Identification of candidate genes associated with resistance against race 0 of *Colletotrichum lentis* in *Lens ervoides*

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Plant Sciences
University of Saskatchewan
Saskatoon

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ABSTRACT

Lens ervoides is a potential source of resistance to anthracnose caused by the pathogen *Colletotrichum lentis*. Transcriptome sequencing was performed on the resistant LR-66-528 and susceptible LR-66-524 recombinant inbred lines (RILs) of *L. ervoides* infected with the aggressive race 0 isolate CT-30 of *C. lentis* to unravel the genetic control underlying the genetic responses against this pathogen. The inoculated samples were harvested at 6, 12, 24, 48, 72, 96 and 144 hours post-inoculation (hpi) for molecular studies. Results of quantitative PCR to estimate fungal biomass revealed that 24, 48, 72 and 96 hpi were interesting time-points for studying disease development because of exponential trends of fungal growth during this period. Subsequent comparison of gene expression based on RNA-Seq at 24, 48, 72 and 96 hpi with that of mock (non-inoculated) samples showed that 3,091 disease responsive genes. Among them, 477 were differentially expressed genes (DEGs) (fold change >2, $P_{adj} < 0.05$) between the resistant and susceptible RILs. Based on expression profiling, these DEGs were clustered into six expression clusters (C1-C6). In Cluster C1, 56 genes were up-regulated in the susceptible RIL whereas in C2, 79 genes were up-regulated in that RIL, mainly at 96 hpi. Cluster C3 contained 91 genes that were up-regulated in the resistant RIL LR-66-528 at 24, 72 and 96 hpi. A total of 97 genes in C4 were significantly up-regulated in LR-66-524 at 24 and 48 hpi. Cluster C5 with 51 genes was the smallest cluster with genes up-regulated in the resistant LR-66-528 and down-regulated in the susceptible LR-66-524, as were 95 genes in Cluster C6. DEGs were functionally annotated to identify those with known functions in disease resistance proteins, such as LRR and NB-ARC domain disease resistance protein, Protein Detoxification, LRR receptor-like kinase family proteins, and Wall-associated Ser/Thr Kinases. The expression of 21 of these genes was validated using RT-qPCR, which confirmed up- or down-regulation as in the RNA-Seq data. Comparison of DEGs and genes in QTLs associated with resistance to anthracnose revealed that nine DEGs were located in the resistance QTL region of chromosome 2, ten in the QTL region of chromosome 5 and three in the QTL region of chromosome 7 of *L. ervoides*. The identified candidate genes associated with resistance should be valuable targets for the future gene function analyses.

ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks to my supervisor, Dr. Sabine Banniza for her support and encouragement throughout. The successfulness of this thesis is also indebted to the suggestions of my committee members, Dr. Helen Booker, Dr. Bunyamin Tar'an and Dr. Curtis Pozniak. I am grateful to my external examiner, Dr. Fengqun Yu for the critical review and inputs for improving this thesis.

I would like to appreciate the guidance and assistance of Pulse Pathology Lab members for helping in making my experiments run successfully.

I am grateful to the project sponsors, University of Saskatchewan, Saskatchewan Pulse Growers, Western Grains Research Foundation, Government of Saskatchewan and NSERC for funding my project.

In the end, I would like to extend my heartfelt thanks to my loving husband, my parents, my sister and my extended family for their moral support, patience and motivation. Also, my roommates for being there with me throughout my degree.

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Chapter 1

1.0 Introduction

Canada is the world's largest exporter of lentil (*Lens culinaris* Medik.). On a global scale, lentil provides nutritional security as it is rich in proteins, vitamins, fiber and micronutrients. It also forms a symbiotic association with *Rhizobium* that fixes atmospheric nitrogen, and breaks disease cycles in mostly cereal-based rotations (Kissinger 2016). In 1970, commercial lentil production was started in Western Canada on 600 hectares, and has increased significantly. About 95% of Canada's lentils are produced in Saskatchewan with an annual production of 2.1 million tonnes in 2018 over an area of 1.3 million hectares (Statistics Canada, 2018). Nevertheless, diseases remain a major impediment for maintaining high seed quality and yields of lentil. As a result, there is a need to develop varieties with durable resistance to control these diseases and complement crop rotation, use of disease-free seeds and fungicide applications.

Several infectious diseases can have devastating effects on lentil production, especially fungal diseases. Ascochyta blight caused by *Ascochyta lentis* and Fusarium wilt caused by *Fusarium oxysporum* are present worldwide (Chen and Sharma, 2011). Major diseases of North America are anthracnose caused by *Colletotrichum lentis*, Botrytis grey mould caused by *Botrytis cinerea* and Sclerotinia white mould caused by *Sclerotinia sclerotiorum* (Chongo *et al*., 2002). Rusts caused by *Uromyces vicia-fabae* and Stemphylium blight caused by *Stemphylium botryosum* are two other foliar diseases prevalent in certain regions of the world (Saha 2009; Chen and Sharma, 2011).

