into the cell lumen (Roundhill et al., 1995). The penetration peg was surrounded by electron-dense materials and the cells remained viable in resistant genotypes. Findings presented here and those of Roundhill et al. (1995) suggest that cell death might have facilitated the colonization of epidermal cells by the fungus. Additional support for the role of cell death in the pathogenicity of *A. lentis* is provided by the fact that cell death was rarely detected in CDC Robin at 60 and 90 hpi. Roundhill et al. (1995) suggested that differences between resistant and susceptible lentil lines may be discerned by the frequency of infection vesicle surrounding electron-dense materials. Rare cases of cell-death were also observed in CDC Robin which might explain the low disease severity

rating. The inconsistent inhibition of cell death in CDC Robin might be due to interactions of defense response genes and environment.

Similar cascades of cellular events were observed in 964a-46 and Eston. The only difference was that relatively higher numbers of cells surrounding the infection site lost viability in 964a-46 compared to Eston. The engagement of non-infected cells in 964a-46 might be caused by a systemic signal transduced to the neighboring cells. Systemic signaling was suggested as the main difference between two genotypes of wheat with resistance to Fusarium head blight (Foroud et al., 2012). Transduction of systemic signals to non-infected cells around the infection site could prime defense response and decrease the aggressiveness of the pathogen, thereby limiting the colonization area as observed for 964a-46. Microscopic studies could not, however, provide conclusive evidence for phenotypic separation of the colonization process of 964a-46 and the susceptible check Eston. Improvement of microscopy techniques may lead to better phenotypic differentiation in future studies.

Analysis of quantitative expression of *PR-1a*, *PR-5*, *PR-4a* and *AOC* suggested that genotypes differentially activated the SA and JA signaling pathways. The rapid increase in *PR-1a* between 18 - 24 hpi and *PR-5* between 12-24 hpi in 964a-46 suggested the involvement of the SA pathway in the interaction with *A. lentis*. However, the role of this pathway in resistance to *A. lentis* is doubtful in this case because of the relatively late timing of associated gene expression compared to hemibiotrophs. In the case of *F. graminearum*, a hemibiotrophic fungal pathogen of wheat, the SA pathway was triggered very early at 6 hpi and the levels of expression were higher in resistant compared to susceptible lines (Ding et al., 2011). No significant activation of SA in CDC Robin and Eston, and relatively late activation of this signaling pathway in 964a-46 makes it less likely that *A. lentis* is a hemibiotrophic pathogen with an early biotrophic phase. The SA-mediated signaling pathway activates defense responses that are effective only against biotrophic and hemibiotrophic fungi (Kunkel and Brooks, 2002). Roundhill et al. (1995) suggested that *A. lentis* is either a necrotroph or hemibiotroph, but data here are more supportive of a necrotrophic life style of *A. lentis*. The activation of HR is always accompanied by a systemic accumulation of SA (Nimchuk et al., 2003). The SA signaling pathway might be triggered by pathogen effectors which induced HR in 964a-46 and at the same time activate a systemic signal that lowered the colonization rate of the pathogen by fast activation of defense genes in neighboring cells. Foroud et al. (2012) also suggested that the transduction of systemic signals to non-infected cells around

the infection site was the main difference between genotypes carrying different resistance genes to Fusarium head blight disease.

Considering that cell death was detected in the descriptive microscopy test both in 964a-46 and Eston, there might be differences between the mechanisms of cell death in Eston and 964a-46. Previous studies showed that there are two distinct types of programmed cell death (PCD) in plants, vacuolar cell death and necrosis, which are morphologically distinct at the histological level (Van Doorn et al., 2011). Vacuolar cell death is mediated by an autophagy-like process induced by hydrolytic enzymes released from the cell vacuoles. Necrosis is induced by early disturbances of the plasma membrane integrity accompanied by the shrinkage of the protoplast. The former is thought to happen in response to biotrophic pathogens upon the SA signaling whereas the latter is a frequent response to necrotrophic pathogens (Van Doorn et al., 2011). Rossi et al. (2011) suggested that *B. cinerea* induces cell death independently of the SA signaling pathway. Another possible difference between Eston and 964a-46 might be suppression of the SA signaling shortly after its induction in Eston through a complicated cross-talk between SA and other signaling pathways such as ABA. The suppressive effect of ABA on SA was documented in *A. thaliana* (Yasuda et al., 2008). The possibility of suppression of SA in Eston can be supported by temporal increase in the expression of *PR-1a* observed at 12 hpi and rapid decline at 18 hpi.

In a recent study of the *A. lentis* transcriptome, the involvement of a complex toxin model was proposed in which the quantitative nature of resistance in lentil is attributed to the interactions of numerous toxins produced by the pathogen with their hypothetical corresponding susceptibility factors in the plant (Lichtenzveig et al., 2012). It is worth noting that reactions to AB in 964a-46 varied from highly to moderately resistant when challenged with two different isolates of *A. lentis* (Tar'an et al., 2003). The activation of the SA pathway in 964a-46 might be due to the recognition of specific pathogen toxins by receptor genes in this genotype, resulting in cell death and successful infection through effector triggered susceptibility (ETS). Previous results suggested that ETS facilitated infection of some host specific necrotrophs following a gene-for-gene interaction between a host specific toxin and a host receptor protein (Oliver and Solomon, 2010). However, activation of HR and systemic accumulation of SA might have sped up the plant basal defense and increased the level of resistance in 964a-46 compared to Eston. The very high levels of *PR-1a* and *PR-5* expression in 964a-46 compared to other genotypes could be attributed to a systemic

induction of SA in intact tissues, resulting in an increase of the transcription levels of *PR-1a* and *PR-5* in non-infected cells.

The putative role of *PR-4a* in lentil resistance to *A. lentis* was described by Mustafa et al. (2009) and Vaghefi et al. (2013). Its antifungal activity was also proven *in vitro* using a recombinant protein (Vaghefi et al., 2013). The involvement of *PR-4* in JA-triggered defense was suggested previously (Thomma et al., 1998), which is why it was selected for analyzing the role of JA in the present study. *PR-4a* expression could potentially explain the differences in resistance levels among genotypes. The expression of *PR-4a* was not induced in Eston until 24 hpi, while it reached a peak in CDC Robin at this time. Similarly, an expression peak occurred in 964a-46, however, this was 12 h later and at significantly lower expression levels. Previous studies suggested that the SA signaling pathway is ineffective against pathogens with a necrotrophic life-style and JA plays a crucial role instead (Glazebrook, 2005). The present results confirmed the role of the JA signaling pathways in the induction of resistance against *A. lentis*. The lower resistance in 964a-46 compared to CDC Robin can be attributed to delayed induction of *PR-4a* (JA pathway). Delayed induction might provide enough time for the pathogen to induce cell death and to colonize tissues. Results showed that *PR-4a* peaked at 36 hpi in 964a-46, which was concurrent with the decline in the expression of *PR-1a* and *PR-5*. This supported the previous cross-talk models suggested for the SA and JA pathways which postulate antagonistic effects of SA on JA signaling (Schenk et al., 2000; Kunkel and Brooks, 2002; Glazebrook et al., 2003). It can be inferred from the accumulated evidence that activation of the SA pathway in this genotype might be the cause of the delay in the activation of the JA signaling and the lower levels of resistance.

The *AOC* expression followed the phenotypic ranking of resistance in the tested genotypes from 12-24 hpi, confirming again the putative role of the JA signaling in resistance to *A. lentis*. Similar to the pattern of *PR-4a* expression, *AOC* expression was increased at a lower rate in 964a-46 compared to CDC Robin, however, it declined faster in CDC Robin than 964a-46. In contrast to *PR-4a*, *AOC* expression peaked at 24 hpi, about 12 h earlier than *PR-4a*. *AOC* is a component of the JA biosynthesis pathway (Vick and Zimmerman, 1987). Usually, PR proteins are expressed downstream of the SA and JA signaling cascades, and a time interval occurs between defense activation and expression. This might explain the 12 h delay in the induction of *PR-4a* compared to *AOC*.

The present results suggest that genotypes were different in their expression of genes related to the SA and JA signaling pathways. Infection by *A. lentis* caused intensive activation of SA-related genes in 964a-46, suggesting differences between it and both AB resistant CDC Robin and the susceptible Eston. Expression levels of genes associated with the JA pathway was associated with differences among genotype in the level of resistance. Microscopy studies suggested that fungal growth was inhibited probably by a lower cell death frequency in CDC Robin; however, this mechanism could not explain the differences between 964a-46 and Eston. Application of more advanced microscopy with modified staining protocols may enable capture of the differences between 964a-46 and Eston. These results together suggest that the reaction of lentil to *A. lentis* was genotype-specific. However, a complete understanding of signal transduction pathways activated upon *A. lentis* infection requires additional analyses of other signals such as ABA, auxin and gibberellic acid and their downstream pathways.

Prologue to Chapter 5

The molecular components of lentil defense reactions to AB have not been intensively investigated due to the absence of both pathogen and plant genome sequences. Recently, several thousand SNP markers polymorphic among lentil genotypes have been discovered, the first drafts of the lentil and *A. lentis* genomes have been released, and several transcriptome analyses have been published. These advances have opened a window for the study of the molecular interaction of lentil with *A. lentis*. Data presented in Chapter 4 highlighted primary cellular and molecular evidence for the existence of different resistance mechanisms in AB resistant lentil genotypes harboring different resistance genes. The inhibition of cell death was suggested as the main mechanism of resistance in CDC Robin. Temporal profiles and expression levels of the JA associated genes varied among resistant genotypes, suggesting the relative importance of this pathway in resistance against *A. lentis*. By contrast, slower colonization in 964a-46 was suggested as the mechanism of resistance. The SA signaling pathway was strongly triggered by *A. lentis* infection in 964a-46, but not in CDC Robin and the susceptible check Eston. The results indicated that large differences may exist in defense mechanisms of different resistant genotypes. However, understanding the quality and quantity of defense responses and the extent to which the mechanisms are different among each requires further analysis. In Chapter 5, the transcriptional regulation of defense responses was compared among the same genotypes used for signal transduction pathway analysis in Chapter 4. A time-series RNA-seq analysis was conducted for samples collected at 6, 12, 18, 24, 36, 48, 60 hpi. The expression levels of a set of candidate defense genes were also measured by quantitative real-time PCR analysis and compared with that of the RNA-seq data. Significant correlation between RNA-seq and qRT-PCR results enabled the identification of candidate defense genes differentially expressed among genotypes.

Chapter 5

Use of RNA-seq analysis to decipher mechanisms of resistance to ascochyta blight in lentil genotypes with different resistance genes

5.1. Introduction & objectives

The biological and economic significance of AB in lentil production was described in the introduction to Chapter 4. In Chapter 4, differences in cellular and molecular mechanisms of AB resistance were observed between CDC Robin and 964a-46. However, more detailed understanding of the scale of differences in defense mechanisms requires comparative analysis at the molecular level of the entire plant defense machinery. The advent of next generation sequencing allows analysis of the transcriptome of plants and pathogen interactions through high-throughput parallel cDNA sequencing, or RNA-seq (Wang et al., 2009). RNA-seq has been used for the study of several plant-pathogen interactions and could successfully identify candidate resistance genes (Faino et al., 2012; Kawahara et al., 2012; Chen et al., 2013; De Cremer et al., 2013; Zhu et al., 2013).

In most plants, innate immunity responses include two typical levels separated by the specificity of responses and activation timing (reviewed by Jones and Dangl, 2006). The first level is triggered by PAMPs or MAMPs, and is referred to as PAMP-triggered immunity (PTI). During PTI, PAMPs or MAMPs are perceived by trans-membrane pattern recognition receptors (PRRs) at the early stage of infection. PAMPs are common, slowly-evolving molecules in pathogens and any mutation is usually detrimental for the pathogens. Flagellin, bacterial cold shock proteins, elongation factors, chitin and plant cell-wall derived oligogalacturonides (OGs) are well-known stimuli of PTI (Boller and Felix, 2009). PRRs are usually receptor-like protein kinases (RLKs) with a trans-membrane domain such as the putative chitin receptor LysM/CERK1 (Wan et al., 2008), peptide receptors (Yamaguchi et al., 2010), the OGs receptor wall-associated kinase 1 (*WAK1*) (Brutus et al., 2010) and brassinosteroid insensitive 1-associated kinase 1 (BAK1) (Chinchilla et al., 2007). Resistance induced by PTI is quantitative and is effective against all pathogens regardless of their life-style (Mengiste, 2012). The second layer of plant defense armoury, effector-triggered

immunity (ETI) usually occurs later than PTI and is the result of specific recognition between plant disease resistance (R) proteins and pathogen effectors. The immune responses activated upon this recognition are faster and stronger than PTI, and usually end in a hypersensitive response (HR). The majority of R proteins belong to NBS-LRR proteins. NBS-LRR proteins are classified into two distinct groups, TIR-domain and CC-domain (McHale et al., 2006). The specific interaction of R proteins with pathogen effectors have been documented in several cases e.g. *AvrRpm1* and *RPM1* (Kim et al., 2009), *AvrRpt2* and *RPS2* (Mudgett and Staskawicz, 1999) and *AvrPto* and *Pto/Prf* (Ronald et al., 1992).

Mechanisms of plant defense against hemibiotrophs and necrotrophs are usually more complex than those against biotrophs and vary with plant species and virulence mechanisms of pathogens (reviewed by Mengiste, 2012). Most defense responses induced by necrotrophs are triggered by transduction of the phytohormone signals ET and JA. A possible role of the SA signaling pathway and systemic acquired resistance (SAR) in defense against necrotrophs has been reported for *A. thaliana* plants with an impaired SA signaling pathway, making them more susceptible to *B. cinerea* (Ferrari et al., 2003). The role of ABA in defense against necrotrophs is controversial and both augmented resistance and susceptibility to pathogens have been reported in ABA-deficient mutants. The ABA pathway triggers multifaceted defense responses in plants which vary with the type of plant tissues, the infection stage and the strategy of the pathogens (Ton et al., 2009). For example, a mutation (sitiens) in the ABA pathway in tomato showed increased resistance to *B. cinerea* by increased accumulation of reactive oxygen species (ROS) (Audenaert et al., 2002; Asselbergh et al., 2007). Callose deposition mediated by the ABA signaling had a positive effect on the resistance of *A. thaliana* to *L. maculans* (Desm.) Ces. & De Not (Kaliff et al., 2007). Other phytohormones such as gibberellic acid (GA) (Luo et al., 2010) and auxin (Kazan and Manners, 2009) are also involved in defense signal transduction against necrotrophs. Accumulation of each or the blend of these phytohormones induces the activation of downstream defense responses including cell-wall reinforcement, accumulation of ROS, the synthesis of PR proteins and biosynthesis of antimicrobial secondary metabolites via the phenylpropanoid pathway (reviewed by Laluk and Mengiste, 2010; Mengiste, 2012).

The mechanisms of lentil resistance to *A. lentis* were previously investigated in resistant genotype ILL 7537 and compared to susceptible genotype ILL 6002 in a cDNA microarray

analysis using 'Pulsechip' that was developed mostly using sequences of several legume relatives (Mustafa et al., 2009). Results indicated substantial differences in type, level and activation time of genes differentially expressed upon *A. lentis* challenge in the resistant and susceptible genotypes. This study succeeded to partially decipher the mechanisms of AB resistance in lentil, however, it was unable to capture all the related genes due to i) presence of limited number of genes in the Pulsechip (156 ESTs from *Lathyrus sativus*, 565 ESTs from chickpea plants challenged with *A. rabiei* and 41 resistance gene analogues (RGAs) from lentil, in total 762 genes) ii) sequence dissimilarity between lentil and relative species iii) technical elaboration of microarray and iv) inclusion of only one resistant genotype in the experiment. More recently, Garcia et al. (2013) conducted a SuperSAGE transcriptome analysis of response to *A. lentis* in lentil genotype ILL 5588. Genes differentially expressed after *A. lentis* infection were annotated as disease resistance genes (31 transcripts), transcription factor (66 transcripts) and kinases (197 transcripts) (Garcia et al., 2013). Both of next generation sequencing (NGS) transcriptome tools superSAGE and RNA-seq analysis have very high sensitivity, but RNA-seq is preferred because of i) a wider dynamic range ii) higher reproducibility and, iii) better estimation of absolute expression (Fu et al., 2009).

Previous microscopic examinations suggested a crucial role for host cell death in the life cycle of *A. lentis* (Chapter 4; Roundhill et al., 1995), suggesting that this pathogen is either a necrotroph or a hemibiotroph with a short biotrophic phase. Microscopic studies in Chapter 4 suggested that fungal growth was likely inhibited by the inhibition of cell death in CDC Robin, but this mechanism could not explain the differences between 964a-46 and Eston. These results also suggested that lentil genotypes CDC Robin, 964a-46 (AB resistant) and Eston (AB susceptible) differed in the expression of genes associated with the SA and JA signaling pathways. Infection by *A. lentis* caused intensive activation of the SA-related genes in 964a-46, suggesting differences between it and the other genotypes. The JA pathway could explain differences among CDC Robin and 964a-46 resistance levels defined in the previous pathogenicity tests (Chapter 3). The results suggested that the reaction of lentil to *A. lentis* was genotype-dependent. However, complete understanding of defense pathways differentially activated by *A. lentis* in AB resistant genotypes with non-allelic resistant genes requires additional analyses of other signals and their upstream and downstream pathways.

The present study was conducted to characterize different AB resistance mechanisms conferred by non-allelic resistance genes in CDC Robin and 964a-46 and the identification of some candidate resistance genes for further mapping and reverse genetic confirmatory analysis.

5.2. Materials and methods

5.2.1. Plant materials and *Ascochyta lentis* inoculation

CDC Robin and 964a-46 are lentil lines derived in the breeding program for improving AB resistance at the CDC, University of Saskatchewan, Canada. CDC Robin is resistant to both AB and race 1 of *C. lentis* causing anthracnose, another economically important disease of lentil. AB resistance in the breeding line 964a-46 is derived from ILL 5588, which is the source of resistance for cultivar Northfield (Ali, 1995). *L. culinaris* cv. Eston was used in this study as a susceptible line (Slinkard, 1981). *A. lentis* isolate AL57 is an aggressive isolate from Landis, Saskatchewan (Banniza and Vandenberg, 2006). A conidial suspension prepared from a monoconidial culture of AL57 was stored in a cryopreservation solution containing 10 % skim milk and 20 % glycerol at -80°C. To prepare fungal inoculum, conidia were revitalized on 50 % oatmeal agar plates (30 g oatmeal [Quick Oats, Quaker Oats Co., Chicago, IL, USA], 8.8 g agar [Difco, BD®, Sparks Glencoe, MD, USA], 1 L H_2O) and incubated for 7 days at room temperature. Plates were then flooded with sterile deionized water and conidia were harvested by scraping the colonies with the edge of a glass microscope slide. The suspension was collected and filtered through one layer of Miracloth into a clean Erlenmeyer flask. The concentration of the conidia suspension was adjusted to 10^6 conidia mL^{-1} using a hemocytometer.

Four seeds of each genotype were sown in 10 cm square pots containing a soilless mixture of Sunshine Mix No. 4 (Sun Grow Horticulture® Ltd., Vancouver, BC, Canada) and Perlite™ (3/1 V/V). In each of three biological replicates, there were 8 pots per genotype, each assigned to one sampling time point. Pots were kept in a greenhouse with average daily temperate of 23.5 °C, relative humidity of 66 % and a light regime of 18/6 h day/night supplied from natural light integrated with an artificial light source provided using high pressure sodium 1000 watt lights. Seedlings with 10 to 15 expanded leaves (21 days after sowing) were inoculated with the conidia suspension at a rate of 2 mL per seedling using an airbrush, and were incubated in a humidity chamber for 48 h. Plants were then incubated on a misting bench, receiving mist for 30 s every 90 min during the day for the remainder of the test. The experiment was conducted as a randomized

complete block design with three biological replicates. One replicate of the experiment was subjected to Illumina sequencing.