One of the major damaging diseases in western Canada is anthracnose caused by *C. lentis.* Anthracnose was first discovered in Manitoba in 1987 (Morrall *et al*., 1988). It has been reported from lentil crops in Canada, the USA, Bulgaria, Pakistan and New Zealand (Kaiser *et al*., 2000). It started spreading into Saskatchewan in 1990 and has since threatened yields with losses of up to 100% in worst scenarios triggered by short crop rotations, frequent rainfall and high temperature (Morrall and Pederson, 1991). Two pathogenic races of *C. lentis* infect the lentil crop in Canada (Buchwaldt *et al*., 2004). Race 0 is more aggressive, and no high levels of resistance have been identified in cultivated lentil, whereas partial resistance to race 1 was found in cultivated lentil. The evolution of new pathogenic races can cause the erosion of available

resistance resulting in resistance breakdown. Wild relatives of crop species have been identified as repositories of allelic diversity for disease resistance, and *Lens ervoides* (Brign.) accessions with superior resistance to anthracnose were discovered (Tullu *et al*., 2010). Using interspecific hybridization involving embryo rescue, transfer of resistance to *C. lentis* race 1 and race 0 from *L. ervoides* accession L01-827A to lentil cultivar Eston was achieved (Fiala *et al*., 2009; Vail *et al*., 2011). However, lack of understanding of the mechanism and control of resistance has prevented the development of markers to trace the introgression of genes into breeding lines. Due to segregation distortion in, and lack of dense linkage maps for interspecific populations genetic studies in these populations have not been possible. Therefore, the intraspecific recombinant inbred line (RIL) population LR-66 was developed from crossing *L. ervoides* accessions IG 72815 and L01-827A. Phenotypic and genotypic analyses of this population led to the identification of QTLs conferring resistance to anthracnose (Bhadauria *et al*., 2017a). RILs identified with highest resistance and susceptibility to anthracnose, parental accessions IG 72815 and L01-827A and the checks *L. culinaris* ssp. *culinaris* cultivars Eston and CDC Robin were previously inoculated with a race 0 isolate of *C. lentis* in a time-course experiment in the greenhouse (Kapoor, 2018). Leaf samples were harvested and studied under the light microscope to identify critical time points when differential resistance responses were triggered in these genotypes. Few differences among the genotypes were visually apparent and were not consistent at later time points.

In recent years, transcriptome-based studies have revolutionized the research into lentil genomics. In this study, the quantitative evaluation of *in planta* fungal growth was implemented using quantitative PCR (qPCR) to identify the specific stage of fungal growth for a transcriptome study. A time-series RNA-seq experiment was conducted on two *C. lentis* infected RILs of the LR-66 RIL population to identify differentially expressed genes (DEGs). Evidence is already available of extensive shared synteny between *L. culinaris*, *L. ervoides* and the model legume *Medicago truncatula* (Verma *et al*., 2014). Synteny-based comparison of the previously identified QTL locations (Bhadauria *et al*., 2017a) with the model legume will help to identify resistance (R-) gene candidates. Once the R-genes are confirmed, perfect markers based on gene sequences can be developed and used to trace the introgression of these genes into hybrid populations from which lentil varieties can be developed.

1.1 Research hypothesis:

It is hypothesized that some of the differentially expressed genes in resistant and susceptible RILs of *L. ervoides* RIL population LR-66 identified through RNA-seq co-localize with QTLs for anthracnose resistance.

1.2 Research objectives:

(i) To determine the critical time-points when the selected genotypes trigger differential resistance responses.

(ii) To identify differentially expressed genes in resistant and susceptible genotypes and determine whether they co-localize with the QTLs for anthracnose resistance.

(iii) To validate the expression of identified genes associated with resistance .

Chapter 2

2.0 Literature Review

2.1 Anthracnose of lentil

Anthracnose is one of the most important limitations for lentil production in North America. Symptoms of this foliar and stem disease start at the seedling stage and appear as tan-colored lesions on lower leaves at the 8 to12-node stage (Chongo and Bernier., 2000). With the progression of the season, the stem base can become covered with lesions causing stem girdling and defoliation, which prevents water and nutrient transport and results in wilting of the plant. Diseased patches occur in the crop that expand rapidly into large yellow or grey patches in an otherwise green field. Dark brown discoloration of these patches indicates dead plants. A minimum duration of 16 h of leaf wetness and temperatures higher than 15°C are essential for anthracnose development.

A primary source of disease inoculum consists of microsclerotia on infected lentil residue which may survive for up to 4 years when residue is buried by tillage (Buchwaldt *et al*., 1996). Microsclerotia can disperse from the soil surface to lower leaflets and stems by rain splash. Rain can induce repeated infection cycles during the growing season, as conidia from infected plants are spread to surrounding plants (Bailey *et al*., 2003). The dispersal of inoculum between fields is air-borne. Microsclerotia are spread to the neighbouring fields through infected lentil debris and dust particles (Buchwaldt *et al*., 1996). Seed-borne infection of *C. lentis* was found to be unimportant, as studies to demonstrate its spread from infected seeds to the seedlings were unsuccessful (Gibson 1993; Morrall 1997). Reaction of lentil cultivars to the causal pathogen, aggressiveness of predominant races and environmental conditions determines the severity of lentil anthracnose.

2.1.1 Management of anthracnose

For the management of lentil crops, use of partially resistant cultivars combined with fungicide applications, e.g. chlorothalonil has been recommended (Chongo *et al*.,1999). To reduce disease pressure, management of infected plant residue is required as it is the primary source of infection. To minimize the risk of anthracnose, new lentil cropping should follow at least a four-

year rotation (Bailey *et al*., 2003). Timely eradication of weeds (e.g. wild vetch) growing in lentil fields is important (Bailey *et al*., 2003). Yield losses caused by anthracnose can be reduced by fungicide application (Buchwaldt 1999; Chongo *et al*., 1999). The optimum growth stage for fungicide applications of chlorothalonil and azoxystrobin was identified as the 10 to 12-node stage to early flowering. The next suitable application was considered at the mid-flowering stage which is 10-14 days after first application (Buchwaldt 1999; Chongo *et al*., 1999). A fungicide decision support system is available to farmers developed by Agriculture and Agri-Food Canada that indicates the appropriate time for fungicide applications based on four risk factors: (A) plant density, (B) number of days with rain in the last 14 days, (C) rain in the 5-day weather forecast and (D) early symptoms of anthracnose. It also provides information on the diagnosis of early disease symptoms as well as on the epidemiology of the disease.

2.2 Causal pathogen

Originally, the causal agent of anthracnose in lentil was identified as *Colletotrichum truncatum* (Andrus and W.D. Moore). *Colletotrichum* isolates from lentil were re-classified as *C. lentis* Damm in the destructivum clade based on recent evidence (Damm *et al*., 2009). *Colletotrichum lenti*s is hemibiotrophic in nature, utilizing sequential biotrophic- and necrotrophic infection approaches to infect the host plant. The changeover from the asymptomatic biotrophic phase to the deadly necrotrophic phase is called the biotrophy-necrotrophy switch and is necessary for anthracnose development. In the Canadian population of *C. lentis*, two pathogenic races were classified, the less virulent race 1 and the more virulent race 0, based on the reaction of 7 host differentials (Buchwaldt *et al*., 2004).

Colletotrichum lentis commences the infection process with single-celled conidia that adhere to aerial parts of the host plants and that germinate to form germ tubes. An appressorium is differentiated at the end of the germ tube, which gradually darkens in colour (O'Connell *et al*., 2000). Appressoria are specialized structures common to many fungal plant pathogens and are involved in the mechanical penetration of the leaf surface. For penetration, *C. lentis* needs to overcome the restrictions posed by plant epidermal cell walls (Bailey *et al*., 2003). Piercing of the host cuticle and cell wall is done by the thin penetration peg emerging from below the appressorium. Penetration pegs in contact with the epidermis penetrate shortly thereafter, resulting in the formation of an infection vesicle in the apoplastic space of epidermal cells that

further elongates into an invasive primary hyphum. This primary hyphum colonizes a single epidermal cell. This phase constitutes the biotrophic phase. The switch from biotrophy to necrotrophy occurs when thin, necrotrophic secondary hyphae develop from the thick primary hyphum and grow through the adjacent cells. This switch results in cell death and tissue disruption on a large scale. Thousands of conidia produced in acervuli, which are developed in anthracnose lesions on the aerial parts of the host plants, restart the disease cycle (O'Connell *et al*., 2000).

After inoculation of partially resistant lentil cultivar CDC Robin, it was shown that isolates of the less virulent race 1 had lower conidial germination, fewer appressoria, and a slower and less destructive necrotrophic phase compared to isolates of the more virulent race 0 (Armstrong-Cho *et al*., 2012). Chongo *et al*. (2002) found that conidia germinated from 3 to 6 hours postinoculation (hpi) on leaflets of resistant and susceptible lentil cultivars, and appressoria formation took place within 6 to 12 hpi. A penetration peg was generated on the underside of the appressorium as the only way to penetrate the cell tissue. At 24 hpi, the primary hyphum had colonized the epidermal cell. Extensive growth of the primary hyphum took place in the epidermal cell but no symptoms were observed until 72 hpi. Initial light greenish lesions were observed between 72 and 144 hpi that turned necrotic, and at 144 hpi severe symptoms were visible. In another study, primary and secondary hyphae were clearly detected at 48 hpi in lentil leaflets (Armstrong-Cho *et al*., 2012).

Colletotrichum lentis does not have as broad of a host range as many other pathogens. It is a pathogen of both *Lens* and *Vicia* (vetch) species and causes anthracnose symptoms on foliar parts of the plants (Gossen *et al*., 2009). Non-sporulating, superficial symptoms are observed rarely under field conditions on *Pisum sativum* L. (pea) and *Cicer arietinum* L. (chickpea).

In the past, several molecular studies have demonstrated virulence differences between race 0 and race 1 isolates of *C. lentis*. A set of *in planta* candidate effectors were identified through expressed sequence tags (ESTs) mining of *C. lentis*-infected lentil tissues (Bhadauria *et al*., 2015). No sign of positive selection pressure was observed in a comparative genomics study of effectors. This indicates that *C. lentis* isolates are under stabilizing selection. A single nucleotide polymorphism in the open reading frame (ORF) of the candidate effector ClCE6 allowed differentiation between pathogenic race 0 and race 1 and was confirmed by testing 52 isolates

with known race-identity (Bhadauria *et al*., 2015). Several genes were identified with involvement in virulence and host colonization. The foreign gene *Arg* encoding argininosuccinate lyase was identified as a bacterial gene acquired by *C. lentis* through horizontal gene transfer to enhance virulence. *Colletotrichum lentis* delivers the effector protein CtNUDIX into lentil cells at the biotrophy-necrotrophy switch signaling a transition in the pathogen to causing cell death (Bhadauria *et al*., 2013). Through *in planta* expression analysis, the toxin protein ClToxB was found to be secreted by *C. lentis* in lentil cells during the biotrophy-necrotrophy switch to intensify cell death signals. As the expression level observed for a race 0 isolate was higher compared to the race 1 isolate, it was concluded that ClToxB secretion contributes to quantitative differences between race 0 and 1 of *C. lentis* (Bhadauria *et al*., 2015).