5.2.2 Illumina sequencing and data analysis

All inoculated leaflets of seedlings were collected at 6, 12, 18, 24, 36, 48, 60 hours after inoculation (hpi) and flash frozen in liquid nitrogen. Leaflets were collected from water sprayed mock-inoculated control plants and sampled prior to inoculation. Leaflet samples were stored at -80 °C. Leaflets pooled for each biological replicate were ground in an RNAse free mortar, pre-cooled by liquid nitrogen. RNA was extracted using a combination of Trizol® reagent (Invitrogen, Carlsbad, USA) protocol and an Ambion® PureLink™ RNA mini kit with on-column PureLink® DNAse treatment (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. To assure complete removal of genomic DNA, total RNA was treated with DNAse I using the DNA-free™ kit (Applied Biosystems, Warrington, UK) after the extraction steps. The purity of RNA was determined using a NanoDrop ND8000 (Thermo Scientific, Wilmington, USA). Samples with an A260/280 ratio less than 2.0 were discarded. The quantity of RNA was determined using a Qubit® 2.0 Fluorometer (Grand Island, NY, USA) and a Qubit™ RNA broad range assay kit (Invitrogen, Carlsbad, USA) following the manufacturer protocol. The integrity of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA).

Library preparation and Illumina sequencing was performed at the National Research Council (NRC) Sequencing Technologies Laboratory, Saskatoon, Canada. Total RNA (~1 µg) for each sample was used for library preparation using Illumina TruSeq® RNA sample preparation v. 2 (Illumina, San Diego, USA). The quality of each library was checked on a DNA 1000 chip by an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA) and the concentration was determined by qPCR using the Kapa SYBR Fast ABI Prism qPCR Kit® (Kapa Biosystems, Wilmington, USA) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Warrington, UK). Equimolar concentrations of the libraries were then pooled and a concentration of 11 pM was used for clustering in one lane of a flowcell on the cBOT (Illumina, San Diego, CA, USA). The samples were then sequenced (2 ×101 cycles, paired-end reads) on the HiSeq 2500 (Illumina, San Diego, CA, USA) using the TruSeq SBS Kit v3-HS 200 cycles Kit (Illumina, San Diego, CA, USA).

Initial processing of reads was performed at the Department of Environment and Agriculture, Curtin University, Australia where the *A. lentis* genome has been sequenced. Raw Illumina reads

were sorted according to adaptor index using the Hiseq 2500 built in software and stored as FASTQ files for each time point per genotype. The reads from each library were trimmed off the index and the universal TruSeq adapter using Cutadapt version 1.1 (https://pypi.python.org/pypi/cutadapt/1.4.2). Resulting reads were filtered to retain only those with a Phred quality score of greater than 30 and a length of at least 25 nucleotides using Prinseq v 0.20.4 (Schmieder and Edwards, 2011; http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi). Paired-reads remaining after trimming were mapped to the *A. lentis* genome of Australian isolate Al-4 (version 130419) using TopHat 2.0.7 (Trapnell et al., 2012; http://tophat.cbcb.umd.edu/). Processed reads were extracted from TopHat bam files using Picard v1.95 (http://picard.sourceforge.net/picard-metric-definitions.shtml) for mapped reads and BamUtils v 1.0.5 (Breese and Liu, 2013; http://ngsutils.org/modules/bamutils/) for unmapped reads.

RNA-seq bioinformatics workflow was conducted in the iPlant Collaborative™ server (Arizona Genomic Institute, Tucson, USA). FASTQ files containing paired-end unmapped reads to the *A. lentis* genome were uploaded in Discovery Environment™ using the iDrop iRODS browser™ (http://iren-web.renci.org:8080/idrop/idrop.jnlp). To assure the quality of reads, FASTQ files were subjected to quality analysis using FastQC v. 0.10.1 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). High quality paired end reads were then mapped to the draft of the 'CDC Redberry' v0.6 lentil genome using the spliced read mapper software TopHat® v. 2.0.9 with built in Bowtie® v. 2.1.0 (Trapnell et al., 2012; http://tophat.cbcb.umd.edu/). Transcripts were constructed for each lentil genotype with the program Cufflink® using the reference genome sequences and bam files developed by TopHat® (Trapnell et al., 2012; http://cufflinks.cbcb.umd.edu/). Normalized gene expression with fragments per kb of exon per million mapped reads (FPKM) values calculated only for uniquely mapped reads in exonic regions, were used for calculation of fold change in expression levels compared to mock-inoculated control plants using Cuffdiff®. Genes with $\log_2$ fold change > 2 were considered differentially expressed. *De novo* expressed genes (with FPKM=0 in the mock-inoculated control) with FPKM > 3 in infected leaflet samples were also considered differentially expressed.

To find functional annotation for differentially expressed genes (DEGs), the corresponding genomic sequences were extracted using the following procedure. Transcript IDs of DEGs were cut from the Cuffdiff output using text manipulation tools of the Galaxy® server

(https://usegalaxy.org/). The coordinates of those transcripts were extracted from GTF annotation files developed by Cuffmerge® from Cufflink® outputs and stored into a new file using filter and sort tools of the Galaxy® server. The genomic sequences corresponding to these coordinates were extracted using gtf_to_fasta script available in TopHat® (http://tophat.cbcb.umd.edu/). The resulting multi-FASTA files were subjected to BLAST analysis in the iPlant Collaborative Atmosphere™ NGS viewer v. 3.2 instance launched with 8 CPUs and 64 GB RAM using standalone BLASTx v. 2.2.29 (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastDocs&DOC_TYPE=Download) against validated and reviewed protein entries in RefSeq release 60 (Pruitt et al., 2012; http://www.ncbi.nlm.nih.gov/refseq) with the following parameters: e-value 1e-5, best hit score edge 0.05, best hit overhang 0.25 and outfmt 5. The gene ontology terms were then extracted for DEGs by analyzing BLAST output using Blast2Go software (http://www.blast2go.com/). Pearson's correlation analysis was conducted for the gene expression values of samples used in the RNA-seq analysis with the hierarchical clustering tool of CLC Genomics Workbench® 7.0.3 using the average linkage distance among samples following the statistical procedure suggested by Eisen et al. (1998).

The candidate defense genes were selected from the list of DEGs obtained for each genotype based on GO terms and prior knowledge of defense pathways in plants (Appendix 7, 8, 9). To determine genes differentially expressed among genotypes, the expression levels of each of those candidate genes were compared among genotypes. If the $\log_2$ fold change of candidate genes differed by more than 2 units (the same threshold used for shortlisting DEGs) among at least two genotypes, they were considered as candidate genes, otherwise they were considered commonly expressed genes.

5.2.3. Sequence homology analysis of differentially expressed NBS-LRR gene analogs

With regard to the relative importance of NBS-LRR genes in resistance/susceptibility to plant pathogens and the induction of cell death, sequence homology analysis was conducted for differentially expressed NBS-LRR genes detected in RNA-seq analysis. DNA sequences belonging to these genes were retrieved from multi-FASTA files containing DEGs sequences using their transcript ids and were translated to protein sequence using the SIXFRAME tool at Biology WorkBench v. 3.2 (http://workbench.sdsc.edu). The predicted protein sequence for the longest open reading frame (ORF) was isolated for further analysis. The accuracy of translation

was confirmed using NCBI BLAST searches. The position of the NB-ARC motif (NBS) of the retrieved sequence was characterized using NCBI conserved domain database and related sequences were isolated using the extractseq tool available in EMBOSS® (http://emboss.bioinformatics.nl/cgi-bin/emboss/extractseq). Alignment was performed using ClustalX v. 2.1 (Thompson et al., 1997) with the default parameters. The alignment output was used to construct a neighbour-joining tree with 2000 bootstrap trials in ClustalX v. 2.1 and the tree was visualized using EVOLVIEW® software (http://www.evolgenius.info/evolview/).

5.2.4. Assessment of selected defense gene expression using quantitative real time PCR

Six genes [orthologs of *Abscisic acid insensitive 1b* (*ABI1b*), *DDB1-Culin 4* (*DDB1-CUl4*), *pathogenesis related homeodomain* (*PRH*), *P. syrngae* pv. *tomato kinase interactor* (*Pto*), *resistance gene analogue 1* (*RGA1*) and *resistance gene analogues 71* (*RGA71*)] which were differentially expressed among genotypes and had contrasting expression levels were selected for expression analysis with qRT-PCR (Table 5.1). Total RNA extracted and used (only one replicate of that) for sequencing, was also used for qRT-PCR. Primers were designed based on lentil cv. 'CDC Redberry' sequences using Primer3 software with default parameters except for maximum product size which was adjusted to 200 bp (Appendix 3, http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The fidelity of the primers was predicted *in silico* using NCBI primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Total RNA was used for reverse transcriptase-dependent first strand cDNA synthesis using the high capacity RNA to cDNA kit™ (Applied Biosystems, Warrington, UK) following the manufacture's protocol. PCR amplifications were conducted in a CFX384 C1000 Touch® real time thermo-cycler (Bio-Rad Laboratories Inc., Singapore) in a 12.5 µl reaction containing 7.1 µl of Applied Biosystems® Fast SYBR® Green Master Mix (Applied Biosystems, Warrington, UK), 0.2 µM of each primer and 5 µl of 1:10 diluted cDNA. The amplification conditions were 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s followed by a melting curve from 60 °C to 95 °C with 0.3 °C intervals. Amplification efficiency was calculated for each primer pair using cDNA stock serially diluted 1:4 (V/V) five times for a total of 6 dilutions for each of the three lentil genotypes, and new primer pairs were re-designed in the case of out of range amplification efficiency. Dilutions were used as template for qRT-PCR following the protocol described above. A linear equation was fitted to the CT values obtained for various cDNA dilutions. Percentile of amplification efficiency (PAE) was calculated from the slope of the regression line using the

equation PAE = 10 (-1/slope) -1. PCR amplifications were conducted in triplicate. PCRs were repeated when the standard deviation of PCR replicates was higher than 0.2. Expression level was reported relative to the mock-inoculated control by calculating fold changes in expression levels following the method suggested by Salzman et al. (2005). Spearman's correlation analysis was conducted between data obtained from RNA-seq analysis and qRT-PCR using PROC CORR of Statistical Analysis System (SAS) version 9.3 (SAS Institute Inc., Cary, NC, USA).

Table 5.1. Description and possible function of six candidate genes assessed using qRT-PCR

Seq. description	ACC[a] of best hit	Gene symbol	Function
Pto kinase interactor	XP_003609869	*Pto*	ETI mediated by Pto kinase
ddb1- and cul4-associated factor	XP_003597148	*DDB1-CUL4*	Ubiquitination in signaling
pathogenesis-related homeodomain	XP_004487993	*PRH*	Transcription regulation of defense
abscisic insensitive 1b	XP_003603175	*ABI1b*	ABA signaling
nbs-containing resistance-like protein	XP_003598562	*RGA1*	CC group, Pathogen recognition
tmv resistance protein n-like	XP_004510225	*RGA71*	TIR group, Pathogen recognition

[a] Ref-seq accession number for the best BLAST hit.

5.3. Results

5.3.1. RNA-seq analysis

5.3.1.1. Analysis of Illumina sequences

The transcription regulation of defense responses to *A. lentis* infection was investigated in the two resistant lentil genotypes CDC Robin and 964a-46, and was compared with the susceptible genotype lentil cv. Eston by RNA-seq analysis. The total number of high quality reads generated by Illumina sequencing was 72,528,007 , 63,031,665 and 69,060,713 for Eston, CDC Robin and 964a-46, respectively (Appendix 4). The average number of high quality reads per sample was 9,066,001, 7,878,958 and 8,632,589 for Eston, CDC Robin and 964a-46, respectively. The average proportion of paired-end reads mapped to lentil CDC Redberry genome sequences was 90.7, 91.0 and 90.5 % of the high-quality reads for Eston, CDC Robin and 964a-46, respectively. The proportion of uniquely mapped reads was 83.8, 83.4 and 82.7 % on average for Eston, CDC Robin and 964a-46, respectively.

The mapping of paired-end reads to the reference genome resulted in the prediction of 23,663, 22,789 and 24,398 genes in Eston, CDC Robin and 964a-46, respectively. To confirm the accuracy

of RNA-seq analysis, the expression levels of a few housekeeping genes including *GAPDH*, *α* and *beta-tubulin*, *DNAj Chaperon* and *transcription elongation factor* (*TEF*) were examined for all sampling times (Appendix 5). Theoretically, the expression levels of housekeeping genes should not be modified by pathogen infection. No significant differences were observed among the expression levels of all the selected house-keeping genes in the infected plants at all the sampling times and the mock-inoculated control, confirming the reliability of RNA-seq analysis (Appendix 5).

The expression levels of genes at all sampling times were used to determine similarity among the samples used for RNA-seq analysis using hierarchical cluster analysis (Fig 5.1). Samples clustered into three main groups with considerable variation in each cluster. Samples of genotypes collected at 60 hpi formed one cluster, indicating similarity of genotypes in gene expression at that time. Samples collected earlier than 60 hpi for all genotypes except for Eston 18h were clustered together, however, variation was also observed among them, resulting in the formation of two sub-clusters. One sub-cluster contained samples collected from mock-inoculated samples (M), those collected from inoculated plants at 6 hpi of all three genotypes, those collected for CDC Robin and Eston at 12 hpi and Eston at 24 hpi, while the other cluster contained samples collected at 36 hpi and 48 hpi for all three genotypes, those collected for CDC Robin and 964a-46 at 18 and 24 hpi and 964a-46 at 12 hpi. These results corroborated that interaction of genotype and time after inoculation is the most relevant cause of differences in the expression profile of samples used for RNA-seq analysis.

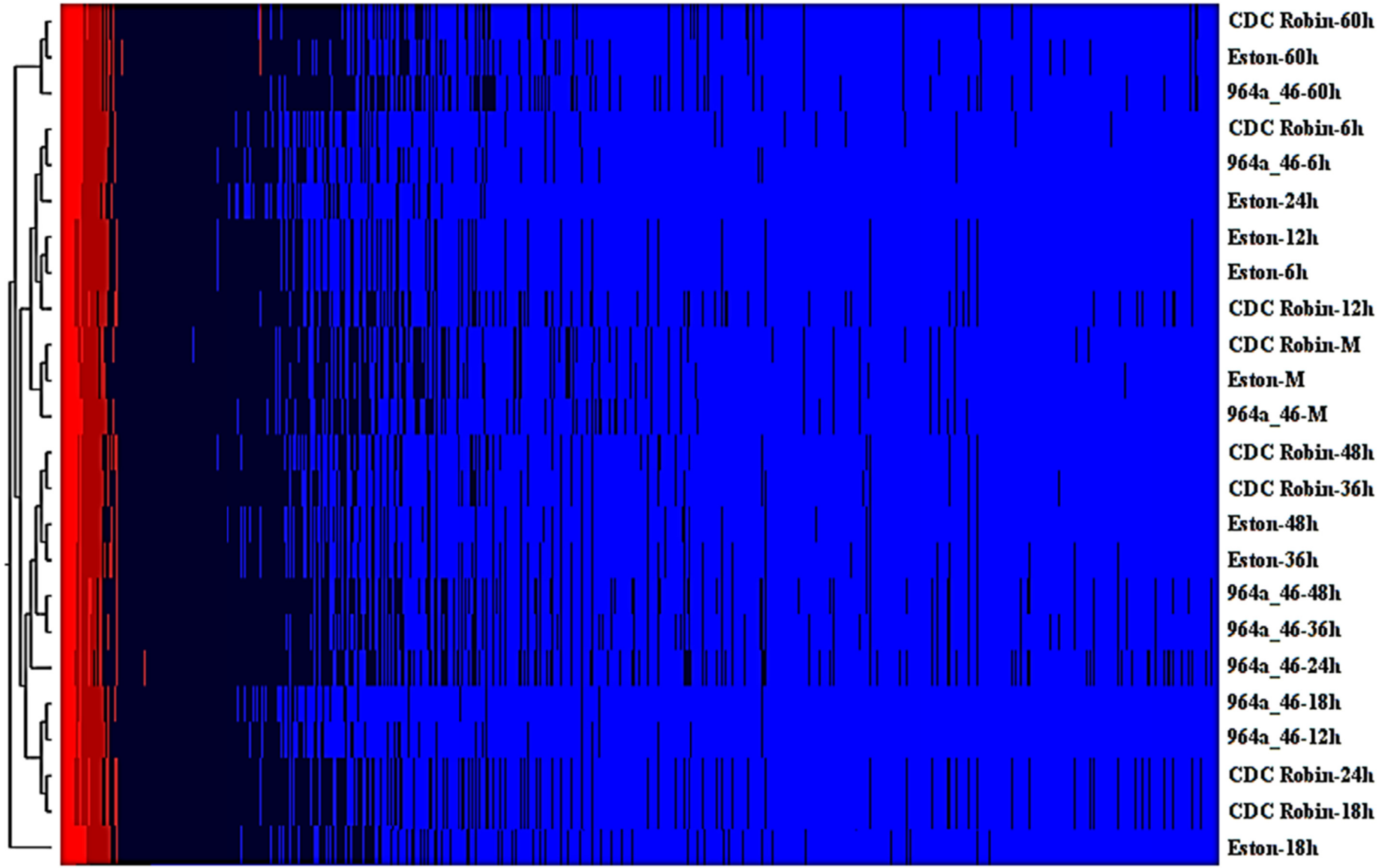

CDC Robin-60h
Eston-60h
964a_46-60h
CDC Robin-6h
964a_46-6h
Eston-24h
Eston-12h
Eston-6h
CDC Robin-12h
CDC Robin-M
Eston-M
964a_46-M
CDC Robin-48h
CDC Robin-36h
Eston-48h
Eston-36h
964a_46-48h
964a_46-36h
964a_46-24h
964a_46-18h
964a_46-12h
CDC Robin-24h
CDC Robin-18h
Eston-18h

Figure 5.1. Cluster analysis of gene expression profiles of lentil genotypes Eston, CDC Robin and 964a-46 measured in mock-inoculated control (M) and inoculated plants at 6, 12, 18, 24, 36, 48 and 60 h post inoculation. Heat map shows the normalized expression levels of transcripts represented by a color spectrum ranging from red (high expression levels) to blue (low expression levels). The dendrogram shows the correlation in the expression profile of samples, calculated using CLC genomics workbench v. 7.0.3, Pearson's correlation analysis and an average linkage distance.

5.3.1.2. Gene ontology analysis of differentially expressed genes (DEGs)

To find functional annotations, DEGs were subjected to BLASTx analysis followed by mapping and annotation analysis in Blast2GO®. The distribution of gene ontology (GO) terms for DEGs was determined from the tabular output of the combined graph analysis in Blast2Go® software. Results suggested that the majority of DEGs in all three genotypes had a role in biological metabolite processes (Fig 5.2). In all sub-categories of biological processes, the resistant genotypes CDC Robin and 964a-46 displayed a higher proportion of DEGs, except for single organism cellular processes, a response to stimuli and regulation of biological processes. Catalytic activity had the highest proportion of DEGs among the GO terms in the molecular function category and 964a-46 had the highest percentage of DEGs in this category. Similar to the biological processes group, resistant genotypes CDC Robin and 964a-46 had higher proportions of DEGs in all sub-categories of the molecular function group, except for the transcriptase activity in which CDC Robin had a slightly lower proportion than the susceptible check Eston. Line 964a-46 had the highest proportion of DEGs in all sub-categories of cellular components except for the membrane category, in which all genotypes had a similar proportion of DEGs. The biggest differences between Eston and CDC Robin, and Eston and 964a-46 were observed in the nucleotide phosphate binding activity and binding activity categories, respectively.

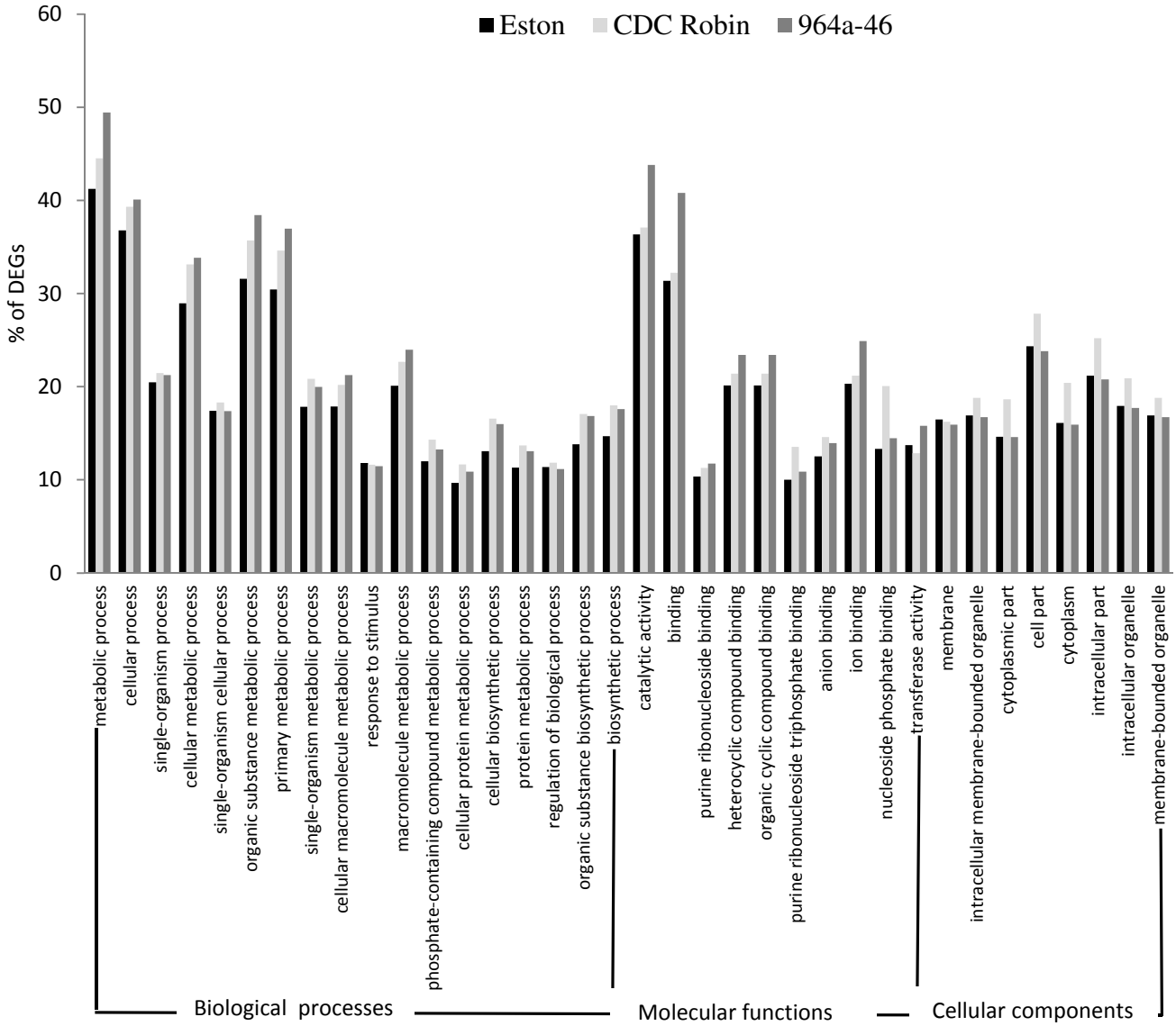


Figure 5.2. Gene ontology terms assigned for differentially expressed genes (DEGs) induced upon *Ascochyta lentis* infection in lentil genotypes Eston, CDC Robin and 964a-46. Gene ontology analysis was conducted using Blast2Go® software.

5.3.2. Assessment of expression levels of selected genes by qRT-PCR

Expression of four defense response genes differentially expressed among lentil genotypes with the highest expression values in the RNA-seq analysis, including *ABI1b*, *PRH*, *DDB1-CUL4* and *Pto* were also assessed by qRT-PCR. *RGA1* and *RGA71* which represented Toll interleukin-1 receptor (TIR) and Non-TIR groups of NBS-LRR genes were also included. Based on the results

of RNA-seq analysis (Appendix 10, Table 2) *ABI1b* was induced more strongly in CDC Robin, while *PRH*, *DDB1-CUL4* and *RGA71*, and *Pto* and *RGA1* were induced more strongly in 964a-46 and Eston, respectively. The expression of *ABI1b*, *DDB1-CUL4*, *PRH*, *Pto*, *RGA1* and *RGA71* peaked at 18, 48, 36, 36, 36 and 48 hpi, respectively in RNA-seq analysis. The expression of these genes based on qRT-PCR analysis peaked at the same sampling time as that in the RNA-seq analysis (Figure 5.3). QRT-PCR results confirmed the differences among genotypes in the expression pattern of the selected defense genes as in all six selected genes, genotypes had differential expression in some of the sampling times. For example, at 12 hpi the down-regulation of *ABI1b* in Eston started, while expression reached a level similar to peak levels at 36 and 48 hpi in CDC Robin at this time. *DDB1-CUL4* and *PRH* expressions were significantly higher in 964a-46 than in the other two genotypes at 24-60 hpi and 24-48 hpi, respectively. Although *PRH* expression was higher in Eston than 964a-46 at 12 hpi, it declined to a significantly lower value at 18 hpi. At this time point, Eston and CDC Robin were not different in *PRH* expression. Compared to *ABI1b* and *PRH*, expression of *DDB1-CUL4* increased later at 24 hpi. Although *DDB1-CUL4* expression was similar in Eston and 964a-46 from 6-24 hpi, there was significantly higher expression in 964a-46 than Eston at both 36 and 48 hpi. *Pto* expression was significantly higher in Eston than in the two other genotypes at all sampling times, except for 60 hpi. The expression of both NBS-LRR genes, *RGA1* and *RGA71* was significantly higher in Eston and 964a-46, respectively than other genotypes in all sampling time points, except for 6 and 60 hpi which supported the differential expression of these genes in Eston and 964a-46, suggested in RNA-seq analysis. Correlation analysis of expression levels estimated from RNA-seq and qRT-PCR suggested significant correlation ($P < 0.001$) between the relative expression levels obtained from these techniques (Figure 5.4). The correlation levels between the RNA-seq and qRT-PCR recorded as 87 %. The significant correlation between two sets of analysis allowed the introduction of candidate defense response genes for each genotype (7, 8, 9, 10) and as a result, finding candidate genes with differential expression among genotypes (Appendix 10, Table 2, 3). The hypothetical role of these candidate genes and their roles in other pathosystem are summarized in Appendix 10. A number of defense genes commonly expressed among genotypes are discussed in Appendix 6.

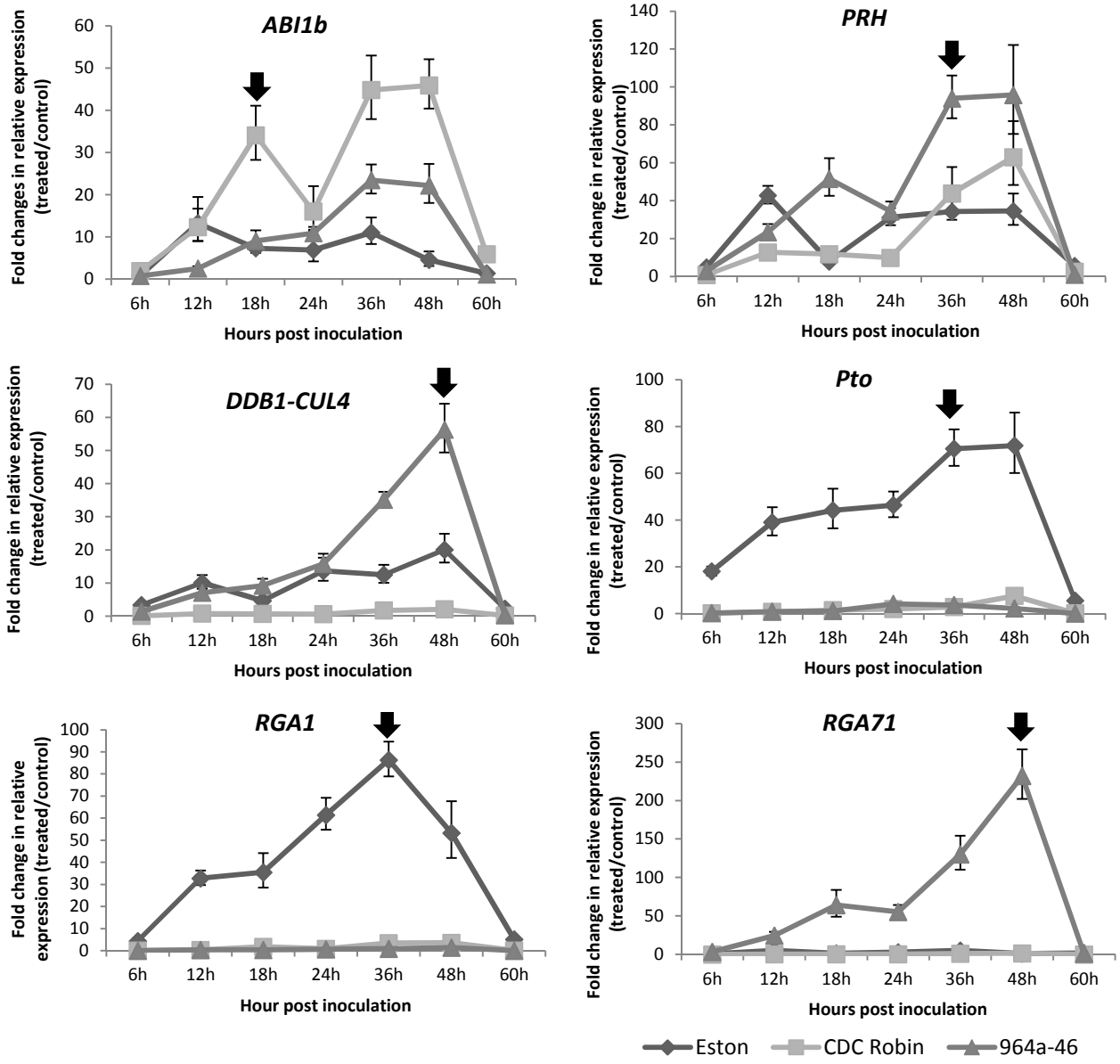


Figure 5.3. Fold-change in the expression of six differentially expressed defense-related genes based on qRT-PCR in lentil genotypes Eston, CDC Robin and 964a-46 inoculated with *Ascochyta lentis*. Data are means of three replicates. Error bars indicate ±SD. Gene expression was reported relative to mock-inoculated samples. Data were normalized using plant *β-actin* (gene banck ACC EU664318) gene expression as a reference gene. Arrow indicate the sampling time with peak gene expression obtained from RNA-seq analysis.

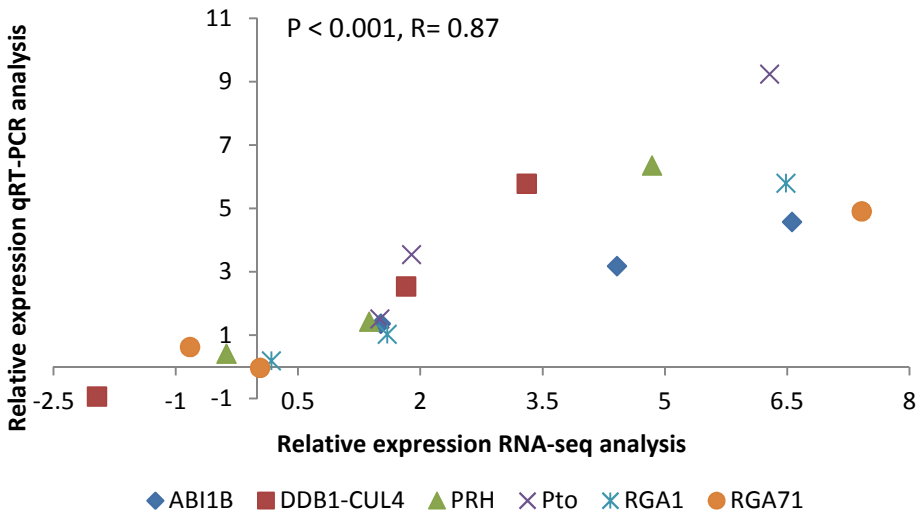


Figure 5.4. Correlation between expression levels estimated from RNA-seq data and qRT-PCR for six candidate defense genes differentially expressed in lentil genotypes Eston, CDC Robin and 964a-46 after *Ascochyta lentis* infection. Data are reported for the sampling times (hpi) representing the peak amount of expression recorded among all three genotypes. RNA-seq and qRT-PCR expression levels were normalized using fragments per kb of exon per million fragments mapped (FPKM) and *β-actin* as reference genes, respectively. The expression levels are relative to the mock-inoculated control samples.

5.4. Discussion

The release of the first lentil draft genome and the use of RNA-seq analysis allowed a more comprehensive image than that from Pulsechip to be developed for the lentil - *A. lentis* interaction. Results of Chapter 3 and 4 showed that AB resistant lentil genotypes carried non-allelic resistance genes, leading to differences in cellular reactions and hormonal signal transduction pathway activation upon *A. lentis* infection. The present study addressed the global transcriptional regulation of defense responses in two AB resistant lentil genotypes with non-allelic AB resistance genes. The results revealed differences between resistant genotypes in pathogen recognition, signal transduction pathways and the activation of downstream defense response genes, and provided a list of candidate genes which will be subjected to further analysis.

According to a previous analysis of lentil resistance mechanisms conducted by Mustafa et al. (2009), differentially expressed genes associated with resistance in ILL 7537 belong to different defense response groups such as ROS, phytohormone signaling and antimicrobial compounds.

Compared to the susceptible genotype ILL 6002, the AB resistant genotype ILL 7537 had a different response profile in terms of activating defense gene involved in pathogen recognition mediated by a serine/threonine protein kinases, faster activation of ROS, earlier accumulation of PR proteins such as PR-3 (β-1,3-glucanase), PR-4a, PR-10 and suppression of the SA pathway induced at early infection. Susceptibility of ILL 6002 was attributed to down-regulation of antimicrobial compounds such as SNAKIN 2, PR proteins such as PR-10, disruption of ABA/GA balance and modification of regulatory genes such as WD-repeat protein, ankyrin protein kinase and polyubiquitination. Some of the resistance associated genes were also induced in ILL 6002, but this occurred too late to suppress pathogen colonization, emphasizing the relevance of timely expression of defense genes in resistance to AB. Some of defense genes described by Mustafa et al. (2009) were also detected in this study, such as *PR-4a* and ankyrin repeat (*AKR*); however, the association between results of the RNA-seq and microarray techniques was generally poor. This was expected since with the selected sequencing depth, the RNA-seq analysis was capable of the simultaneous monitoring of about 25000 genes. RNA-seq analysis is not affected by drawbacks of microarray analysis such as reproducibility and limited range of gene expression detection (Fu et al., 2009). In addition, the analysis in the current study was based on lentil sequences, not related legumes. The inclusion of two resistant genotypes with different resistance mechanisms enabled the development of a more detailed image of resistance mechanisms against *A. lentis*. The differences between mechanisms of resistance in the ILL 7537 lentil genotype used by Mustafa et al. (2009) and the genotypes used in this study, sampling size and the technical drawbacks of cDNA microarray analysis with Pulsechip might be the causes of poor correlation between the detected genes in the two studies.

Cluster analysis of samples used for RNA-seq analysis suggested a divergent gene expression pattern among genotypes especially at 12, 18 and 24 hpi. Samples of all three genotypes collected from mock-inoculated plants and those collected from infected plants at 6 and 60 hpi were clustered together. Results of Chapter 4 suggested that *A. lentis* AL57 spores were germinated at 6-12 hpi. Clustering of all inoculated samples at 6 hpi as well as mock samples might reflect pathogen inactivity at these time points. Results of qRT-PCR here and in Chapter 4, indicated that expression of all genes at 60 hpi reached a basal level almost equal to that of samples collected at 6 hpi. This might explain the clustering of samples of all genotypes at 60 hpi.

5.4.1. Assessment of some candidate genes using qRT-PCR

To confirm the results obtained from RNA-seq analysis, a recommended method is to pick a small set of candidate genes and analyze the gene expression using qRT-PCR. Here candidate genes with different roles were selected including orthologs of *Pto, RGA1* and *RGA71* with putative roles as a receptor, orthologs of *ABI1b* and *DDB1-CUL4-* with roles in signal transduction pathways and ortholog of *PRH* which is a transcription factor involved in transcription activation of PR proteins. *Pto* encodes a protein kinase which is involved in the specific recognition of *AvrPto* secreted from *P. syringae* pv. *tomato* in tomato, however kinase activities have not been reported for this gene (Oh and Martin, 2011). *RGA71* encodes a TIR-NBS-LRR gene that is an ortholog of *N* resistance genes. *N* is a TIR-NBS-LRR resistance gene, mediating resistance against Tobacco Mosaic Virus in tobacco through the induction of a hypersensitive reaction (Marathe et al., 2002). *RGA1* is a homologue of a NBS-LRR gene in *M. trancatula* (gene bank ACC: XP_003598558) which was highly expressed in Eston. *ABI1b* encodes a serine/threonine protein phosphatase 2C, reported to lower responsiveness to ABA in plants (Merlot et al., 2001). *DDB1-CUL4* is a type of E3 ubiquitin ligase and is involved in the SA signaling pathway (Santner and Estelle, 2010; Magori and Citovsky, 2012). *PRH* is a transcription factor involved in transcriptional regulation of defense genes such as *PR-2* (Korfhage et al., 1994).

Statistical analysis implied a significant correlation between the expression levels obtained from RNA-seq and qRT-PCR, suggesting the reliability of results obtained from RNA-seq analysis. Data of qRT-PCR were correlated as high as 87 % with those of RNA-seq. A similar level of correlation was also observed by De Cremer et al. (2013), while using RNA-seq for dissecting interaction of lettuce with *B. cinerea*. Final conclusions about the genes playing a role in AB resistance should be drawn only after mapping such genes in the LR-18 mapping population and/or reverse genetic tools such as gene silencing and mutation analysis. In the following sections, efforts are made to discuss the potential roles of some of the candidate genes. Detailed elaboration of defense pathways, differentially expressed among genotypes will be postponed until data from two more replications are supplied in future.

5.4.2. Defense response genes induced differentially among lentil genotypes upon *Ascochyta lentis* infection

5.4.2.1. Pathogen recognition

PTI-related genes differentially expressed among genotypes were orthologs of *NIMA-related kinase 6* (*NEK6*), *receptor like kinase* (*RLK*), *lectin s-receptor-like serine threonine-protein kinase* (*LECRK*), *EFR3 receptor kinase* (*EFR3*), *BRI1-associated receptor kinase* (*BAK1*) and *wall-associated protein kinase 1* (*WAK1*). *NEK6* encodes a precursor of ethylene and is involved in abiotic stress response in *A. thaliana* (Zhang et al., 2011). A few genes with receptor like kinase activity were expressed after *A. lentis* infection. Receptor like kinases are generally reported to have a positive role in plant resistance against necrotrophs e.g. *ERECTA receptor like kinase* mediated resistance to necrotrophs such as *Plectosphaerella cucumerina* in *A. thaliana* (Llorente et al., 2005). However, receptor like protein30 was a receptor of *S. sclerotiorum* culture filtrate elicitor1 (SCFE1) and mediated susceptibility to both *S. sclerotiorum* and *B. cinerea* (Zhang et al., 2013). One of the QTLs detected for AB resistance in the LR-18 population (developed from CDC Robin × 964a-46) was co-localized with a receptor like protein kinase gene explaining about 28.7 % of phenotypic variation (Chapter 3). These results suggested the importance of receptor like protein kinases in resistance to AB. *LERK*, isolated initially from legumes, contains an extracellular lectin domain (Singh et al., 2012). However, this receptor was not required for the induction of basal resistance to *B. cinerea* (Singh et al., 2013). *EFR* was proven to be a target for *AvrPto* bacterial effectors, resulting in suppression of PTI during the infection of *P. syringae* pv. *tomato* (Xiang et al., 2008). Present results also indicated an up-regulation of protein kinase *Pto* in Eston. *WAK1* is a well-known cell wall associated protein kinase in *A. thaliana* with an extracellular domain linked to the pectin fraction of cell walls (Decreux and Messiaen, 2005). Previous studies indicated the association of *WAK1* with the SA signaling pathway through amelioration of the lethal effects of very high doses of SA which occurs in plants upon the recognition of a pathogen.