2.3 The cultivated lentil (*Lens culinaris*)

Lens culinaris is a pulse crop with a lens-shaped grain. Lentil plants are generally short but can range from 20 to 75 cm in height, depending on growing conditions. As a cool season crop, lentil is moderately resistant to high temperatures and drought. It is grown in many temperate areas such as the Mediterranean basin, Central, Western and South Asia, Ethiopia, temperate regions of North and South America and regions with Mediterranean climate in Australia (Durán *et al*., 2004). It is annual, short-statured, self-pollinated and has an indeterminate growth habit that requires heat or nutrient stress to cease flowering. It has a thin taproot system and many lateral roots, but *Lens* species differ prominently in taproot length and number of lateral roots (Sarker *et al*., 2006). The lentil stems are thin, and leaves are alternate, compound and pinnate with elliptical leaflets. It flowers after a juvenile period of vegetative growth, which varies among genotypes. Lentil has an important role in biological nitrogen fixation, as a result of which the inclusion of this crop in cropping systems is of great advantage to the succeeding crop by enhancing physical, chemical and biological properties of the soil.

Lentil is a diploid (2n=2x=14) species and its estimated genome size is approximately 4.2 Gb (Bett *et al*., 2016). The karyotype of lentil is symmetric with three pairs of metacentric and submetacentric chromosomes, three pairs of acrocentric chromosomes and one metacentric pair with secondary constrictions near the centromere (Ladizinsky, 1993).

The first genetic maps of lentil were developed using restriction fragment length polymorphisms (RFLPs) and some morphological markers (Havey and Muehlbauer, 1989). Subsequently, efforts were made to map the lentil genome using random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) markers (Eujayl *et al*., 1998), inter-simple sequence repeats (ISSRs) and resistance gene analog (RGA) markers in addition to RAPDs (Rubeena *et al*., 2003), simple sequence repeats (SSRs) (Tullu *et al*., 2008) and EST-SSR markers to enrich the intraspecific linkage map (Gupta *et al*., 2012). Sharpe *et al*. (2013) identified 44,879 single nucleotide polymorphism (SNP) markers using Ilumina genome analyzer and used these markers to map the genomes of multiple genotypes from wild and cultivated lentil species. Another set of 50,960 SNPs were identified by Temel *et al*. (2014) to construct a SNP-based linkage map of *L. culinaris*. Genotyping by sequencing (GBS)-based mapping of a RIL population developed from a cross between the two *L. ervoides* accessions L01-827A and IG 72815 at the F_9 on the Illumina Hiseq 2500 platform led to the discovery of 2,180 high quality SNP markers distributed across seven linkage groups with a cumulative spanning distance of 740.95 cM and an average inter- marker distance of 1.36 cM (Bhadauria *et al*., 2017a).

2.4 The genus *Lens*

The genus *Lens* (Miller) is nested within the tribe Viciae, which comprises cool-season legumes belonging to the sub-family Papilionaceae and the family Fabaceae (Leguminoseae). The genus *Lens* was originally classified by Miller in 1740 but species differentiation in this genus is still the subject of discussion (Cubero *et al*., 2009). Earlier, the genus *Lens* was classified by Ladizinsky (1979) into the four species *L. culinaris, L. orientalis, L. nigricans* and *L. ervoides*. However, later some accessions of *L. nigricans* were reclassified as *L. odemensis* thus a new classification of *L. culinaris* into the cultigen subspecies *culinaris* and the wild ssp. *orientalis* and *odemensis* was established, whereas *L. nigricans* obtained the two subspecies *nigricans* and *ervoides* (Ladizinsky *et al*., 1984). *Lens* species were reclassified again as *L. culinaris*, with subspecies *culinaris* and *orientalis*, *L. odemensis*, *L. ervoides*, and *L. nigricans* (Ladizinsky 1993), followed by the addition of two more species to the genus, *L. tomentosus* (Ladizinsky 1997) and *L. lamottei* (Van Oss *et al*., 1997). According to the most recent species classification developed by Ferguson *et al*. (2000) there are seven taxa grouped into four species, namely *L.*

culinaris ssp. *culinaris*, *L. culinaris* ssp. *orientalis*, *L. culinaris* ssp. *tomentosus*, *L. culinaris* ssp. *odemensis*, *L. ervoides*, *L. lamottei*, and *L. nigricans* based on molecular and morphological studies. Each of the species belonging to *Lens* has its own morphological characteristics and exhibit specific ecological affinities and typical geographic distributions. Irrespective of systematic arrangements of taxa, all studies concurred that *L. culinaris* ssp. *orientalis* is the most closely related wild progenitor of *L. culinaris* ssp. *culinaris* whereas *L. nigricans* is the most distant relative.

The genus *Lens* has been subdivided into three different gene pools based on the ability of species to hybridize with cultivated lentil as an expression of their relatedness to *L. culinaris* ssp. *culinaris* (Muehlbauer *et al*., 2005). The cultivated lentil *L. culinaris* spp. *culinaris* and *L. culinaris* spp. *orientalis* are placed in the primary gene pool. The secondary gene pool includes *L. odemensis* (Ladiz.) and *L. tomentosus* (Ladiz.), while the tertiary gene pool includes *L. nigricans* (M. Bieb.) Godr. and *L. ervoides* (Brign.) Grande and *L. lamottei* Czefr. In another study, genotyping by sequencing (GBS) analysis was used to classify gene pools (Wong *et al*., 2015). This study showed that *L. culinaris*, *L. orientalis* and *L. tomentosus* belong to primary gene pool as they can form fertile hybrids with cultivated lentil species. *Lens lamottei* and *L. odemensis* are in secondary gene pool as partial fertilization is possible through embryo rescue techniques. *Lens ervoides* is included in the tertiary and *L. nigricans* in the quaternary gene pools as these are not able to form hybrids with cultivated lentil. Based on evolutionary studies of *Lens*, the genus is considered "active" and interchanges are possible among the genepools (Cubero *et al*., 2009).