Genotypes had differential expression of genes involved in signaling cascades involved in PTI including various types of mitogen activated protein kinases (MAPKs) e.g. *MEK2*, *MAPKL*, *MAPKK* and *MAPK-ntf6*. This indicated a diversification of MAPK signaling in the AB resistant genotypes. Previous reports have suggested the presence of 24 MAPK genes in *A. thaliana* possibly indicating a great diversity in this pathway. In addition, MAPKs are among the targets of

pathogen effectors responsible for suppression of PTI and are required in compatibility reactions (Shan et al., 2007; Zhang et al., 2007; Krachler et al., 2011).

NBS-LRR genes form the second layer of pathogen recognition and are capable of perception of effectors which suppress PTI during incompatible reactions (Métraux et al., 2009). A total of 32 NBS-LRR genes were up-regulated in response to *A. lentis* infection, among which 13 genes were exclusively up-regulated in Eston and 12 were shared between Eston and other genotypes. Similarly, 31 NBS-LRR genes were differentially expressed in lentil genotype ILL 5588 after *A. lentis* infection (Garcia et al., 2013), emphasizing the involvement of these genes as a response to *A. lentis* infection in lentil. The number of uniquely expressed NBS-LRR genes in each genotype negatively correlated with their resistance levels. A closer look at the expression levels of NBS-LRR genes shared among genotypes suggested a contrasting expression pattern among resistant genotypes for most of them. These results suggest that NBS-LRR genes could have a role in differential responses of resistant genotypes to *A. lentis*.

Many of the NBS-LRR gene-mediated downstream defense responses such as the activation of an oxidative burst, ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis related proteins and the HR were detected in the present study. None of the NBS-LRR genes discovered to date, except for *RLM3*, a TIR-NBS-LRR protein which mediates resistance to hemibiotroph *L. maculans*, and necrotrophs *B. cinerea*, *Alternaria brassica* and *A. brassicicola* in *A. thaliana* have been associated with resistance against necrotrophs (Staal et al., 2008). By contrast, NBS-LRR genes mediated susceptibility to host-specific necrotrophs such as *Pyrenophora tritici-repentis* and *S. nodorum* in wheat through an inverse gene-for-gene interaction with a host-specific effector (toxin) (Faris et al., 2010). A recent effort to identify pathogenicity factors in *A. lentis* resulted in the identification of a large numbers of necrotrophic effectors, orthologs to host-specific toxins present in *S. nodorum* (Lichtenzveig et al., 2012). The presence of a host-specific toxin in the *A. lentis* secretome and the larger number of NBS-LRR genes uniquely expressed in the susceptible genotype might indicate the involvement of a complex toxin model for the reaction of lentil to *A. lentis*.

5.4.2.2. Phytohormone signaling

Results of RNA-seq analysis suggested the involvement of ABA signaling in the interaction of *A. lentis* with lentil genotypes. A role for ABA in the induction of resistance against plant

pathogens is controversial. Comparison of gene expression among resistant and susceptible lentil genotypes suggested an alteration in sensitivity to ABA in CDC Robin through stronger activation of *ABI1b* in this genotype compared to the other genotypes. *ABI1b* encodes a serine/threonine protein phosphatase 2C, reported to lower responsiveness to ABA in plants (Merlot et al., 2001). Meanwhile, the induction of *ABI5* in Eston suggested the activation of the ABA pathway in this genotype. Additional support for the role of ABA insensitivity in the CDC Robin response came from the higher expression of *pentatricopeptide repeat-containing protein* (*PRP*) in this genotype. The ortholog of this gene in *A. thaliana*, *ABA overly sensitive 5* (*ABO5*) is involved in the regulation of ABA, however, its role in response to pathogen attack is unclear. Previous studies indicated an antagonistic effect of ABA on the SA and JA/ET signaling pathways (Anderson et al., 2004; Yasuda et al., 2008). As an example, ABA promoted the susceptibility of *A. thaliana* to *F. oxysporum* through suppression of the JA/ET-induced defense genes such as *PDF1.2* and *PR-4a* (Anderson et al., 2004). The same strategy might be in effect in Eston as its expression of *PR-4a* (JA-associated gene) was much lower than in the two resistant genotypes. Also, the expression levels of ET-associated genes such as *Ethylene responsive transcription factor 1b* (*ERF1b*) and *ethylene responsive transcription factor* (*ERF*) increased earlier in 964a-46 than Eston, supporting a delay in activation of the ET signaling pathway in Eston.

Results in Chapter 4 suggested a strong induction of the SA signaling pathway in 964a-64, but not in CDC Robin, which correlated with the up-regulation of SA-related genes in the current study. Generally, the SA pathway is actively involved in the induction of resistance against biotrophs, and induced during the biotrophic phase in hemibiotrophs (Kunkel and Brooks, 2002; Ding et al., 2011). Other reports showed that some necrotrophs hijacked resistance mechanisms effective against biotrophs to induce cell death and promote host cell colonization (Hammond-Kosack and Rudd, 2008; Grant and Jones, 2009). For example, a recent transcriptome analysis of lettuce after *B. cinerea* infection revealed that similar defense pathways are activated in compatible reactions to this pathogen and incompatible reaction to *Bremia lactucae*, a biotroph that causes powdery mildew disease in lettuce (De Cremer et al., 2013). Therefore, the SA pathway might be induced by *A. lentis* effectors, promoting cell death in 964a-46 and Eston. Results in Chapter 4 indicated the initial activation of *PR-1a* in Eston early during infection by *A. lentis* and down-regulation thereafter. This may support the hypothesis of suppression of the ET and SA signaling pathways in Eston by ABA. The induction of numerous defense response genes by SA could be

responsible for inhibiting colonization as observed for 964a-46 (Chapter 4). In support of this, Ferrari et al. (2003) proposed a role for the SA pathway in resistance to *B. cinerea*. *DDB1-CUL4* was induced stronger in 964a-46 than in Eston and CDC Robin. This gene is involved in ubiquitin-proteasome regulation of plant hormone signaling (Santner and Estelle, 2010), and could be associated with strong activation of SA in 964a-46. Similar to results in Chapter 4, *PR-1a* was induced in Eston but at lower expression levels than 964a-46. On the other hand, fewer of the SA-associated genes were observed in CDC Robin, indicating no SA involvement in resistance to AB in this genotype. The role of the SA signaling pathway in 964a-46 warrants further research.

Present results suggested that the JA/ET pathway might be supressed by heightened levels of ABA in Eston. The *de novo* expression of an ET signaling suppressor, *protein reversion-to-ethylene sensitivity1-like* (*AtRTE1*) observed in Eston supported this hypothesis (Ma et al., 2012). The time difference in the induction of the ET pathway could be the cause of the lower colonization levels in 964a-46 observed (Chapter 4). The other ET-associated genes differentially expressed among genotypes were *ethylene responsive transcription factor 1* (*ERF1*) and *gaga transcription factor* (*GAGA-TF*). Previous studies suggested the presence of two branches in the ET/JA signaling pathway in *A. thaliana*. One was regulated by apetala2/ethylene response factor domain transcription factor 59 (ORA59) and ERF1 that converge the ET and JA pathways. The other was regulated by MYC2 transcription factor, integrating the ABA and JA pathways (Adie et al., 2007; Dombrecht et al., 2007). Some of the ET/JA associated genes such as defensin-like protein were uniformly expressed in all three genotypes, regardless of differences in detected upstream ET-associated gene expression. This might be due to the activation of part of the JA/ET pathway as a common response to *A. lentis* infection. None of the ET/JA-associated genes detected in the present study have been previously characterized in lentil. There might also be differences between model (e.g. *A. thaliana*) plants genes involved in the ET/JA pathway and those in lentil, explaining the discrepancies observed in this study.

5.4.2.3. Cell death regulation

DEGs directly or indirectly associated with cell death were *autophagy-related* (*ATG*)18g, *ATG8d*, *poly polymerase like* (*PARP*), *programmed cell death* (*PDCD)*, *cyclin-dependent kinase* (*CDK*) and *constitutive subtilisin 3* (*CSB3*). The expression patterns of these genes supported cell death inhibition mechanisms described in CDC Robin (Chapter 4). Previous results suggested a

crucial role of autophagy in plant resistance to necrotrophs through negative regulation of cell death (Lai et al., 2011; Kabbage et al., 2013). The inhibitory role of *ATG18g* was dependent on the JA signaling and a WRKY transcription factor in *A. thaliana* (Lai et al., 2011). In CDC Robin, *ATG18g* and *ATG8d* were up-regulated and *de novo* expressed, respectively. Induction of the JA pathway and *de novo* expression of *WRKY51* and *WRKY21* in CDC Robin suggests a similar pathway for the induction of the autophagosome in lentil and supported the involvement of *ATG18g* and *ATG8d* in the resistance of CDC Robin to *A. lentis*.

CSB3 mediates resistance to biotrophs through a SA-dependent regulation of a hypersensitive reaction (Gil et al., 2005), supporting the possibility of hijacking biotrophic mechanisms of resistance by *A. lentis* in 964a-46. *CDK* is a typical component of mammalian apoptosis (Castedo et al., 2002). This gene was induced in Eston, suggesting a similarity between mammalian apoptosis and the plant cell death induced in Eston.

5.4.2.4. Cell wall reinforcement

Three DEGs associated with cell wall reinforcement were detected in the RNA-seq analysis including *cellulose synthase* (*CESA*), *xyloglucan glycotransferase* (*CSLC6*) and *callose synthase* (*CALS*). Previous results indicated a role for *CESA* in resistance against necrotrophs in *A. thaliana* through an ABA-dependent mechanism (Hernández-Blanco et al., 2007). ABA signaling was shown to change the cell wall make up, specifically decreasing he amount of cellulose and uronic acid (Sánchez-Vallet et al., 2012), increasing the susceptibility of *A. thaliana* plants to the necrotrophic pathogen *Plectosphaerella cucumerina* (Sánchez-Vallet et al., 2012). Studies by Lorenzo et al., (2003) indicated activation of this gene by ERF1, a transcription factor that integrates responses to the ET and JA signaling pathways. *CSLC6* is involved in the synthesis of xyloglucans responsible for forming the matrix polymers which are dynamic parts of the plant cell wall (Nishitani, 1995). *PARP* encodes the poly(ADP-Rib) polymerase enzyme which is involved in numerous defense responses to biotic stresses such as callose and lignin deposition, pigment accumulation and phenylalanine ammonia lyase activity (Adams-Phillips et al., 2010). The *PARP*-deficient *A. thaliana* showed higher susceptibility to *B. cinerea*, suggesting the involvement of this gene in resistance to necrotrophs (Adams-Phillips et al., 2010). Ellinger et al., (2013) reported *CALS* involvement in callose deposition as an ABA-dependent defense response. The involvement

of these genes in the response of resistant genotypes to *A. lentis* highlighted a possible role for cell wall dynamics in resistance.

5.4.2.5. Pathogenesis related proteins

A wide spectrum of PR proteins were induced upon *A. lentis* infection including PR-1a, PR-3 (endo β-1,3-glucanase), PR-4a, PR-9 (peroxidases), TLPs (thaumatin-like protein), PR-10, PR-10a (aba-responsive protein abr18), hevein-like protein (Hel), defensin-like protein and Hva22. The expression of *PR-1a* was only up-regulated in 964a-46, suggesting the induction of the SA signaling pathway. Results presented in Chapter 4 also suggested differential expression of *PR-1a* among lentil genotypes.

TLPs belongs to the PR-5 family and is a well-known SA-responsive gene (Nawrath and Métraux, 1999). However, some studies have indicated that the expression of *TLPs* is an integrated response to both the SA and JA signaling pathways (Jayaraj et al., 2004). Results of Chapter 4 indicated a heightened expression of *PR-5* only for 964a-46. *In silico* analysis of primer pairs used in Chapter 4 for amplifying *PR-5* and sequence obtained for *TLP* in RNA-seq analysis suggested that there may be other copies of these genes in the lentil genome with an annealing site recognized by the primer pairs used in Chapter 4, but not captured during RNA-seq analysis.

PR-4a is the only PR protein studied in lentil to date. Previous transcriptome analysis of lentil suggested an up-regulation of *PR-4a* upon *A. lentis* challenge in a resistant but not in a susceptible genotype (Mustafa et al., 2009). Vaghefi et al. (2013) demonstrated the antifungal activity *in vitro* of a recombinant lentil PR-4a protein (product of *LcPR4a*) on *A. lentis.* Hence, the previous results in Chapter 4, which suggested that *PR-4a* expression levels were correlated with the resistance levels of genotypes, were confirmed. The expression of *Hel* is a typical defense response triggered by JA/ET signaling.

Gene expression studies of resistant genotypes carrying non-allelic resistance genes had different gene expression patterns in response to *A. lentis* infection.

Many of the genes differentially expressed among genotypes have a role downstream of pathogen recognition receptors, along the signaling pathways, but several differentially expressed genes involved in pathogen recognition were also identified. The pathogen recognition genes are of primary interest and may provide an opportunity for breeders to exploit these for gene pyramiding. Future research might also include the application of reverse genetic tools to

determine the contribution of these genes to resistance to AB in the resistant genotypes used in this study, and other resistant genotypes used in the breeding program.

CHAPTER 6

GENERAL DISCUSSION

6.1. Genetic dissection of ascochyta blight resistance genes in lentil

AB is an economically important disease of lentil in Canada and elsewhere, affecting both, the quality and quantity of lentil crops. A widely preferred method of control is to introduce disease resistance through integration of AB resistance genes into high yielding varieties. Recent advancements in the introgression of genes from wild lentil germplasm into cultivated lentil have provided an opportunity to increase both genetic diversity and genetic gain. Durability of resistance is a critical issue in breeding programs, as erosion of a newly released source of resistance undermines the costs and efforts involved. Combining available resistance genes in a gene pyramiding program may increase the time required for pathogens to evolve virulence factors, and therefore, decrease the risk of resistance break-down. The present study was conducted to elucidate the relationships among the AB resistance genes which have been widely used for AB resistance improvement. Results suggest that these sources of resistance carried unique AB resistance genes. This might partially explain satisfactory progress in developing AB resistance in the lentil breeding program of the CDC, University of Saskatchewan (Vandenberg, pers. comm.). An important achievement in this study was the characterization and confirmation of a new source of resistance in *L. ervoides* L-01-827A, which conditioned an unprecedented level of resistance to AB. The successful transfer of these resistance genes to cultivated lentil was confirmed through phenotyping LR-59, a RIL population derived from *L. culinaris* cv. Eston × *L. ervoides* L-01-827A. Previous research on L-01-827A highlighted the potential of this genotype for high levels of resistance to anthracnose pathogen race 0 (Fiala et al., 2009), stemphylium blight (Podder et al., 2012) as well as broomrape (*Orobanche crenata*)(Bucak et al., 2014).

Finding markers closely linked to AB resistance genes is an essential step for an efficient gene pyramiding program. The first comprehensive SNP-based linkage map developed for LR-18 (CDC Robin × 964a-46) (Sharpe et al., 2013) was used for QTL analysis and confirmed the differences in AB resistance QTLs between the parents. Closely linked SNP markers, suitable for using in marker-assisted gene pyramiding were identified.

6.2. Different resistance genes and different mechanisms of resistance

Do the non-allelic resistance genes identified through allelism tests trigger different mechanisms of resistance in their downstream pathways? It had been suggested previously that different resistance genes manifested resistance through different resistance mechanisms (Bai et al., 2005; Foroud et al., 2011), and for lentil, this is the first time different mechanisms of resistance have been reported in response to AB. Microscopic studies revealed how resistance was manifested at the cellular levels. Primary observation showed that the growth of pathogen was inhibited in CDC Robin, ILL 7537 and L-01-827A. Non-significant difference among genotypes in conidial germination proved that *A. lentis* conidia germinated uniformly on all tested genotypes, suggesting post-germination inhibition of pathogen growth. Viability tests indicated that cell death inhibition might be a mechanism of resistance in CDC Robin, while a lower colonization rate was suggested as a mechanism of resistance in 964a-46. At the gene expression level, these differences were supported by activation of different phytohormone signaling pathways. The SA pathway was strongly triggered in 964a-46, while JA pathway activation was a common response to infection in both of the resistant genotypes CDC Robin and 964a-46. In the susceptible genotype Eston, the JA and SA pathways were triggered much later, indicating suppression of defense mechanisms by *A. lentis* in this genotype or slower activation of these pathways. These differences highlight the importance of activation time and expression levels of the JA pathway in the outcome of *A. lentis*-lentil interaction.

Resistance to plant pathogens involves many defense response genes that are active in pathogen recognition, transduction of hormonal signals and the activation of downstream defense response genes. In Chapter 4, only a few genes associated with different signal transduction pathways were used to investigate the possibility of different mechanisms of resistance in the genotypes. However, understanding fully the details of different mechanisms of resistance in genotypes harboring different resistance genes required monitoring the global transcriptome of these genotypes. Efforts were already made to obtain an insight into the defense mechanisms responsible for AB resistance (Mustafa et al., 2009). Microarray technology equipped with probes designed based on the sequences of related species such as chickpea*, L. sativus* and a few lentil resistance gene analogues available at the time were used for this purpose. The data from that study provided only a preliminary understanding of defense mechanisms. The study only evaluated gene

expression in one resistant lentil resistant genotype, and therefore did not capture variation in the resistance mechanisms among genotypes.

Lentil was considered an orphan legume in terms of genetic and genomic resources when this study was initiated. Recent developments in lentil genomics have significantly changed this situation with several thousands of SNP markers being discovered, the first drafts of the lentil and *A. lentis* genomes have been released and several transcriptome analyses have been published. These advances have facilitated the application of RNA-seq for a new analysis to capture a high-resolution image of lentil interaction with *A. lentis*. Furthermore, comparison of genotypes harboring different resistance genes enabled identification of the levels of divergence in the mechanisms of resistance present in these genotypes. Differences among genotypes were recorded to be at the pathogen recognition levels as both the number and expressions of a few NBS-LRR and receptor-like protein kinase genes varied among genotypes. Analysis of the *A. lentis* transcriptome at the Department of Environment and Agriculture, Curtin University, Australia suggested homology between *A. lentis* effectors and those characterized in *S. nodorum* (Lichtenzveig et al., 2012). *S. nodorum* is a narrow-host range necrotroph that is dependent on a few host-selective toxins for successful infection. Similar to *S. nodorum*, *A. lentis* has a very narrow host range, which may indicate the role of host selective toxins in this pathogen as well (Kaiser, 1997; Hernandez-Bello et al., 2006). Host susceptibility genes interacting specifically with *S. nodorum* toxins have been cloned and shown to be members of the NBS-LRR genes family (Faris et al., 2010). A large number of NBS-LRR genes in lentil genotypes were up-regulated upon *A. lentis* infection in the current study as well. The number of up-regulated NBS-LRR genes was negatively correlated with resistance levels of genotypes, indicating the possible involvement of these genes in the susceptibility of lentil. QTL analysis conducted for LR-18 showed co-localization of SNPs with a receptor-like protein kinase and an ethylene transcription factor gene, but not with the NBS-LRR genes. Usually, the composite interval mapping procedure is not capable of detecting QTLs with high epistasis effects and inclusive composite interval mapping (ICIM) should be used instead (Zhang et al., 2012). Susceptibility NBS-LRR genes show large epistasis effects because the specific recognition between one host-selective toxin and the corresponding susceptibility gene results in cell death and susceptibility, regardless of allelic diversity at other loci. A few hundred SNP markers used for linkage mapping which had been developed previously using a limited number of ESTs from non-inoculated plants may have

hampered the detection of pathogen-responsive genes such as NBS-LRR genes, simply due to inadequate resolution of the available map. Recent genome-wide screening for NBS-LRR genes have indicated the presence of several hundred NBS-LRR hypothetical genes in the genome of lentil cv. CDC Redberry (Perumal Vijayan, Dept. of Plant Sciences, University of Saskatchewan, pers. comm.). Efforts are ongoing to enrich the current lentil linkage map with these newly characterized genes which might help to decipher the roles of NBS-LRR genes in AB resistance.

A few receptor-like protein kinases were also up-regulated upon *A. lentis* infection and were differentially expressed between resistant genotypes. Detection of orthologs of well-known receptor kinases such as BAK1, EFR3, WAK1, CERK and FLS2 in the lentil genome was an indicator of high homology between lentil and the model plant *A. thaliana* in the PTI pathway. Some of these receptors were up-regulated in the susceptible genotype Eston (e.g. an ortholog of *BAK1*). This was not surprising as many of the PTI responses are activated at the beginning of the infection process and suppressed by pathogen effectors in compatible reactions later. Most of the resistance genes characterized for necrotrophs belonged to the PTI pathway (Mengiste, 2012), emphasizing the importance of this pathway in resistance to necrotrophs. The present results showed that a different set of PTI-associated genes were up-regulated exclusively in CDC Robin. In addition, none of the PTI-associated genes were exclusively up-regulated in 964a-46. Taking into account that cell death occurred both in Eston and 964a-46; it seems that the PTI pathway was suppressed in both genotypes resulting in successful infection. It can be hypothesized here that compatibility was the result of specific recognition of host-selective toxins with NBS-LRR genes which induced cell death and facilitated infection. The lower colonization in 964a-46 compared to Eston might be associated with i) activation of the ET/JA pathway early during infection ii) transmission of a systemic signal to intact cells and priming resistance for subsequent infection, and iii) activation of the SA pathway and accumulation of large amounts of antimicrobial compounds which slowed down colonization. Only one isolate of *A. lentis* was used in this study, therefore no conclusions can be drawn on variation in the pathogen population. However, previous assessments of 964a-46 after inoculation with two isolates of *A. lentis* indicated a significant difference in reactions of this line to these isolates (Tar'an et al., 2003). Banniza and Vandenberg (2006) reported that the host reaction of 16 lentil genotypes to 65 isolates of *A. lentis* collected in Canada resulted in a continuum of severity of infection, indicating natural variation of aggressiveness in the population without any distinct pathotypes. By contrast, six pathovars of *A.*

lentis were reported in another study with 39 isolates of *A. lentis* and 22 lentil lines (Nasir and Bretag, 1997). The insight into molecular host defense mechanisms obtained in this study and molecular studies of *A. lentis* effectors supports the hypothesis of a complicated reverse gene-for-gene (toxin) model and at the same time significant diversity in the mechanisms of resistance among genotypes. Results of current and previous studies have suggested the possibility of pathovar specification in this system. Drawing a final conclusion about the nature of the *A. lentis* - lentil interaction requires integration of genomic data from host plant and those from pathogen-side.

A complicated signal transduction network was present downstream of the pathogen recognition machinery that involved phytohormones SA, JA/ET, ABA and MAPK signaling pathways. Results suggest the possibility of a hijacking of the ABA pathway by *A. lentis*. Taking into account the suppression of the SA and JA/ET pathways by ABA, this could be an efficient mechanism of virulence in *A. lentis*. CDC Robin possessed an ABA suppression mechanism which might impede the deteriorating effect of ABA in this genotype, thereby allowing it to largely escape infection.

6.3. Suggestions for future research

Allelism tests suggested the presence of non-allelic AB resistance genes in the resistant genotypes used in the breeding programs at the CDC. A QTL analysis was only conducted for the LR-18 population, the only population with a genetic linkage map. QTL analysis of LR-03 (ILL 1704 × ILL 7537) and comparison between AB resistance QTLs detected in LR-18 and LR-03 (using anchor markers) could be used in future for confirming the allelism tests. Results confirmed successful transfer of AB immunity genes present in L-01-827A to cultivated lentil. QTL analysis conducted using LR-59 interspecific linkage map identified QTLs where there were >10 cM between loci probably due to segregation distortion (Kirstin Bett, pers. comm.). Introgression of AB immunity genes through backcross breeding can be accelerated by using a closely linked marker.

Results presented here could not conclusively determine the life style of *A. lentis*. Previous microscopic studies suggested the disruption of cell protoplast before colonization by the pathogen (Roundhill et al., 1995), suggesting a necrotrophic life style of this pathogen. The power of the current microscopic study was not enough to exclude the presence of a short hemibiotrophic life

style. Future research, involving the application of different staining protocols will address this. Previous studies suggested the activation of the SA signaling pathway during the biotrophic phase in hemibiotrophs (Ding et al., 2011). Therefore, the activation of SA only in 964a-46 could not be associated with a hemibiotrophic life-style of the pathogen. Future availability of genomic data for *A. lentis* effectors might also improve the understanding of this pathosystem.

Line 964a-46 was different from Eston in terms of activating the SA pathway and an earlier activation of ET. The time difference between the activation of the ET pathway in Eston and 964a-46 was about 30 h, sufficient for the pathogen to progress with the infection before activation of the JA/ET associated defense responses in Eston. Reverse genetics studies and exogenous application of SA and JA analogues might be used in future to address the hypotheses made for the role of each of these signaling pathways in resistance of lentil to AB.

RNA-seq analysis was applied to find candidate AB resistance genes responsible for diverse mechanisms of defense in CDC Robin and 964a-46. Conducting RNA-seq analysis using additional replicates is required to confirm the reproducibility of postulated candidate genes. In the meantime, one step forward is to find polymorphism in these candidate genes and use LR-18 mapping population to map them. Linkage between AB resistance QTLs detected in Chapter 3 and these candidate genes can support the involvement of these genes in lentil resistance to AB. It may shorten the list of candidate genes however; the final conclusion concerning the role played by each of candidate genes can only be drawn using reverse genetic tools such as mutation analysis and gene silencing.

6.4. Conclusions

Evidence from the allelism tests of RIL populations indicated that genes conditioning AB resistance in lentil genotypes CDC Robin, ILL 7537, 964a-46 and ILL 1704 were non-allelic. The transfer of AB resistance genes, available in *L. ervoides* L-01-827A, to hybrid lines through the development of interspecific hybrid RILs was confirmed in this study. Evidence from QTL analysis confirmed that CDC Robin and 964a-46 carried two distinct AB resistance QTLs.

Genotypes were different in their expression of genes related to the SA and JA signaling pathways. Infection by *A. lentis* caused intensive activation of the SA-related genes in 964a-46, suggesting major differences between this and the other genotypes. The JA pathway could explain

differences among genotype resistance levels defined in the pathogenicity tests. Microscopy studies suggested that *A. lentis* growth was inhibited in CDC Robin probably by preventing cell death induced by pathogen effectors; however this mechanism could not explain the differences between 964a-46 and Eston.

Using RNA-seq analysis, the expression profile after infection by *A. lentis* of two AB resistant genotypes and the susceptible genotype Eston was compared. Results suggested major difference among resistant genotypes at different layers of the plant defense machinery, including pathogen recognition, an interwoven network of defense signaling pathways, cell death regulation, and reinforcement of the plant cell wall and the expression of antimicrobial PR proteins.

In conclusion, results imply the co-existence of two strategies of resistance in lentil against *A. lentis*; one inhibited the colonization of the plant by preventing pathogen-induced cell death, present in CDC Robin but absent in 964a-46. The second decreased the colonization of tissue by limiting the growth of the pathogen after successful penetration of dead cells and was specific to 964a-46. The results suggest that 964a-46 and CDC Robin mediated resistance to AB through different resistance mechanisms, making them ideal candidates for resistance gene pyramiding. Gene pyramiding could be accelerated using closely linked markers to resistance genes identified by QTL analysis.

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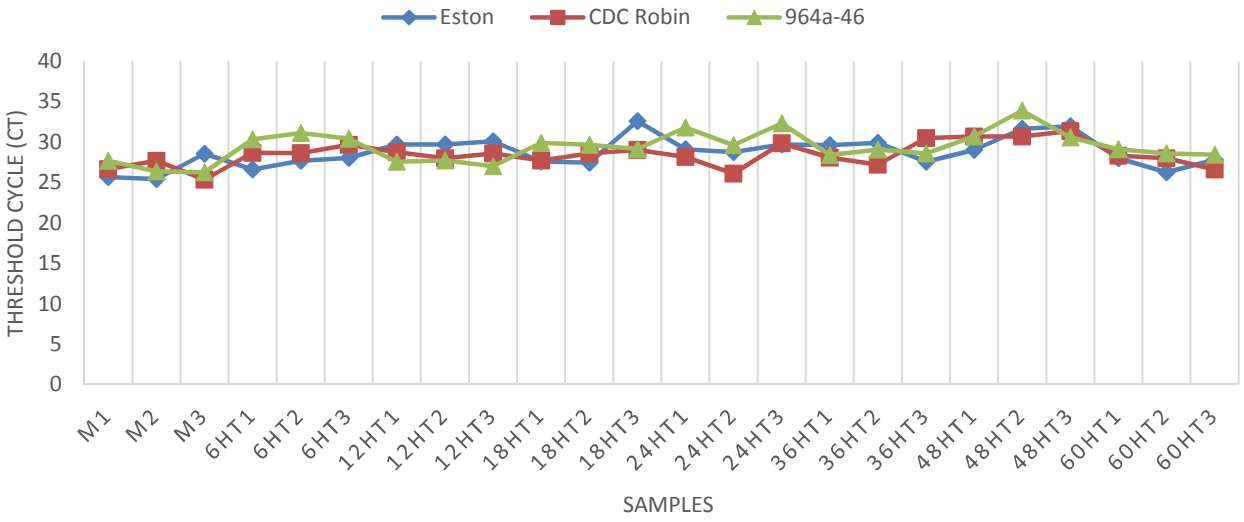
APPENDICES

Appendix 1. Results of analysis of variance obtained from Proc mixed of SAS for the expression analysis of *PR-1a*, *PR-5*, *PR-4a* and *AOC* using qRT-PCR

Effects	Genes name											
	PR-1a			PR-5			PR-4a			AOC		
	DF	F	P value	DF	F	P value	DF	F	P value	DF	F	P value
Genotype	2	4.5	0.0175	2	2.7	0.0786	2	0.6	0.5530	2	32.5	<.0001
Hpi	6	110.0	<.0001	6	69.2	<.0001	6	28.5	<.0001	6	71.0	<.0001
Genotype×hpi	12	16.8	<.0001	12	13.7	<.0001	12	3.2	0.0027	12	13.7	<.0001

Appendix 2. Threshold cycle (CT) values obtained for *β-actin* gene, used for normalization of qRT-PCR data of PR-1a gene. CT values are reported for mock (M) and infected samples collected at 6, 12, 18, 24, 36, 48 and 60 hpi in three biological replicates.



Appendix 3. Primer pairs used for assessment of expression levels of selected candidate genes by qRT-PCR

gene name	Sequence 5'->3'
ABI1b	F: ATCCGAGGTACAATCGCAAC
	R: CCTTGGAAACGAAACAGGAG
DDB1-CUL4	F: CTCATCCACAGGGAACAAAAA
	R: GATTAGGTGACGAGGGCAAA
PRH	F: TCATCTGAGGGCCATTCTTC
	R: CATTCCTCCTGGAGACCAAG
Pto	F: GAGTTAAAATCGCCGTTGGA
	R: TCCAAGAACACGGGTAGAATG
RGA1	F:AGGAAAGAACGCTTGACTGG
	R: ACGGCTAGTAGCTGGGAATG
RGA71	F: ACCCAACGATTTTGATCAGG
	R: ATCTCCAATGGACGGGTGTA

Appendix 4. Output summary generated by TopHat software of mapping Illumina reads against the *Lens culinaris* cv. CDC Redberry genome

Genotypes	samples	high-quality paired-end reads	Reads mapped to reference genome	%	Reads mapped uniquely to reference genome	%
Eston	M	8775806	8032644	91.5	7431349	84.7
	6h	10893300	9730089	89.3	9001605	82.6
	12h	7060316	6399674	90.6	5870057	83.1
	18h	7549602	6961493	92.2	6398986	84.7
	24h	6724400	6130984	91.2	5693931	84.7
	36h	13169988	11906877	90.4	11024060	83.7
	48h	5598228	5034382	89.9	4669141	83.4
	60h	12756367	11541071	90.5	10687367	83.8
	Average	9066001	8217152	90.7	7597062	83.8
	Total	72528007	65737214	-	60776496	-
CDC Robin	M	7525507	6836741	90.8	6347415	84.3
	6h	15013507	13685136	91.2	12570779	83.7
	12h	6286050	5702624	90.7	5274034	83.9
	18h	6307549	5688398	90.2	5188774	82.3
	24h	7948399	7505332	94.4	6686352	84.1
	36h	6722494	6084783	90.5	5594456	83.2
	48h	7340280	6615538	90.1	6115844	83.3
	60h	5887879	5293015	89.9	4839965	82.2
	Average	7878958	7176446	91.0	6577202	83.4
	Total	63031665	57411567	-	52617619	-
964a-46	M	10262515	9305129	90.7	8664323	84.4
	6h	5521013	5036930	91.2	4600664	83.3
	12h	6195310	5572593	89.9	5055440	81.6
	18h	16085923	14696895	91.3	12865561	80.0
	24h	8905902	8032511	90.2	7375933	82.8
	36h	4754922	4276008	89.9	3919104	82.4
	48h	8863682	7980617	90.0	7330438	82.7
	60h	8471446	7682607	90.9	7133521	84.2
	Average	8632589	7822911	90.5	7118123	82.7
	Total	69060713	62583290	-	56944984	-

Appendix 5. Expression levels of house-keeping genes in lentil genotypes Eston (a) CDC Robin (b) and 964a-46 (c) relative to mock-inoculated control. All the changes in house-keeping genes possessed non-significant false discovery rate P values. Identical gene name represents different copies of selected housekeeping genes found in RNA-seq analysis output.

a)

Gene name	Hours post inoculation						
	6	12	18	24	36	48	60
GAPDH[a]	-0.32	0.65	1.12	0.28	0.46	0.57	0.98
α-tubulin	-1.11	-1.24	0.08	-1.62	-1.76	-1.42	-0.98
α-tubulin	0.04	-0.75	-0.82	-0.60	-1.16	-0.59	-1.55
β-tubulin	-0.89	-1.54	-0.87	-0.55	-1.20	-0.54	-0.52
β-tubulin	-0.44	-0.72	-0.07	-0.60	-1.14	-1.29	-1.87
DNAj Chaperon	0.08	0.13	0.16	-0.11	0.28	0.10	-0.11
DNAj Chaperon	-0.26	0.05	-0.78	0.51	-0.49	-0.48	0.65
DNAj Chaperon	1.26	0.84	1.56	1.20	0.75	0.20	-1.74
TEF[b]	1.17	1.39	1.27	0.78	1.03	1.41	0.86
TEF	1.76	1.51	0.10	1.34	0.69	1.38	0.15
TEF	-0.59	-0.42	-1.26	-1.67	-0.34	-0.20	-0.14

b)

Gene name	Hours post inoculation						
	6	12	18	24	36	48	60
GAPDH[a]	-0.58	0.01	0.27	-0.17	0.56	-0.07	0.01
α-tubulin	-0.90	0.89	-1.50	0.05	0.85	-0.58	0.63
α-tubulin	-0.20	-0.69	-1.70	-1.74	-1.08	-1.26	-1.5
β-tubulin	-1.68	-1.14	-1.10	-1.24	-0.86	-1.04	-0.33
β-tubulin	-0.79	-0.88	-0.18	-1.44	-0.83	-0.93	-2.74
DNAj Chaperon	0.33	0.16	0.47	0.60	0.30	0.27	-0.14
DNAj Chaperon	0.01	-1.90	0.50	0.80	-1.65	-0.81	0.73
DNAj Chaperon	1.01	1.08	0.45	0.20	0.35	1.40	-1.02
TEF[b]	-0.29	0.91	0.77	1.30	1.08	0.61	0.11
TEF	-0.51	-0.67	-1.06	-0.13	-0.51	-0.21	-2.14
TEF	-1.47	-0.62	-0.96	-0.03	-0.56	-0.50	-0.22

c)

Gene name	Hours post inoculation						
	6	12	18	24	36	48	60
GAPDH[a]	-0.39	0.26	0.14	0.13	-0.46	-0.39	1.22
α-tubulin	0.41	0.42	0.35	1.89	0.97	0.02	0.07
α-tubulin	0.67	-0.19	-0.65	-0.69	-1.34	-1.50	0.67
β-tubulin	-0.78	-0.86	-1.17	-1.67	-1.01	-1.24	0.33
β-tubulin	-0.63	-0.43	-1.02	-1.14	-0.96	-1.02	-1.56
DNAj Chaperon	-0.02	-0.09	0.08	0.25	0.36	0.04	2.23
DNAj Chaperon	0.20	0.17	0.38	0.80	0.40	-0.55	0.02
DNAj Chaperon	0.28	0.24	0.50	0.71	0.77	0.43	0.12

TEF[b]	0.13	0.24	0.14	1.19	1.17	1.01	-0.08
TEF	0.23	-0.18	-0.55	0.98	-0.94	-0.32	0.37
TEF	-1.09	-0.95	-0.74	-0.30	-0.94	-0.48	-0.36

[a] Glyceraldehyde-3-Phosphate Dehydrogenase, [b] Translation elongation factor. [c] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control.

Appendix 6. Highly up-regulated defense response genes commonly expressed among lentil genotypes Eston, CDC Robin and 964a-46.

Highly up-regulated defense response genes commonly expressed among genotypes included PAMP receptors, genes associated with ABA signaling, oxidative burst, flavonoid biosynthesis pathway and PR proteins (Table 1). The PAMP receptors included receptor- like protein kinases such as *flagellin sensitive 2* (*FLS2*) and *somatic embryogenesis receptor kinase* (*SERK*). *FLS2* was characterized as a PAMP receptor for bacterial flagellin (Gómez-Gómez and Boller, 2000) and has been shown to induce PTI in collaboration with *BAK1*, the other well-characterized PAMP receptor (Chinchilla et al., 2007). Gene orthologous to *FLS2* has not yet been characterized in fungal-plant interactions. However, *FLS2*-triggered defense responses conferred resistance to *B. cinerea*, corroborating the presence of convergent responses downstream of PTI that are induced by fungi and bacterial (Asai et al., 2002). *SERK* is also a PAMP receptor mediating PTI in conjunction with *BAK1* (Heese et al., 2007). Research suggested the formation of a molecular complex between *SERK3/BAK1* and *FLS2* and their inevitable role in resistance to various bacteria and oomycetes (Heese et al., 2007), hence a *FLS2* and *SERK* complex may also be involved in PTI in the lentil-*A. lentis* interaction.

Some commonly expressed genes among all genotypes were those associated with ABA signaling such as *SNF1 related protein kinase* (*SNRK*), *pyrobactin Resistance like* (*PYL6*), *cyctein rich-repeat secretory protein* (*CRRS*) and *guanine nucleotide-binding protein* (*GP*). *SNRK* is a protein kinase involved in ABA signaling in *A. thaliana* (Fujii and Zhu, 2009). Disruption of triple *SNRK* genes in *A. thaliana* suggested a role in the phosphorylation of b-zip transcription factors involved in the transcription activation of ABA-responsive genes (Fujii and Zhu, 2009). The *PYL* gene family in *A. thaliana* encodes a JA-responsive protein involved in the JA-ABA cross-talk, through interaction with protein phosphatase 2C, a key regulator of ABA sensitivity in plants (Lackman et al., 2011). *CRRS* is an ABA-responsive gene found in the apoplast of poplar (*Populus alba* L.) upon abiotic stresses and infection with *Melampsora* and has a putative role in ameliorating deteriorating effects of various stresses (Pechanova et al., 2010). *GP* is involved in the transduction of hormone and light signals from numerous receptors with a proposed role in the ABA signal transduction pathway (Weiss et al., 1994; Pandey et al., 2006). An ortholog of this gene negatively regulated the ABA pathway in *A. thaliana* by altering ABA sensitivity and was

required for negative regulation of ABA, occurring during seed germination and early development of seedlings (Pandey et al., 2006).