2.5 Wild relatives as potential sources of genetic resources

Crop wild relatives represent an abundant source of novel genes for crop improvement. Due to a narrow genetic base of the cultivated lentil, the wild relatives have been exploited to widen the genetic base in order to improve resistance to various biotic and abiotic stresses. Accessions of *L. culinaris* ssp. *odemensis*, *L. culinaris* ssp. *orientalis*, *L. nigricans* and *L. ervoides* were found to be resistant to vascular wilt (Bayaa and Erskine, 1991). Desirable traits such as winter hardiness at high altitudes were identified in accessions of *L. culinaris* ssp. *orientalis* originating from high altitudes (Hamdi *et al*., 1996). Considerable yield losses are caused by parasitic broomrape (*Orobanche crenata*), and *L. ervoides*, *L. odemensis* and *L. culinaris* ssp. *orientalis* were found

to be resistant to this parasitic weed (Farnandes-Aparcio *et al*., 2009). Resistance to the foliar disease Ascochyta blight was identified in some accessions of *L. culinaris* spp. *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides* (Taylor *et al*., 2007). Superior resistance for anthracnose was identified in accessions of *L. ervoides* (Tullu *et al*., 2006). All *Lens* species were screened for Stemphylium blight, another fungal disease, and *L. lamottei* and *L. ervoides* accessions were found to be resistant (Podder *et al*., 2013).

2.6 *Lens ervoides*

Lens ervoides is a wild relative of lentil present in the tertiary gene pool. Leaves are 1.5-3 cm long and consist of two to four pairs of leaflets, terminating occasionally in a simple tendril. It has semi-hastate or obliquely lanceolate stipules. Peduncles are occasionally longer than subtending leaves. Pods are ovate-rhomboid and puberulent. Compared to the other wild relatives of lentil, *L. ervoides* has smaller leaves, calyx teeth, pods and seeds. The natural habitat of *L. ervoides* are shady or partially shady niches, among bushes or under trees such as pines and oaks with stony soils. It can be found growing with other annual legumes such as *Trigonella, Trifolium*, *Vicia* and *Pisum* species (Ladizinsky and Abbo 2015).

Geographically, *L. ervoides* is found in countries bordering the Mediterranean Sea, including Israel, Jordan, Lebanon, Turkey, some regions of Croatia, Italy and Algeria. In Ethiopia and Uganda, it has a somewhat different habitat, and grows on basalt soil in open habitats among perennial grasses. Ecologically, it differs from other wild relatives of lentil but may grow close to them when their habitats coincide (Ladizinsky and Abbo, 2015).

2.7 Resistance to anthracnose of lentil

Some studies have been carried out to investigate the resistance of lentil to anthracnose and to identify sources of resistance that can be introgressed into new cultivars. Among 1,767 germplasm accessions of *L. culinaris* screened for resistance to *C. lentis*, 16 accessions were found to be resistant to race 1 (Buchwaldt *et al*., 2004). Cultivar CDC Robin is the first anthracnose-resistant variety derived through a crossing program to transfer anthracnose resistance into lentil with appropriate seed characteristics by the breeding program of the Crop Development Centre, University of Saskatchewan (Vandenberg *et al*., 2002). The resistance was transferred from the variety ‘Indianhead’, also known as plant introduction PI 320952. Screening

for resistance to race 1 has been carried out routinely in western Canada and about one-third of the lentil varieties carry some resistance (Government of Saskatchewan, 2017). In the variety Indianhead and accession PI 320937 a combination of a major and minor resistance genes for race 1 resistance was described (Buchwaldt *et al*., 2001; Tullu *et al*., 2003). Resistance to race 0 and race 1 were described for the three *L. culinaris* lines VIR2633 (Georgia), VIR2058 and VIR2076 (Czech Republic) (Shaikh *et al*., 2013).

Germplasms of some *Lens* species have been screened extensively in the past few years and accessions of *L. lamottei* and *L. ervoides* have been identified with superior resistance to race 0 and race 1 of *C. lentis* (Tullu *et al*., 2006). *L. ervoides* accession PI 72847 was crossed with the susceptible *L. culinaris* variety 'Eston', and a single hybrid plant was selfed to develop a population of 85 F_8. Among these 85 lines, 25% showed a resistant response after inoculation with a *C. lentis* race 0 isolate and 22% after inoculation with a race 1 isolate. A population developed from a cross between resistant *L. ervoides* line IG 72815 and Eston was screened at the F_8 generation and 29% of the lines showed resistance to race 0 (Tullu et al., 2013).

2.8 Genetic and QTL mapping of anthracnose resistance in lentil

Tullu *et al*. (2003) reported the dominant gene *LCt-2* for resistance to *C. lentis* race 1 whose effect was influenced by several minor genes in a RIL population derived from a cross between lentil cv. Eston and PI 320937. Locus *LCt-2* was linked to two RAPD and three AFLP markers. Tar'an *et al*. (2003) confirmed marker-assisted selection for *C. lentis* race 1 resistance in a RIL population derived from a cross between CDC Robin and breeding line 964a-46 using RAPD marker $OPEO6_{1250}$. Tullu *et al*. (2006) reported linkage between resistance genes for Ascochyta blight and anthracnose as a QTL on linkage group 6, which explained 41% variation for Ascochyta blight resistance was localized between an AFLP marker and anthracnose resistance gene *LCt-2*. The genetics of anthracnose resistance for race 1 and race 0 in the interspecific RIL population LR-59 developed by crossing *L. ervoides* accession L01-827A and Eston was studied by Fiala *et al*. (2009). In this study, two recessive genes were found to be involved in resistance to both races; however, results were skewed due to segregation distortion because of loss of some RILs during population advancement. Specific RILs derived from the cross between Eston and L01-827A were identified which conferred resistance to race 0 under field conditions (Vail *et al*., 2011). Two recessive genes were reported for anthracnose resistance in the interspecific