Table 1. Sequence description and expression levels of highly up-regulated genes commonly expressed in lentil genotypes Eston, CDC Robin and 964a-46 after *Ascochyta lentis* infection

Sequence description[a]	Gene symbol[b]	Genotypes					
		Eston		CDC Robin		964a-46	
		Peak time	Log_2 fold change[c]	Peak time	Log_2 fold change	Peak time	Log_2 fold change
abscisic acid receptor pyl6	*Pyl6*	6h	5.7	12h	6.3	24h	7.7
Peroxidase	*POX*	6h	3.4	6h	3.6	60h	3.1
cysteine-rich repeat secretory protein 38	*CRRS*	6h	4.2	6h	5.6	6h	4.0
chalcone synthase	*CHS*	12h	5.2	6h	5.7	6h	3.8
peroxidase 12-like	*POX12*	12h	7.6	18h	6.8	24h	7.4
glutathione s-transferase gstu6-like	*GSTU6*	12h	6.1	6h	5.3	12h	5.4
pathogenesis-related protein STH2	*PR-10*	12h	4.7	6h	4.2	6h	3.3
aba-responsive protein abr17	*PR-10*	12h	6.9	18h	7.6	12h	5.5
pathogenesis-related protein pr10	*PR-10*	12h	9.0	18h	9.1	24h	9.9
linoleate 13s-lipoxygenase	*LOX*	12h	7.1	6h	5.9	12h	5.5
acidic mammalian chitinase	*CHI*	12h	6.7	12h	5.9	6h	7.5
endo-beta-1 3-glucanase	*BG*	12h	5.7	18h	6.7	18h	5.8
isoflavone reductase	*IR*	12h	5.6	6h	5.1	12h	3.7
2-succinylbenzoate-CoA ligase	*SBCL*	12h	4.2	12h	3.3	24h	4.3
guanine nucleotide-binding protein	*GP*	12h	3.6	18h	4.7	12h	3.9
cellulose synthase-like protein e1	*CSL*	12h	3.7	12h	4.0	24h	2.8
pleiotropic drug resistance protein 3-like	*PDR*	12h	3.7	6h	4.0	6h	2.1
hypothetical protein MTR_2g007950	-	12h	3.7	24h	4.4	24H	3.6
Dihydroflavonol-4-reductase	*DFR*	12h	5.6	18h	6.0	12h	5.5
Disease resistance response protein pi49	*PR-10*	12h	7.2	6h	6.9	6h	5.5
flavonoid 3 -monooxygenase-like	*FOX*	18h	3.0	48h	3.4	36h	2.1
peroxidase 53-like	*POX-53*	24h	6.1	48h	4.5	18h	5.0
flavonoid 3 -hydroxylase	*FH*	24h	3.4	24h	2.1	48h	1.4
endoplasmic reticulum membrane	*ERM*	36h	3.3	18h	3.7	24h	5.1
snf1-related protein kinase	*SNRK*	48h	5.6	24h	5.5	24h	6.3
peroxidase 4-like	*POX4*	12h	4.4	6h	5.3	6h	4.3
somatic embryogenesis receptor kinase	*SERK*	6h	3.4	6h	4.0	6h	2.9
receptor-like serine threonine kinase fls2	*FLS2*	6h	3.7	6h	3.4	6h	3.0
phenylalanine ammonia-lyase class 3-like	*PAL*	6h	3.7	6h	3.2	6h	1.9
alcohol dehydrogenase-like 5-like	*ADH*	12h	4.3	6h	4.5	6h	2.9
f-box protein pp2-a13-like	*PP2-A13*	36h	3.7	6h	3.5	24h	5.6
hypothetical protein MTR_042s0018	-	12h	4.4	24h	4.9	24h	5.5
cytosolic fructose-1 6-bisphosphatase	*FBP*	12h	4.6	24h	4.9	24h	7.1
ethylene receptor 2-like	*ERL*	36h	3.8	48h	4.3	6h	2.7
auxin response factor	*ARF*	60h	13.9	18h	13.5	12h	13.8
potassium transporter 5-like	*KUP*	12h	5.8	18h	6.4	24h	5.6
wrky transcription factor 51-like	*WRKY-51*	36h	2.9	36h	3.2	36h	3.1
ethylene-overproduction protein 1-like	*ETO1*	36h	3.3	24h	4.0	24h	4.4

[a] Sequence description inferred from the top BLAST hit, [b] gene symbols were obtained from TAIR (the *Arabidopsis* information resource) website (http://www.arabidopsis.org). For genes with no ortholog in *A. thaliana*, gene symbols were the abbreviation of sequence description. [c] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples with that of mock-inoculated control.

A few commonly expressed genes associated with other phytohormone signals were also commonly expressed among genotypes. Two genes associated with the SA signaling were among commonly expressed genes including hypothetical protein MTR_2g007950 and *endoplasmic reticulum membrane* (*ERM*). *LOX* is involved in the first step of the JA biosynthesis pathway and mediates oxygenation of α-linolenic acid. The up-regulation of this gene in all lentil genotype suggested that regulation of JA might occur further downstream.

PR proteins commonly expressed among genotypes included numerous isoforms of the PR-10 family such as STH2, disease resistance response pi4, *aba-responsive protein 17* (*ABR17*) and others such as endo-beta-1,3-glucanase and acidic mammalian chitinase-like gene. The up-regulation of *PR-10* genes after *A. lentis* infection was previously suggested (Mustafa et al., 2009). Up-regulation of PR-10 genes was also reported in different plant species such as pea (*Pisum sativum)*, rice (*Oryza sativa*) and potato (*Solanum tuberosum*) in response to pathogens infection and abiotic stresses such as wounding and cold-hardening (McGee et al., 2001; Kav et al., 2004). Transgenic canola (*B. napus*) plants overexpressing pea PR-10 showed an augmented tolerance to salt stress (Srivastava et al., 2004). In addition, a proteome analysis of *M. truncatula* plants indicated the up-regulation of a PR-10 member in response to the soil-borne pathogen *Aphanomyces euteiches* (Colditz et al., 2005). Further studies on this system revealed a negative effect of PR-10 on the expression of other PR proteins involved in tolerance to *A. euteiches* such as PR-5b, thereby suppressing plant resistance (Colditz et al., 2007). Endo-beta-1,3-glucanase and acidic mammalian chitinase-like genes are members of the PR-2 and PR-3 families that are involved in plant defense through hydrolyzing fungal cell wall components (van Loon and van Strien, 1999).

The role of *f-box protein pp2-al3 like* (*PP2-A13*) in resistance to necrotroph *B. cinerea* was recently demonstrated (Segarra et al., 2013). This gene encoded an F-box domain, suggesting the involvement of this gene in hormonal ubiquitination process. Most of genes associated with oxidative burst such as different members of the peroxidase family (peroxidase 12 and 53), *gluthathione s-transferase 6* (*GSTU6*) and potassium transporter (*KUP*) were commonly expressed in all three genotypes, suggesting the occurrence of genotype-independent oxidative stress responses after *A. lentis* infection. Other defense response genes commonly expressed in all

genotypes were those involved in flavonoid biosynthesis, including several key enzymes in the biosynthesis of lignin and phenolic compounds.

Appendix 7. Defense response genes up-regulated upon *Ascochyta lentis* infection in lentil cv. Eston. SA=salicylic acid, JA=jasmonic acid, ET=ethylene, ABA= abscisic acid, PAMP=Pathogen Associated Molecular Patterns, ETI= effector triggered immunity.

Gene ID[a]	Seq. description[b]	ACC[c] of best hit	Peak time	log_2 fold change[d]	Function
XLOC_022171	efr3-like protein	XP_003597513	6h	15.2	PAMP-triggered immunity-receptor
XLOC_008064	abscisic acid-insensitive 5-like protein	XP_003618910	6h	14.7	ABA signaling
XLOC_010439	autophagy-related protein	XP_004505205	6h	13.6	Cell-death inhibition
XLOC_004835	abscisic acid receptor pyl6	XP_003600988	6h	5.7	ABA/JA crosstalk, *ABI1* inhibition
XLOC_019535	Peroxidase	XP_003602463	6h	3.4	Response to oxidative stress
XLOC_006674	cysteine-rich repeat secretory protein 38	XP_003543377	6h	4.2	Response to ABA
XLOC_000825	tga transcription factor	XP_003624993	6h	7.9	Transcription regulation SA signaling
XLOC_013188	leucin-rich receptor serine threonine protein kinase bak1	XP_003550961	6h	11.8	PAMP-triggered immunity-perception and signaling
XLOC_001260	chalcone synthase	XP_003621532	12h	7.2	Flavonoids synthesis
XLOC_014704	pathogenesis-related protein pr-4a (Copy#2)	XP_004495803	12h	2.7	Antifungal activities
XLOC_003128	pathogenesis-related protein pr-4a (Copy#1)	XP_004495803	12h	2.7	Antifungal activities
XLOC_007085	peroxidase 12-like	XP_004496443	12h	7.6	Response to oxidative stress
XLOC_008040	glutathione s-transferase gstu6-like	XP_003613500	12h	6.1	Response to oxidative stress
XLOC_006548	pathogenesis-related protein STH2	XP_003609710	12h	4.7	Response to wounding and elicitors
XLOC_004217	aba-responsive protein abr17 (PR-10)	XP_003594849	12h	6.9	ABA signaling
XLOC_017955	mitogen-activated protein kinase 3	XP_003610715	12h	12.2	PAMP-triggered immunity-signaling
XLOC_012879	pathogenesis-related protein pr10	XP_003594834	12h	9.0	Antifungal activities
XLOC_021764	linoleate 13s-lipoxygenase	XP_004501999	12h	7.1	JA biosynthesis
XLOC_009222	acidic mammalian chitinase	XP_004501097	12h	6.7	Antifungal activity, Chitinase class V
XLOC_005264	endo-beta-1 3-glucanase	XP_003607319	12h	5.7	Antifungal activity
XLOC_011061	isoflavone reductase	XP_003612053	12h	5.6	Flavonoids synthesis
XLOC_023581	2-succinylbenzoate-CoA ligase	XP_003599556	12h	4.2	Defense response to fungus
XLOC_023601	guanine nucleotide-binding protein	XP_003612703	12h	3.6	ABA and ET signaling
XLOC_014720	cellulose synthase-like protein e1	XP_003600354	12h	3.7	Cell-wall reinforcement
XLOC_000779	pleiotropic drug resistance protein 3-like	XP_004298258	12h	3.7	Secretion of antimicrobial metabolites
XLOC_011532	hypothetical protein MTR_2g007950	XP_003593113	12h	3.7	systemic acquired resistance (SAR)
XLOC_011956	Dihydroflavonol-4-reductase	XP_003623706	12h	5.6	Flavonoids synthesis
XLOC_006722	Disease resistance response protein pi49	XP_003594836	12h	7.2	Unknown
XLOC_003676	flavonoid 3 -monooxygenase-like	XP_003627305	18h	3	Flavonoids synthesis
XLOC_018612	peroxidase 53-like	XP_004497888	24h	6.1	Response to oxidative stress
XLOC_008039	flavonoid 3 -hydroxylase	XP_003598945	24h	3.4	Flavonoids synthesis
XLOC_000780	Pto kinase interactor	XP_003609869	36h	15.7	ETI mediated by Pto kinase
XLOC_011456	ET-responsive transcription factor wri1	XP_004489377	36h	12.6	ET signaling pathway
XLOC_022252	cyclin-dependent kinase g-2-like	XP_003601812	36h	11.4	Programmed cell-death
XLOC_013025	senescence-associated protein sag102	XP_004493689	36h	4.1	SA signaling
XLOC_006191	endoplasmic reticulum membrane	XP_004514829	36h	3.3	SAR, secretory pathway
XLOC_009624	ethylene-overproduction protein 1-like	XP_004506795	36h	3.3	ET signaling pathway
XLOC_022775	snf1-related protein kinase	XP_003608836	48h	5.6	PAMP -perception and signaling
XLOC_006002	arginine amidohydrolase	XP_006288104	48h	3.6	Defense response to *B. cinerea*
XLOC_015820	cbl-interacting protein kinase	XP_004490472	48h	12.1	Calcium signaling, Oxidative burst
XLOC_012382	f-box lrr-repeat protein	XP_003604167	60h	13.7	Ubiquitination during signaling
XLOC_009300	programmed cell death protein	XP_003606813	60h	10.1	Programmed cell-death

[a] Gene ID assigned to transcripts in Cufflink® output, [b] sequence description inferred from the top BLAST hit, [c]Ref-seq accession number for the best blast hit. [d]Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control.

Appendix 8. Defense response genes up-regulated upon *Ascochyta lentis* infection in lentil cv. CDC Robin. JA=jasmonic acid, ET=ethylene, ABA= abscisic acid, PAMP=Pathogen Associated Molecular Patterns, AOC=Allen Oxide Cyclase.

Gene ID[a]	Seq. description[b]	ACC of best hit[c]	Peak time	log_2 fold change[d]	Function
XLOC_004871	autophagy-related protein 18g-like	XP_004496058	6h	14.2	Cell-death inhibition
XLOC_004950	pleiotropic drug resistance protein	XP_003597818	6h	10.9	Secretion of secondary metabolites
XLOC_015767	cellulose synthase protein h1-like	XP_004506254	6h	9.7	Cell-wall reinforcement
XLOC_020965	linoleate 13s-lipoxygenase	XP_004501999	6h	5.9	JA signaling
XLOC_001206	chalcone synthase	XP_003621532	6h	5.71	Flavonoids synthesis
XLOC_015514	peroxidase 4-like	XP_003615995	6h	5.3	Response to oxidative stress
XLOC_006320	pathogenesis-related protein STH2-like	XP_003609710	6h	4.1	Antifungal activity
XLOC_005229	somatic embryogenesis receptor kinase	XP_004485853	6h	4.0	PAMP-triggered immunity
XLOC_020286	receptor-like serine threonine kinase fls2	XP_003532650	6h	3.4	PAMP-triggered immunity
XLOC_014882	phenylalanine ammonia-lyase class 3-like	XP_004491627	6h	3.2	lignin and flavonoids synthesis
XLOC_013982	isoflavone 2 -hydroxylase-like	XP_004509096	6h	3.0	Flavonoid synthesis
XLOC_010835	alcohol dehydrogenase-like 5-like	XP_004516585	6h	4.5	Lignin synthesis
XLOC_017493	ser thr protein kinase	XP_003624374	6h	15.2	PAMP-triggered immunity
XLOC_017656	f-box protein pp2-a13-like	XP_004499949	12h	3.5	Ubiquitination during signaling
XLOC_021944	snf1-related protein kinase	XP_003608836	12h	5.6	ABA signaling
XLOC_003872	ankyrin repeat domain-containing protein	XP_003618797	12h	12.3	PAMP-triggered immunity- signaling
XLOC_014902	f-box protein skip16	XP_003606823	12h	9.9	Ubiquitination during signaling
XLOC_015339	auxin-repressed kda protein	NP_001237812	12h	7.1	Negative regulation of ABA
XLOC_003454	hevein-like pre-protein	XP_004494704	12h	5.0	JA response gene
XLOC_006660	thaumatin-like protein	XP_003589504	12h	4.0	Antifungal activity
XLOC_000549	serine threonine-protein kinase nek6	XP_004513642	12h	15.0	PAMP/ET signaling
XLOC_005620	abscisic insensitive 1b	XP_003603175	18h	11.2	ABA signaling
XLOC_012444	pathogenesis-related protein pr10	XP_002262970	18h	9.1	Antifungal activities
XLOC_014173	pathogenesis-related protein pr-4a (Copy#1)	XP_004495803	18h	7.8	Antifungal activities
XLOC_003025	pathogenesis-related protein pr-4a (Copy#2)	XP_004495803	18h	8.6	Antifungal activities
XLOC_006869	peroxidase 12-like	XP_004496443	18h	6.8	Response to oxidative stress
XLOC_004078	aba-responsive protein abr17 (PR-10)	XP_003594849	18h	7.1	ABA signaling
XLOC_010964	endo-beta-1 3-glucanase	XP_003607319	18h	6.7	Antifungal activity
XLOC_010718	isoflavone reductase	XP_003612053	18h	5.2	Flavonoids synthesis
XLOC_003065	isoflavone 4 -o-methyltransferase-like	XP_004505107	18h	8.0	Flavonoid synthesis
XLOC_013355	xyloglucan glycosyltransferase 6-like	XP_004510233	18h	14.4	cell wall reinforcement
XLOC_009564	glutathione s-transferase-like	XP_003591636	18h	3.6	Response to oxidative stress
XLOC_014101	mitogen activated protein kinase 20-1	XP_003617448	18h	14.3	MAPK cascade
XLOC_016502	ankyrin repeat-containing protein	XP_004515011	24h	12.4	PAMP-triggered immunity
XLOC_015474	hypothetical protein MTR_042s0018	XP_003636475	24h	4.9	Response to oxidative stress
XLOC_001686	cytosolic fructose-1 6-bisphosphatase	XP_003593121	24h	4.9	Response to abscisic acid
XLOC_009336	ethylene-overproduction protein 1-like	XP_004506795	24h	4.0	ET signaling

XLOC_009997	mitogen-activated protein kinase MK2	XP_004503681	24h	14.3	MAPK cascade
XLOC_003546	receptor-like protein kinase	XP_003614437	24h	14.9	PAMP-receptor
XLOC_010332	map kinase-like protein	XP_003617971	24h	11.6	MAPK cascade
XLOC_019682	2-hydroxyisoflavanone synthase	XP_004505108	36h	7.53	Flavonoid synthesis
XLOC_009078	pentatricopeptide repeat protein	XP_003602712	36h	10.7	Negative regulation of ABA signaling
XLOC_012925	hva22-like protein i	XP_004494238	36h	15.6	Response to ABA signaling
XLOC_017987	peroxidase 53-like	XP_004497888	48h	4.5	Response to oxidative stress
XLOC_011462	g-type lectin s-receptor-like serine threonine-protein kinase at4g03230-like	XP_004513553	48h	13.6	PAMP-triggered immunity, ABA signaling
XLOC_003499	poly polymerase-like	XP_004501091	48h	12.6	Negative regulation of hypersensitive response, response to ET and ABA
XLOC_014043	isoflavone reductase protein	XP_003612049	60h	3.5	Flavonoid synthesis
XLOC_010127	autophagy-related protein	XP_004505205	60h	11.5	Cell-death inhibition

[a] Gene ID assigned to transcripts in Cufflink® output, [b] sequence description inferred from the top BLAST hit, [c]Ref-seq accession number for the best blast hit. [d] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control.

Appendix 9. Defense response genes up-regulated upon *Ascochyta lentis* infection in lentil genotype 964a-46. SA=salicylic acid, JA=jasmonic acid, ET=ethylene. ABA= abscisic acid, SAR=Systemic acquired resistance.

Gene ID[a]	Seq. description[b]	ACC of best hit[c]	Peak time	log_2 fold change[d]	Function
XLOC_002873	ethylene-responsive transcription factor 1b	XP_004495104	6h	17.4	ET signaling
XLOC_003898	ddb1- and cul4-associated factor	XP_003597148	6h	12	Ubiquitination, SA signaling, SAR
XLOC_015940	ethylene receptor-like	XP_003605421	6h	10.8	ET signaling pathway
XLOC_016680	peroxidase 4-like	XP_003615995	6h	4.3	Response to oxidative stress
XLOC_012150	ethylene receptor 2-like	XP_004495309	6h	2.7	ET signaling pathway
XLOC_012407	isoflavone reductase homolog	XP_004509553	6h	3.5	Flavonoids synthesis
XLOC_013875	cbl-interacting protein kinase	XP_003616004	6h	14.1	Calcium signaling, Oxidative burst
XLOC_016372	pleiotropic drug resistance protein 1-like	XP_003588699	12h	8.7	Secretion of secondary metabolites
XLOC_020877	auxin response factor	XP_003597256	12h	13.8	ABA signaling pathway
XLOC_010966	gaga-binding transcriptional activator	XP_003589387	12h	11.3	ET signaling pathway
XLOC_013107	glutathione s-transferase f9-like	XP_004499970	12h	3.5	Response to oxidative stress
XLOC_005624	endo-beta-1 3-glucanase	XP_003607319	18h	5.2	Antifungal activities
XLOC_019286	peroxidase 53-like	XP_004497888	18h	5	Response to oxidative stress
XLOC_021664	mitogen-activated protein kinase kinase	XP_003604135	18h	12.5	PAMP-triggered immunity-signaling
XLOC_003234	pathogenesis-related protein pr-4a (copy#1)	XP_004495803	24h	9.36	Antifungal activities
XLOC_007321	peroxidase 12-like	XP_004496443	24h	7.4	Response to oxidative stress
XLOC_013379	pathogenesis-related protein pr10	XP_003594834	24h	9.9	Antifungal activities
XLOC_020603	Peroxidase	XP_003596717	24h	5.8	Response to oxidative stress
XLOC_005074	potassium transporter 5-like	XP_004508966	24h	5.6	Ion flux, oxidative burst
XLOC_001307	chalcone synthase	XP_003592044	24h	4.6	Flavonoids synthesis
XLOC_024314	2-succinylbenzoate- ligase	XP_003599556	24h	4.3	Defense response to fungus
XLOC_013915	hva22-like protein i	XP_004494237	24h	12.4	ABA signaling response
XLOC_021257	Heat shock protein	XP_003611429	24h	11.6	defense response against fungi
XLOC_018932	f-box protein pp2-a13-like	XP_004499949	24h	5.6	Ubiquitination during signaling
XLOC_012965	f-box family-3	XP_003608305	24h	5.5	Ubiquitination during signaling
XLOC_009985	ethylene-overproduction protein 1-like	XP_004506795	24h	4.4	ET signaling pathway
XLOC_007089	thaumatin-like protein	XP_003589504	24h	4.1	Antifungal activity
XLOC_006601	pathogenesis-related homeodomain	XP_004487993	36h	16.2	Transcription regulation of defense
XLOC_021008	myb-like dna-binding protein bas1	XP_004512739	36h	10.3	ABA signaling pathway
XLOC_018116	ethylene-responsive transcription factor	XP_004491090	36h	11.0	ET signaling pathway
XLOC_016503	auxin-repressed kda protein	NP_001237812	36h	7.5	Negative regulation of ABA signaling
XLOC_022622	callose synthase 11-like isoform x1	XP_004485779	36h	11.4	cell-wall reinforcement
XLOC_019040	map kinase homolog ntf6-like	XP_003606524	36h	12.9	PAMP-triggered immunity-signaling
XLOC_014515	wrky transcription factor 51-like	XP_004502873	36h	3.1	SA/JA crosstalk

XLOC_004067	pathogenesis-related protein 1a	XP_003545770	36h	11.3	SA defense responses
XLOC_013533	senescence-associated protein sag102	XP_004493689	48h	5.7	SA signaling
XLOC_020283	f-box fbd lrr-repeat protein at3g14710-like	XP_004488684	60h	18.2	Ubiquitination during signaling

[a] Gene ID assigned to transcripts in Cufflink® output, [b] sequence description inferred from the top BLAST hit, [c] Ref-seq accession number for the best blast hit. [d] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control.

Appendix 10. Candidate defense response genes differentially expressed among lentil genotypes upon *A. lentis* infection

NBS-LRR genes differentially expressed after *A. lentis* infection among lentil genotypes

Due to the crucial role of NBS-LRR genes in plant-pathogen interactions and considering the relative abundance of these genes among DEGs, the data for this group were analyzed separately. Results indicated that a relatively higher number of these genes were induced by pathogen infection in the susceptible genotype Eston compared to the other two genotypes (Figure 5.3.A). Only two NBS-LRR genes were up-regulated in all three genotypes, suggesting that differences exist among genotypes in recognition of *A. lentis* effectors. The number of NBS-LRR genes shared between Eston and 964a-46 and Eston and CDC Robin was equal (five genes), whereas CDC Robin and 964a-46 only shared two genes.

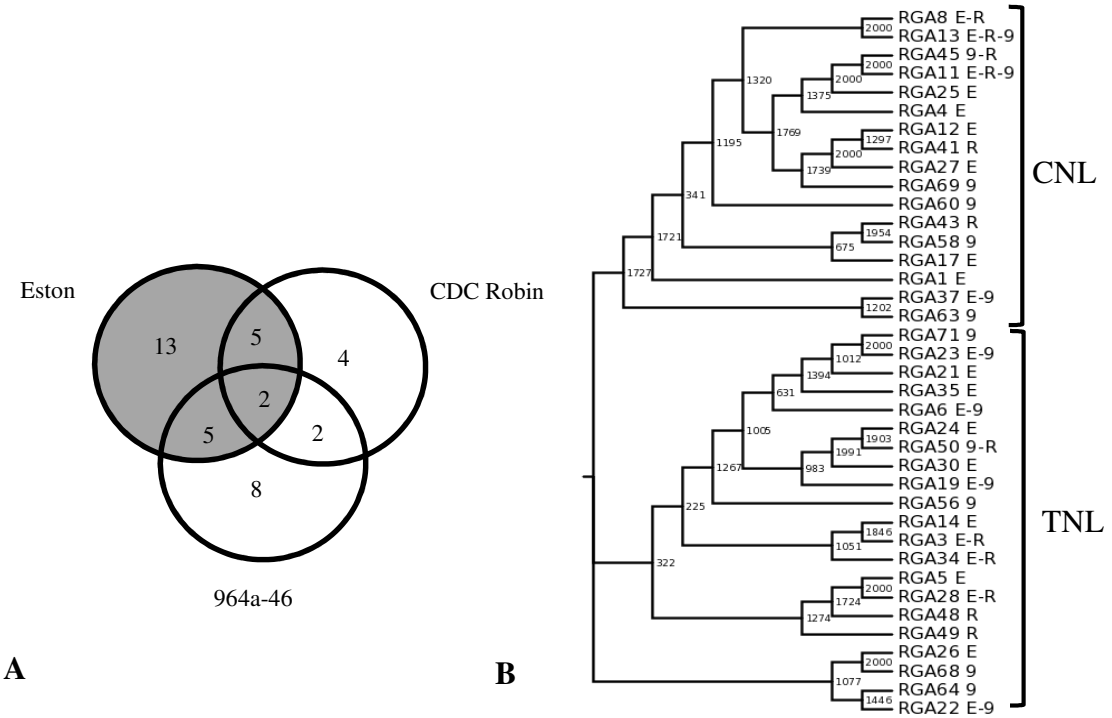


Fig 1. Differentially expressed NBS-LRR genes induced upon *Ascochyta lentis* infection in lentil genotypes Eston, CDC Robin and 964a-46. (A) Venn diagram showing the number of differentially expressed NBS-LRR genes and their distribution among the lentil genotypes. (B) Sequence homology analysis conducted using the protein sequence of NBS domains for

differentially expressed NBS-LRR genes. E, R and 9 represents differentially expressed genes in Eston, CDC Robin and 964a-46, respectively. Numbers on the tree branches represent boot strap scores calculated by ClustalX® software. TNL and CNL represent TIR-domain and CC-domain types of NBS-LRR genes, respectively.

Sequence homology analysis suggested the existence of two main groups of NBS-LRR genes with TIR-domain and CC-domain, providing some evidence of the accuracy of domain isolation and sequence homology analysis (Figure 5.3.B). Genes with homology to *N* R-gene were very frequent. These were up-regulated in Eston and 964a-46 and shared between Eston and CDC Robin, but were otherwise absent in CDC Robin (Table 1). *N* is a TIR-NBS-LRR resistance gene, mediating resistance against TMV in tobacco through the induction of a hypersensitive reaction (Marathe et al., 2002). The occurrence of *N* ortholog only among DEGs induced in Eston and 964a-46 genotypes, both of which responded with cell death to *A. lentis* infection (see Chapter 4), suggested that these genes may be involved in the compatible interaction.

All NBS-LRR genes shared between Eston and CDC Robin had contrasting expression levels except for *RGA34*, for which peak expression levels occurred at different times after inoculation in these genotypes (Table 1). *RGA3* and *RGA4* were up-regulated in Eston but down-regulated in CDC Robin. Similar contrasting expression levels were also observed for *RGA6*, *RGA19* and *RGA37*, three RGAs shared between Eston and 964a-46. By contrast, the expression of two NBS-LRR genes shared between CDC Robin and 964a-46 were up-regulated upon pathogen infection in both genotypes, although the expression levels peaked earlier in CDC Robin than 964a-46. The expression of *RGA11* was down-regulated in Eston and up-regulated in both resistant genotypes, whereas contrasting expression was not observed for *RGA13,* the other NBS-LRR gene shared among all three genotypes.

Table 1. Expression levels of NBS-LRR genes up-regulated upon *Ascochyta lentis* infection in lentil genotypes Eston, CDC Robin and 964a-46.

Gene name	Gene ID[a]	Seq. description[c]	Peak time	Log_2 fold change[d]
Eston				
RGA12	XLOC_010480	nbs resistance protein	12h	14.5
RGA14	XLOC_001857	tmv resistance protein n-like	12h	13.0
RGA17	XLOC_011776	disease resistance rpp13-like protein	12h	4.8
RGA5	XLOC_003044	tmv resistance protein n-like	18h	10.5
RGA21	XLOC_016994	cc-nbs-lrr resistance protein	18h	13.0
RGA4	XLOC_009244	cc-nbs-lrr resistance protein	24h	11.0
RGA24	XLOC_020622	nbs resistance protein	24h	12.6
RGA25	XLOC_005511	cc-nbs-lrr resistance protein	24h	10.3
RGA27	XLOC_014883	nbs-lrr resistance protein	24h	10.1
RGA1	XLOC_022532	nbs-containing resistance-like protein	36h	13.9
RGA26	XLOC_006729	resistance protein	36h	10.8
RGA30	XLOC_001306	nbs resistance protein	36h	11.0
RGA35	XLOC_016995	tmv resistance protein n-like	48h	12.2
CDC Robin				
RGA43	XLOC_017292	nbs-containing resistance-like protein	18h	11.7
RGA49	XLOC_007325	tir-nbs-lrr disease resistance protein	24h	10.9
RGA41	XLOC_014194	disease resistance rpp13-like protein	48h	15.3
RGA48	XLOC_014767	tir-nbs-lrr disease resistance protein	48h	15.3
964a-46				
RGA56	XLOC_005904	tir-nbs-lrr resistance protein	12h	14.8
RGA68	XLOC_011347	resistance protein	12h	15.6
RGA69	XLOC_023616	nbs-lrr resistance protein	18h	12.4
RGA60	XLOC_021221	resistance protein	36h	13.0
RGA63	XLOC_020855	cc-nbs-lrr resistance protein	48h	4.6
RGA58	XLOC_020905	disease resistance protein rpm1-like	60h	11.5
RGA64	XLOC_012096	disease resistance-like protein	60h	13.9
RGA71	XLOC_013741	tmv resistance protein n-like	48h	13.7
Eston-CDC Robin				
RGA3				
Eston	XLOC_006880	tir-nbs-lrr resistance protein	24h	13.0
CDC Robin	XLOC_006664		60h	-7.8
RGA8				
Eston	XLOC_022229	nbs-lrr type disease resistance protein	48h	7.5
CDC Robin	XLOC_021419		36h	-14.1
RGA28				
Eston	XLOC_020814	tmv resistance protein n-like	24h	2.8
CDC Robin	XLOC_020070		48h	9.6
RGA32				
Eston	XLOC_006592	tmv resistance protein n	36h	2.5
CDC Robin	XLOC_006363		12h	11.8
RGA34				
Eston	XLOC_020498	disease resistance protein	48h	14.5
CDC Robin	XLOC_019781		24h	14.5
Eston-964a-46				
RGA6				
Eston	XLOC_014501	tmv resistance protein n-like	6h	9.1
964a-46	XLOC_015056		60h	-11.5
RGA19				
Eston	XLOC_004928	tir-nbs-lrr rct1-like resistance protein	48h	-13.5

964a-46	XLOC_005097		12h	11.2
RGA22				
EStON	XLOC_003983	resistance protein	18h	12.4
964a-46	XLOC_004121		18h	14.4
RGA23				
Eston	XLOC_013232	tmv resistance protein n-like isoform	24h	13.8
964a-46	XLOC_013744		24h	2.8
RGA37				
Eston	XLOC_012650	cc-nbs resistance protein	48h	-16.0
964a-46	XLOC_013140		18h	8.2
CDC Robin-964a-46				
RGA45				
CDC Robin	XLOC_021889	cc-nbs-lrr resistance protein	24h	11.1
964a-46	XLOC_023433		12h	12.1
RGA50				
CDC Robin	XLOC_012240	nbs-lrr resistance protein	36h	11.2
964a-46	XLOC_013153		48h	8.0
Eston-CDC Robin-964a-46				
RGA11				
Eston	XLOC_022720	cc-nbs-lrr resistance protein	6h	-12.0
CDC Robin	XLOC_021889		24h	11.1
964a-46	XLOC_023433		12h	12.1
RGA13				
Eston	XLOC_001518	nbs-lrr type disease resistance protein	12h	13.5
CDC Robin	XLOC_001457		48h	10.4
964a-46	XLOC_001572		12h	9.3

[a] Gene ID assigned to transcripts in Cufflink® output, [c] sequence description inferred from the top BLAST hit, [d] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control.

Highly up-regulated defense response genes differentially expressed among lentil genotypes upon *A. lentis* inoculation

A series of PTI-associated genes including receptors and signaling components were differentially expressed among genotypes. PTI-associated genes exclusively induced in Eston included *BAK1*, *EFR3* and *CDK* (Table 5.2). *BAK1* deficient plants lost their ability to contain programmed cell death and then were more susceptible to necrotrophic pathogens (Kemmerling et al., 2007). The peak of *BAK1* expression occurred at 6 hpi in Eston and declined soon after that. This suggested that *BAK1* expression might be suppressed by pathogen virulence effectors targeting the PTI immunity pathway. *NEK6*, *RLK, LERCK* and *RSTK* were receptor like protein kinases up-regulated exclusively in CDC Robin. *NEK6* is a serine/threonine protein kinase previously shown to be induced by 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene which is involved in stress response in *A. thaliana* (Zhang et al., 2011). Analogues of

RLK and *RSTK* have also been characterized either as PAMP receptors or resistance genes eg. *Xa21* and *PBS1* (Wang et al., 1996; Swiderski and Innes, 2001). *LERCK* is an important member of the receptor like protein kinases with a trans-membrane lectin domain, initially characterized in legumes and is required for β-aminobutyric acid (BABA) mediated priming of resistance in *A. thaliana* (Singh et al., 2012). *CBI interaction protein kinase* (*CIPK*) was highly up-regulated in Eston and 964a-46, but remained unchanged in CDC Robin. This gene encodes a protein kinase with calcium binding domain and is involved in ROS-mediated calcium signaling (Kolukisaoglu et al., 2004). An ortholog of *Pto* was among the genes only up-regulated in Eston. This gene encodes a protein kinase which is involved in the specific recognition of *AvrPto* secreted from *P. syringae* pv. *tomato* in tomato, however kinase activities have not been reported for this gene (Oh and Martin, 2011).

Several orthologs of MAPK genes were differentially expressed among genotypes. MAPK is a common signaling pathway in eukaryotes and is responsible for transduction of abiotic and biotic stresses in plants (Pedley and Martin, 2005). This pathway transfers signals generated from both NBS-LRR and PAMP receptors during plant immunity responses, turning on the expression of many downstream defense pathways such as ET synthesis, ROS production, PR gene expression and cell death (Pedley and Martin, 2005). All of *MAPKs* except for *MAPK3* were up-regulated in one or both of the resistant genotypes, suggesting the association between MAPK activities and resistance to AB in lentil. *MEK2* was only up-regulated in CDC Robin. *MEK2* has a function in protein phosphorylation during PAMP signaling and is interacting with SNRK which was uniformly induced in all genotypes in the present study.

A few differentially expressed genes were associated with protein-protein interaction including *AKR*, *DDB1-CUL4*, and three F-box genes including *SKIP16*, *FBD* and *FLR*. An ortholog of *AKR* gene was up-regulated in CDC Robin but not in the other genotypes. Orthologs of *AKR* play a role in protein-protein interaction in plants and are key part of hormonal signaling in the development of resistance in plants. NPR1 and non-expressor of PR1 and non-inducible immunity1 (NIM1) are examples of ankyrin repeat domain-containing proteins which are involved in the regulation of plant defense to numerous pathogen through the SA/JA crosstalk (Cao et al., 1997; Spoel et al., 2003). *DDB1-CUL4*, *FBD* and *SKIP16* were also differentially expressed among lentil genotypes. These genes are involved in the regulation of various signaling pathways at translational levels.

DDB1-CUL4 and *FBD* were exclusively up-regulated in 964a-46, while *SKIP16* expression was specific to CDC Robin.