RIL population LR-26 developed from a cross between Eston and *L. ervoides* accession IG 72815. RIL population LR-66 derived from a cross of *L. ervoides* accessions L01-827A and IG 72815 showed varying levels of resistance to *C. lentis* (Bhadauria et al., 2017a). QTL analysis of this RIL population identified five QTLs conferring resistance to *C. lentis* race 0 and six QTLs to race 1. QTLs for resistance to race 0 account for 48 % and race 1 for 59 % of the variance in resistance response. QTLs for resistance to *C. lentis* races 0 and 1 co-localized on LG3 (qANTH0-3 and qANTH-3.1) and LG-5 (qANTH0-5.1 and qANTH0-5.2; qANTH1-5.1 and qANTH1-5.2) suggesting that a large proportion of resistance in LR-66 to the two races of *C. lentis* is controlled by genes located at the same or closely linked loci. Association of two exclusive loci (qANTH0-2 and qANTH0-7) with differential responses of RILs to races 0 and 1 accounted for the remaining 20 % of the variance in race 0 resistance, whereas three exclusive loci (qANTH1.2-1, qANTH1.2-2 and qANTH1.3-2) accounted for 34 % of variance in race 1 resistance (Bhadauria *et al*., 2017a).

2.9 Resistance gene discovery for anthracnose in lentil

A total of 2,852 expressed sequence tags (ESTs) of lentil derived from *C. lentis*-infected leaf tissues were analyzed during the biotrophy-necrotrophy switch of a race 1 isolate of *C. lentis* infecting susceptible lentil cv. Eston with the objective to catalogue defense related genes (Bhadauria *et al*., 2013). These ESTs were assembled into 1,682 unigenes, among which 387 unigenes were predicted to be stress- and defense-related proteins. The largest class of defense-related proteins contained pathogenesis-related proteins. A high number of transcripts encoding defense related proteins suggested that lentil cells recognize *C. lentis* at the biotrophy-necrotrophy switch and in response, mount an inducible defense (Bhadauria *et al*., 2013). Bhadauria *et al*. (2017b) also developed a cDNA plasmid library from susceptible lentil cv Eston infected with an isolate of virulent race 0 of *C. lentis*. The library was sequenced and generated a total of 11,094 expressed sequence tags representing 3,488 unigenes. Mapping of unigenes onto the *C. lentis* and the *L. culinaris* genomes led to the identification of 2,418 unigenes of fungal origin and 1,070 unigenes of plant origin. Gene ontology analysis of the unigenes revealed that the transcriptome contained 22 candidate effectors and 26 resistance genes.

2.10 Concept of disease resistance

The study of the genetics of plant disease resistance started in the late 1800s and since then many resistance genes have been identified. Disease resistance can be qualitative or quantitative. Qualitative resistance is explained by the gene-for-gene theory (Flor, 1947) where, following Mendelian concepts, the resistant phenotype is explained by the interaction of a resistance gene of the host with a matching avirulence gene of the pathogen. Quantitative disease resistance is difficult to assess in absolute terms and is not as well understood as qualitative (monogenic) disease resistance (Corwin and Kliebenstein, 2017). The concept of quantitative resistance can be intricate, but a fair understanding is required for its utilization in various crop improvement programs.

The probability of a pathogen to infect two plant species decreases as the phylogenetic distance between the plant species increases. The immunity of plant species that prevents infection by most pathogens is called non-host resistance (Schulze-Lefert and Panstruga, 2011). This type of resistance has gained a lot of attention as it is durable and effective against almost all genetic variants of a pathogen species thus resulting in plant susceptibility to a relatively small number of adapted pathogens. Non-host resistance establishes the host range of pathogenic microorganisms. It appears to be stable and is therefore referred to as basal resistance. Both, constitutive barriers as well as inducible reactions contributing to the protective mechanisms of plant species are proposed to contribute to stability of non-host resistance.

The plant defense mechanism is categorized into two main groups, passive and active defenses (Guest and Brown, 1997). Natural barriers are present in healthy plants and represent passive defense mechanism. These are physical barriers such as wax, cuticle, cell wall, stomata and lenticels. The defense mechanism that is only activated upon pathogen recognition is active defense. It is a rapid response such as changes in membrane function, oxidative burst, cell wall reinforcement, hypersensitive cell death and phytoalexin accumulation.

Extensive genetic and molecular work has been done that led to the grouping of plant immune responses in two major types: Pathogen-associated molecular pattern (PAMP)-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) both of which are accompanied by a set of induced defenses that usually repels the attack by pathogens (Bigeard *et al*, 2015).

2.10.1 PAMP-Triggered Immunity (PTI)

In addition to PAMPs (or more generally, microbe-associated molecular patterns-MAMPs), PTI can also be triggered by damage-associated molecular patterns (DAMPs), which are the degradation products of plants or endogenous or newly synthesized peptides released by plants due to pathogen invasion (Boller and Felix, 2009).