Table 2. Sequence description and expression levels of highly up-regulated genes differentially expressed among lentil genotypes Eston, CDC Robin and 964a-46 after *Ascochyta lentis* infection

Seq. description[a]	Gene symbol[b]	Genotypes					
		Eston		CDC Robin		964a-46	
		Peak time	Log_2 fold change[c]	Peak time	Log_2 fold change	Peak time	Log_2 fold change
serine threonine-protein kinase nek6-like	*NEK6*	12h	1.0	12h	15.0	36h	1.0
receptor-like protein kinase	*RLK*	48h	2.0	24h	14.9	60h	0.9
ser thr protein kinase	*RSTK*	18h	6.0	6h	15.2	12h	4.5
Pto kinase interactor	*PTO*	36h	15.7	24h	2.3	48h	-0.2
g-type lectin s-receptor-like serine threonine-protein kinase	*LECRK*	12h	4.6	48h	13.6	18h	-0.1
leu-rich receptor serine threonine protein kinase bak1	*BAK1*	6h	11.8	12h	1.9	6h	1.0
efr3-like protein	*EFR3*	6h	15.2	36h	1.2	24h	0.8
cbl-interacting protein kinase	*CIPK*	48h	12.1	24h	3.0	6h	14.1
mitogen-activated protein kinase 3 (ERK1)	*MAPK3*	12h	12.2	6h	-0.5	6h	-0.3
mitogen activated protein kinase 20-1	*MAPK 20-1*	36h	3.4	18h	14.3	12h	11.2
map kinase-like protein	*MAPKL*	36h	1.3	24h	11.6	24h	1.1
map kinase homolog ntf6-like	*MAPK-ntf6*	24h	0.5	36h	1.0	36h	12.9
mitogen-activated protein kinase kinase	*MAPKK*	12h	2.0	6h	2.3	18h	12.5
mitogen-activated protein kinase MEK2	*MEK2*	18h	0.2	24h	14.3	6h	1.1
ET-responsive transcription factor 1b-like	*ERF1b*	12h	3.4	6h	1.5	6h	17.4
ET-responsive transcription factor wri1	*WRI1*	36h	12.6	18h	14.2	6h	1.7
ET-responsive transcription factor	*ERF*	18h	0.5	36h	1.1	36h	11.0
ethylene receptor-like	*ER*	36h	1.7	24h	9.7	6h	10.8
gaga-binding transcriptional activator	*GAGA-TF*	36h	0.3	12h	0.1	12h	11.3
abscisic insensitive 1b	*ABI1b*	24h	0.9	18h	11.2	36h	-0.7
abscisic acid-insensitive 5-like protein	*ABI5*	6h	14.7	60h	0.1	60h	0.9
pentatricopeptide repeat-containing protein	*PRP*	36h	-0.4	36h	10.7	24h	2.1
f-box protein skip16	*SKIP16*	18h	0.3	12h	9.9	12h	0.2
f-box fbd lrr-repeat protein at3g14710-like	*FBD*	60h	3.4	60h	4.8	60h	18.2
f-box lrr-repeat protein	*FLR*	60h	13.7	18h	0.7	36h	2.7
ddb1- and cul4-associated factor	*DDB1-CUL4*	48h	2.5	24h	-0.7	48h	12.0
ankyrin repeat domain-containing protein	*AKR*	12h	-0.6	12h	12.3	18h	-0.2
pathogenesis-related protein 1a	*PR-1a*	18h	6.8	12h	3.4	36h	11.3
Heat shock protein	*HSP*	12h	13.5	18h	1.3	24h	11.6
Hevein-like protein	*Hel*	60h	1.9	12h	8.0	12h	2.0
Hva-22	*Hva-22*	12h	0.7	36h	15.6	24h	12.4
pathogenesis-related protein pr-4a- Copy#1)	*PR-4a*	12h	2.7	18h	8.6	24h	9.4
pathogenesis-related protein pr-4a (Copy#2)	*PR-4a*	12h	2.7	18h	7.8	AB[c]	AB
thaumatin-like protein	*TLP*	12h	1.7	12h	5.9	24h	6.0
calcium-transporting atpase	*CTA*	12h	0.6	12h	0.8	36h	13.3

Programmed-cell death protein	*PDCD*	60h	10.1	60h	-0.3	18h	11.1
cyclin-dependent kinase g-2-like	*CDK*	36h	11.4	24h	0.7	24h	0.4
autophagy-related protein	*ATG*	6h	13.6	12h	12.6	6h	1.1
autophagy-related protein 18g-like	*ATG18g*	18h	1.9	6h	14.2	36h	1.8
cellulose synthase h1-like	*CESA*	12h	1.7	6h	9.7	24h	0.7
xyloglucan glycosyltransferase 6-like	*CSLC6*	6h	6.4	18h	14.4	12h	10.4
callose synthase 11-like isoform x1	*CALS*	36h	2.0	18h	1.1	36h	11.4
pathogenesis-related homeodomain	*PRH*	12h	0.7	24h	1.9	36h	16.2
tga transcription factor	*TGA*	6h	7.9	12h	1.9	48h	-0.3
myb-like dna-binding protein bas1	*MYB*	12h	10.7	60h	6.5	24h	10.3
myb-like dna-binding protein bas1	*MYB*	24h	11.0	6h	2.9	24h	10.3
poly polymerase-like	*PARP*	48h	5.7	48h	12.6	6h	0.7
arginine amidohydrolase	*ARGAH*	48h	3.6	12h	7.2	36h	1.9

[a] sequence description inferred from the top Blast hit, [b] gene symbol was obtained from TAIR (the Arabidopsis information resource) website (http://www.arabidopsis.org). For genes with no orthologs in *A. thaliana*, gene symbols were the abbreviation of sequence description. [c] Fold change in gene expression was calculated by Cuffdiff® software by dividing FPKM value of infected samples to that of mock-inoculated control. [d] Absence of this copy of PR-4a gene.

A few ABA-associated genes were also differentially expressed among genotypes including *ABI1b*, *ABI5* and *PRP*. *ABI5* was only induced in Eston (Brocard et al., 2002). *ABI1b* which is a negative regulator of ABA was not up-regulated in Eston and 964a-46, which might cause an increase in sensitivity to ABA, and activation of ABA-responsive genes through *ABI5* transcription factor activity in this genotype. *PRP* which is an ortholog of *ABO5* in *A. thaliana*, was exclusively induced in CDC Robin. The negative regulation of the ABA signaling pathway was also suggested for this gene. *MYB* is a MY transcription factor, which is involved in the activation of ABA-responsive genes in plants (Abe et al., 1997). This gene was not induced in CDC Robin, but was highly up-regulated in Eston and 964a-46, corroborating the negative regulation of ABA signaling in CDC Robin.

The ET signaling pathway was induced earlier in 964a-46 than Eston (Appendix 6,8) and ET-associated genes had higher expression levels in 964a-46 than Eston. Four ET-responsive transcription factors were differentially expressed among genotypes, including *ERF1b*, *WRI1, GAGA-TF* and *ERF*. *ERF1b*, *GAGA-TF* and *ERF*, were expressed exclusively in 964a-46, whereas *WRI1* was expressed in Eston and CDC Robin. This suggested a higher participation of the ET signaling pathway in developing resistance in 964a-46 than CDC Robin.

PDCD was up-regulated in Eston and 964a-46 but not in CDC Robin. The expression of this gene with the expression of a *CDK* ortholog in Eston indicated the induction of cell death mechanisms in this genotype. The expression of *PDCD* peaked at different times in Eston and 964a-46. Considering the low expression levels of *CDK* in 964a-46 and the difference between Eston and 964a-46 in *PDCD* expression, different manifestation of cell death could be suggested for these two genotypes. *ATG18g* is involved in the cell death inhibition process through formation of autophagosomes (Lai et al., 2011). This gene was exclusively induced in CDC Robin and may contribute to resistance against AB in lentil. By contrast, *ATG* was up-regulated in Eston and CDC Robin but not in 964a-46, suggesting that not all *ATG*s are important for resistance of lentil to AB.

Some of the genes involved in cell wall modification were also differentially expressed among genotypes. *PARP* was induced both in Eston and CDC Robin, however the expression level was much higher in CDC Robin than Eston. The product of this gene regulates various defense responses such as callose deposition, negative regulation of cell death and the ABA and ET signaling pathways. Higher *PARP* expression level in CDC Robin could be associated with resistance to AB. The higher expression of *CSLC6* in CDC Robin and 964a-46 indicates the greater potential of these genotypes in the reinforcement of cell wall. Besides this gene, *CESA* and *CALS* also have a role in cell wall reinforcement. *CESA* was only expressed in CDC Robin while *CALS* only in 964a-46, which suggested different mode of cell wall alteration in these genotypes during *A. lentis* infection.

Downstream defense response genes, mainly PR proteins, were also among the genes differentially expressed among genotypes. *PR-4a* and *PR-1a* expressed differentially among genotypes. Reads associated with *PR-4a* genes were mapped to two different loci in genotype Eston and CDC Robin, whereas in 964a-46 reads mapped to one of those loci were absent. This might be either due to large polymorphism between CDC Redberry and 964a-46 at that locus, impeding the mapping of the read to the reference genome, or possibly the presence of two copies of PR-4 gene in CDC Robin and Eston. Regardless of the hypothetical differences in the copy number or large polymorphism, *PR-4a* expression at the shared locus was higher in CDC Robin and 964a-46 than Eston. *PR-1a* expression was the highest in 964a-46, however it was also up-regulated in Eston. The expression of this gene declined earlier in Eston compared to 964a-46, suggesting negative regulation of the SA pathway in Eston. Other downstream defense response genes differentially expressed among genotypes include *TLP*, *HEL* and *HVA22*. *TLP* and *HVA22*

were up-regulated in both resistant genotypes CDC Robin and 964a-46. *HEL* was only up-regulated in CDC Robin and might be involved in CDC Robin-specific mechanisms of resistance. *HEL* is a JA-responsive genes used frequently for monitoring the JA pathway. Expression of this gene in CDC Robin confirmed the crucial role of the JA signaling in resistance response mediated by resistance genes in this genotype. *PRH* was only induced in 964a-46. This gene encodes a transcription factor regulating the expression of PR proteins in response to pathogen attacks (Korfhage et al., 1994). *HSP* was induced in Eston and 964a-46, but not in CDC Robin. HSPs are common genes expressed in plants upon the exposure to various stresses and are involved in cell homeostasis (Al-Whaibi, 2011).

ARGAH expression was higher in CDC Robin than the other genotypes. This gene is a key component of polyamine biosynthesis in plants and is involved in the defense responses induced by the JA signaling pathway in *A. thaliana* (Chen et al., 2004). Knock-out and constitutive expression of *ARGAH* in *A. thaliana* confirmed a positive role of this gene in resistance against *B. cinerea* (Brauc et al., 2012).

Defense gene induced *de novo* by *A. lentis* in lentil genotypes Eston, CDC Robin and 964a-46

A few genes associated with phytohormone signaling were expressed de-novo in lentil genotypes after *A. lentis* infection (Table 3). *WAK* is a trans-membrane receptor like protein with an extra-cellular domain linked to cell wall pectin (Decreux and Messiaen, 2005). The expression of this gene was up-regulated upon the infection by plant pathogens and exogenous treatment of SA analogues in *A. thaliana* (He et al., 1998).This gene was induced only in 964a-46 and Eston which might indicate the induction of the SA pathway in these genotypes, but not in CDC Robin. *AtRTE1* was among the *de novo* up-regulated genes in Eston. This gene encodes a protein which negatively regulates the ET signaling pathway (Ma et al., 2012) and may be involved in the susceptibility of this genotype. An ortholog of *Gasa5*-like protein was induced in Eston, but not the other two genotypes. Previous studies showed that *Gasa5* negated the expression of *NPR-1*, a key component of the SA signaling pathway (Zhang and Wang, 2011). Another interesting gene *de novo* expressed in CDC Robin was a member *NPR* gene family called *NPR-3*. It has a role in regulation of *NPR-1* expression and, unlike *NPR-1*, directly interacts with SA (reviewed in Dharmasiri et al., 2013). Expression of this gene in CDC Robin may inhibit the SA signaling

pathway through deactivation of *NPR-1*. None of the *de novo* expressed genes in 964a-46 had SA suppressing activity, while orthologs of many genes associated with the SA signaling pathway were *de novo* expressed in this genotype, such as tga transcription factor, senescence-associated protein din1, anthranilate phosphoribosyl transferase, ser thr protein kinase, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate chloroplastic-like and *WAK1*. The strong expression of *PR-1a* and the occurrence of many genes associated with the SA pathway support the activation of the SA pathway in 964a-46.

Table 3. *De novo* induced defense response genes upon *Ascochyta lentis* infection in lentil genotypes Eston, CDC Robin and 964a-46. JA=jasmonic acid, ET=ethylene, ABA= abscisic acid, PAMP=Pathogen Associated Molecular Patterns. Bolded genes are differentially induced among genotypes.

Gene ID[a]	Seq description[b]	ACC of best hit[c]	Peak time	FPKM[d]	Function
Eston					
XLOC_001273	mlo-like protein	XP_003607521	6h	3.8	Ion-flux, cell-death inhibition
XLOC_000852	defensin-like protein	XP_003543246	12h	41.3	JA mediated defense responses
XLOC_004287	**ethylene-responsive transcription factor erf110**	**XP_003602514**	**12h**	**25.8**	**Ethylene signaling pathway**
XLOC_001439	molybdenum cofactor sulfurase-like	XP_004506463	12h	5.3	MAPK cascade
XLOC_018108	ser thr protein kinase	XP_003624374	12h	3.7	MAPK cascade, response to chitin, SAR, regulation of HR
XLOC_008787	**respiratory burst oxidase-like protein**	**XP_003602726**	**18h**	**8.6**	**peroxidase activity**
XLOC_005382	**ethylene-responsive transcription factor erf118**	**XP_004497097**	**18h**	**3.7**	**ET signaling pathway**
XLOC_008369	tyrosine-protein phosphatase	XP_003610492	18h	3.8	Negative regulation of MAPK
XLOC_002375	aba-responsive protein abr18 (PR-10a)	XP_004508175	24h	7.4	ABA induced defense response
XLOC_009925	proteinase inhibitor type-2 cevi57-like	XP_004516042	36h	55.5	Proteinase inhibitory effect
XLOC_008126	**elmo domain-containing protein a-like**	**XP_004493259**	**36h**	**3.1**	**ABA signaling pathway**
XLOC_019402	**g-type lectin s-receptor-like serine threonine-protein kinase rlk1-like**	**XP_004488803**	**36h**	**3.2**	**MAPK signaling cascade**
XLOC_010544	nep1-interacting 2-like	XP_003524730	48h	7.0	Chitin-induced defense response
XLOC_005034	**protein reversion-to-ethylene sensitivity1-like (*AtRTE1*)**	**XP_004498900**	**48h**	**6.4**	**Negative regulation of ET signaling**
XLOC_000653	tga transcription factor	XP_003627926	60h	6.3	Transcription activity, SA signaling
XLOC_002550	protease inhibitor	XP_003617314	60h	88.7	Proteinase activity
XLOC_004411	e3 ubiquitin-protein ligase	XP_004503690	60h	4.5	Signaling by ubiquitination
XLOC_006642	wall-associated receptor kinase 2-like	XP_004502549	60h	9.0	Response to SA signaling
XLOC_013051	**gasa5-like protein**	**XP_003616284**	**60h**	**4.3**	**Response to SA signaling**
CDC Robin					
XLOC_007391	**ethylene-responsive transcription factor 1b**	**XP_003541350**	**6h**	**4.8**	**ET signaling pathway**
XLOC_015031	**wrky transcription factor 22-like**	**XP_002278221**	**6h**	**9.9**	**PAMP triggered immunity**

XLOC_002301	aba-responsive protein abr18 (PR-10a)	XP_004508175	12h	14.0	ABA defense response
XLOC_019332	**NPR-3 protein**	**XP_003545296**	**12h**	**17.7**	**Negative regulation of SA pathway, SA and JA cross-talk**
XLOC_001380	molybdenum cofactor sulfurase-like	XP_004506463	18h	3.5	MAPK cascade
XLOC_002471	protease inhibitor	XP_003617314	18h	264.9	Proteinase activity
XLOC_021446	defensin-like protein-like	XP_004487695	18h	56.1	JA induced defense responses
XLOC_003952	**autophagy-related protein 8d-like (ATG8d)**	**XP_004515097**	**18h**	**3.1**	**Cell death inhibition**
XLOC_008124	tyrosine-protein phosphatase	XP_003610492	18h	6.4	Regulation of MAPK cascade
XLOC_010230	nep1-interacting 2-like	XP_003524730	24h	10.9	Chitin-induced defense response
XLOC_007391	**pentatricopeptide repeat-containing protein**	**XP_004515322**	**24h**	**10.4**	**suppression of ABA signaling**
XLOC_013777	e3 ubiquitin-protein ligase	XP_004511021	24h	14.7	Signaling by ubiquitination
XLOC_005961	chitinase domain-containing protein	XP_004512315	24h	5.3	Chitinase activity
XLOC_006955	**iws1-like protein**	**XP_003612709**	**24h**	**25.8**	**Brassinosteroid signaling,**
XLOC_013839	**4-coumarate- ligase-like 7-like**	**XP_002523698**	**24h**	**5.2**	**JA biosynthesis process**
XLOC_021559	mlo-like protein	XP_004510198	24h	4.7	Ion-flux, cell-death inhibition
XLOC_022239	Peroxidase	XP_003616748	36h	37.5	Oxidative stress
XLOC_015627	**wrky transcription factor 51-like**	**XP_003588914**	**48h**	**23.2**	**PAMP triggered immunity**
XLOC_006500	proteinase inhibitor type-2 cevi57-like	XP_004516042	60h	151.3	Proteinase activity
XLOC_017493	ser thr protein kinase	XP_003624374	60h	10.4	MAPK cascade, response to chitin, SAR, regulation of HR
964a-46					
XLOC_000671	tga transcription factor	XP_003627926	6h	5.7	SA signaling pathway
XLOC_006365	chitinase domain-containing protein	XP_004512315	6h	10.6	Chitinase activity
XLOC_022781	**oxidative stress 3**	**XP_004513063**	**6h**	**14.1**	**Oxidative burst**
XLOC_004480	**map3k delta-1 protein**	**XP_003617693**	**18h**	**4.3**	**MAPK cascade**
XLOC_017770	mlo-like protein 11-like	XP_004487692	18h	3.1	Ion-flux, cell-death inhibition
XLOC_006932	proteinase inhibitor type-2 cevi57-like	XP_004516042	24h	60.0	Proteinase activity
XLOC_009548	**acidic mammalian chitinase-like**	**XP_004501097**	**24h**	**222.2**	**Chitinase activity**
XLOC_022964	defensin-like protein-like	XP_004487695	24h	25.0	JA induced defense responses
XLOC_018595	**senescence-associated protein din1**	**XLOC_018595**	**24h**	**9.5**	**SA signaling pathway**
XLOC_010696	mlo-like protein 2-like	XP_004515994	24h	27.5	Ion-flux, cell-death inhibition
XLOC_021257	**Heat Shock protein**	**XP_003611429**	**36h**	**9.5**	**Stress responses**
XLOC_008670	Tyrosine-protein phosphatase	XP_003610492	36h	3.8	regulation of MAPK cascade
XLOC_012140	**anthranilate phosphoribosyltransferase (*PAT1*)**	**XP_003601245**	**36h**	**3.8**	**SAR**
XLOC_018767	ser thr protein kinase	XP_003624374	48h	11.2	MAPK cascade, response to chitin, SAR, regulation of HR
XLOC_023806	Peroxidase	XP_003616748	48h	11.2	Oxidative stress
XLOC_016043	**cationic peroxidase 1-like**	**XP_004514549**	**48h**	**4.1**	**Oxidative stress**
XLOC_003713	**4-hydroxy-3-methylbut-2-en-1-yl diphosphate (*CSB3*)**	**XP_004487120**	**60h**	**10.4**	**Regulation of hypersensitive reaction**
XLOC_004554	e3 ubiquitin-protein	XP_004503690	60h	8.0	Signaling by ubiquitination
XLOC_006844	wall-associated receptor kinase 2-like	XP_004502549	60h	5.8	Response to SA signaling

[a] Gene ID assigned to transcripts in Cufflink® output, [b] sequence description inferred from the top BLAST hit, [c]Ref-seq accession number for the best blast hit, [d] fragments per kilobase of exon per million mapped reads.

Two orthologs of WRKY transcription factors, *WRKY 21* and *WRKY 51* were exclusively induced in CDC Robin but not in the other two genotypes. Previous research indicated the involvement of WRKY transcription factors in different hormonal signaling pathways (Pandey and Somssich, 2009). Marchive et al. (2013) suggested a role of a WRKY transcription factors in the induction of the JA-responsive genes in grapevine. Exclusive induction of these two genes in CDC Robin supports different pattern in induction of defense genes in this genotype and 964a-46.

The *de-novo* expression genes were also included downstream defense response genes. Defensin-like genes encode small antimicrobial proteins (45 to 54 amino acids) and are a prevalent protein family in the plant kingdom. *PDF1.2* which is a JA/ET-induced defense gene is a defensin like protein 16 in *A. thaliana* (Penninckx et al., 1998). A few protease inhibitor genes were also among the *de novo* expressed genes in all three genotypes. These genes are important components of plant responses to wounding and are effective in defense against insects, nematodes and plant pathogens (Hisash et al., 1997; van der Hoorn and Jones, 2004).

Another important gene expressed *de novo* in all three genotypes was a mutation-induced recessive alleles (*mlo)* gene. The inhibition of cell death mediated by the product of this gene caused susceptibility to powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* in barley (Büschges et al., 1997). Mutation in this gene caused a durable type of resistance to this disease in barley, whereas it increased the susceptibility to a hemibiotrophic pathogen *M. grisea* (Jarosch et al., 2003), suggesting the involvement of this gene in resistance to pathogens with necrotrophic life-style (necrotrophs and hemibiotrophs). The expression of *mlo* orthologs in all lentil genotypes may suggest the involvement of this gene as common response to *A. lentis* infection. Nevertheless, two copies of the *mlo* orthologs were detected in 964a-46 assuming greater potential in 964a-46 to produce this protein upon *A. lentis* infection. Another gene associated with autophagy-mediated cell death inhibition *ATG8d* was exclusively induced in CDC Robin, providing further support for the involvement of autophagy in AB resistance in CDC Robin. In addition *CSB3*, which is involved in the regulation of hypersensitive responses in plants, was only induced in 964a-46.