Upon detection of the pathogen (or cell damage caused by pathogens), plants induce defense mechanisms which restrict the entry of microbes through the production and secretion of antimicrobial compounds, the generation of hypersensitive responses and reactive oxygen species (O'Brien *et al*., 2012). Examples for general PAMPs/MAMPs are bacterial flagellin, elongation factor Tu and fungal chitin (Felix *et al*., 1999; Kunze *et al*., 2004; Albert 2013). DAMPs are products of cell wall damage or endogenous peptides (Serrano *et al*., 2014). Pattern recognition receptors (PRRs) are cell membrane bound receptor-like kinases (RLKs) with extracellular domains allowing perception of PAMPs/MAMPs (Böhm *et al*., 2014). *Arabidopsis* flagellin-sensitive 2 (FLS2) is a PRR, which recognizes the N terminus of flagellin from *Pseudomonas aeruginosa* (Gómez-Gómez and Boller, 2000). *Arabidopsis* EF-Tu receptor (EFR) is a PRR, which recognizes elongation factor Tu (EF-Tu) from *Escherichia coli* (Zipfel *et al*., 2006).

2.10.2 Effector-Triggered Immunity (ETI)

The other component of the plant immune system is triggered when plants recognize specific virulence proteins (effectors) produced by pathogens and is known as Effector-Triggered Immunity (ETI). A complex network of defense pathways is correlated with activation of ETI. This activation corresponds to defensive responses at the cellular level and involves a large-scale transcriptional reprogramming (Wu *et al*., 2014). In order to recognize effectors, the plant defense systems have developed resistance (R) proteins during plant-pathogen coevolution. The plant defense system is triggered by avirulent pathogens and results in rapid programmed cell death (PCD) upon infection, called a Hypersensitive Response (HR) (Dangl *et al*., 1996). In response to pathogen recognition, expression of plant defense-related genes and transcriptional reprogramming takes place (Bhattacharjee *et al*., 2013).

The direct interaction of R proteins and Avr or effector proteins is well explained by the gene-for-gene hypothesis (Flor, 1947). In *Arabidopsis*, the effector protein PopP2 of *Ralstonia solanacearum*, the causal pathogen of bacterial wilt, is an avirulence protein and is recognized by

the R protein RRS1-R. The interaction between RRS1-R and PopP2 leads to the resistance reaction (Deslandes *et al*., 2003). Furthermore, indirect interactions of R proteins and effector proteins can also lead to ETI. This is well explained by the "guard hypothesis" model. According to this model, R proteins (guards) are associated with endogenous host proteins (guardees), which are the target proteins of the pathogen effectors. The guardee is modified by the pathogen effector in its quest to create a favourable environment and this perturbation of the guardee is recognized by the guard leading to a resistance response (Dodds *et al*., 2006). A classical example for the guard hypothesis is *Arabidopsis* RIN4 (RPM1-INTERACTING PROTEIN 4) which is a guard that triggers plant resistance when it is degraded by AvrRpt2 which is an effector avirulence protein from *Pseudomonas syringae* (Mackey *et al*., 2003). Another mechanism that is based on the indirect interaction is called the "Decoy model" where a decoy is an effector target without a role in host resistance or susceptibility in the absence of its related R protein (Hoorn *et al*., 2008). ZED1 in *Arabidopsis* is thought to have evolved as a decoy which "lures" HopZ1a, an effector of *Pseudomonas syringae* to the ZAR1 resistance complex, which is a nucleotide-binding leucine-rich repeat (NB-LRR) protein resulting in ETI activation (Lewis *et al*., 2013).

In the past few years, R-genes have been discovered and cloned from several crops and model species. These characterized R-genes have been used effectively in crop improvement research programs (Gururani *et al*., 2012). Functional R-genes that have been isolated, confer resistance to bacteria, viruses, fungi, oomycetes, as well as nematodes and insect pathogens. Plant disease resistance genes have been classified into eight groups based on their amino acid motif arrangement and membrane spanning domains (Gururani *et al*., 2012). Most R-genes belong to the first class characterized by a nucleotide binding site (NBS), a C-terminal leucine rich repeat (LRR) and a putative coiled coil domain (cc) at the N-terminus. The NBS domain carries a number of motifs such as P-loop, kinase-2, kinase-3a and GLPL motifs, which are highly conserved in most of the characterised R-genes, such as *RPS2* and *RPM1* (*Arabidopsis*). The LRR domain facilitates protein-protein interactions and is the major determining factor in recognition specificity (Palomino *et al*., 2006). In the second class, the cytoplasmic proteins with homology to the intracellular signalling domain of *Drosophila* Toll and mammalian interleukin-1 receptor (TIR) are present whereas those proteins lacking TIR contain a putative coiled coil domain (CC-NBS-LRR) (Meyers *et al*., 2003). The resistance gene *L* in flax against *Melampsora*

lini (flax rust) and *N* in tobacco against the tobacco mosaic virus (Lawrence *et al*. 1995) belong to this class. The third class with LRR-transmembrane (LRR-TM) domains comprise resistance genes *Cf-2, Cf-4* and *Cf-9* of tomato against the pathogen *Cladosporium fulvum* (Jones, 2001). The LR-RTM protein kinase (LRR-TM-PK) is the fourth class, comprising the resistance gene *Xa21,* which causes resistance in rice against *Xanthomonas oryzae* (Song *et al*., 1995). This consists of an extracellular domain, a transmembrane domain and intracellular serine-threonine kinase domain. The fifth class contains putative extracellular LRRs, along with a PEST (Pro-Glu-Ser-Thr) which have a role in protein degradation accompanied by short protein motifs (Thomma *et al*., 2011). *RPW8* (*Arabidopsis*) belongs to the sixth class, which contains a membrane protein, along with a putative coiled coil domain (Wang *et al*., 2009). The seventh class contains R-genes such as *RRS1-R* (*Arabidopsis*), which provides resistance to the bacterial phytopathogen *Ralstonia solanacearum*. It contains a C-terminal extension with a putative nuclear localization signal (NLS) along with a WRKY domain (Deslandes *et al*., 2003). Maize *Hm1,* which confers resistance against Southern corn leaf blight, belongs to the eighth class of R-genes. It is an enzymatic R-gene, and neither contains LRR nor NBS groups (Johal *et al*., 1992).

2.11 Signal transduction pathways in plant defense

A complex network of signal molecules and transcriptional regulators are involved in plant defense responses. Overlapping signaling networks are used by ETI and PTI, which transfer the resistance signal initiated at the receptor, to the cell nucleus. These signaling networks are mediated mainly by plant hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) (Glazebrook, 2005). Mutational studies have been conducted to identify the role of these hormones in plant defense response.

SA signaling pathways were found to have a role in biotrophs, hemibiotrophs and in systemic acquired resistance (Kumar, 2014). It is the central regulator of defense in plants as it interacts with other signaling pathways to induce disease resistance response (Thaler *et al*., 2012). Mutants have been used to study the role of SA in signal transduction. Some mutants showed constitutive expression of Pathogenesis-related (PR) genes, SA accumulation and hypersensitive response symptoms, which resulted in resistant reactions (Clarke *et al*., 2000). Non Expressor of *PR-1* (*NPR1*) is an essential component of this pathway. Monomers of *NPR1,* upon dissociation caused by an increase in SA, enter the nucleus and interact with TAG transcription factors,

which activates *PR-1* expression (Johnson *et al*., 2003). There are several other important components which play a role in the SA pathway such as *NPR3*, *NPR4* and *EDS5* (Enhanced disease susceptibility 5).

The SA signaling pathway is ineffective against necrotrophs and instead JA plays an important role in the induction of plant resistance to necrotrophic plant pathogens (Kumar, 2014). PR gene activation is not always correlated with increased SA levels, indicating that JA and ET also lead to the induction of PR genes. *PDF1-2* and *Thi2-1*, which code for defensin and thionin, respectively, are expressed upon exogenous application of JA or ET (Penninckx *et al*., 1998). Mutants that are insensitive to JA and ET have been used to dissect and study these pathways. ET both positively and negatively modulates the SA signaling pathway (Pieterse *et al*., 2012).

Another signaling pathway that plays an important role in defense responses is the ABA signaling pathway. Varied defense responses triggered by ABA have been observed in plants that differ with type of plant tissues, the infection stage and pathogen approach (Ton *et al*., 2009). A high level of resistance to necrotrophs and JA/ET-responsive gene expression is observed when *ABI1* and *ABI2* (abscisic insensitive 1 and 2), both of which are negative regulators of ABA, are mutated (Anderson *et al*., 2004; Hernández-Blanco *et al*., 2007). Thus, ABA signaling has been identified as a complex modulator of plant defense responses (Sánchez -Vallet *et al*., 2012).

2.12 Transcriptome analysis

Traditional genetic approaches are not enough to understand the dynamics of the intertwined downstream defense signaling pathways. With the increasingly diverse approaches of molecular biology, the study of complex host-pathogen interactions has become easier. The high-throughput transcriptome analysis is an important first step for interpreting gene expression in both plants and pathogens. The transcriptome is a complete set of messenger RNAs produced in a particular cell or tissue type. Using deep-sequencing techniques, the sequencing of mRNA (RNA-Seq) has revolutionized the way in which eukaryotic transcriptomes are analyzed. Generally, a population of RNAs (total or fractionated as poly (A) +) is converted to a cDNA library with one or both ends ligated to adapters. Each cDNA molecule, using an amplification approach or not, is sequenced in a high-throughput way to attain short sequencing from either one end (single-end) or both ends (paired-end). Based on the sequencing platform used the reads are usually 30-400 base pairs long (Wang *et al*., 2009).

Transcriptome analyses require robust, efficient and statistically principled bioinformatic tools (Garber *et al*., 2011). The tools are categorized as: 1) those for read alignment 2) those for transcript assembly and 3) those for transcript and gene expression quantification.

The alignment of the sequenced reads to a reference genome is the primary step in Next-generation sequencing. The pre-requisite step prior to mapping of the reads is to check the quality of the sequenced reads. This is done by filtering reads with poor sequence qualities and removing the ligated adapters, thereby minimizing the computational time required for alignment or assembly (Garber *et al*., 2011). The subsequent step of analysis consists of assembly and quantification. The aligned sequences are quantified in order to measure gene expression. The transcript expression is measured by estimation of the number of fragments generated by each transcript using bioinformatic tools. Thus, measuring the number of fragments in each condition signifies the expression level of the transcript (Trapnell *et al*., 2012). Furthermore, the expression level is compared among different treatments. Various computational tools are used for analysis of RNA-Seq expression profiles for different treatments to determine overall effects. This leads to downstream analyses, such as gene annotations and enrichment in order to identify candidate genes.

2.12.1 Transcriptome of infected lentil plants

In the past few years, RNA-Seq studies have been conducted in lentil to assemble the expressed transcriptomes and to identify genes expressed at different developmental stages (Sudeesh *et al*., 2016). One study was conducted to identify candidate genes for resistance to ascochyta blight in three different genotypes of *L. culinaris* using their transcriptomes after inoculations with *A. lentis* (Sari *et al*., 2018). In that study, many NBS-LRR genes were up-regulated in the susceptible genotype suggesting their contribution to susceptibility of lentil to *A. lentis*. The RLK (receptor-like kinase) gene family involved in pathogen recognition was also found to be differentially expressed among the genotypes. Different sets of RLKs were up-regulated in the resistant genotype suggesting these to be candidate genes for resistance against *A. lentis*. Another transcriptome study was conducted in lentil to uncover the genetic basis of resistance to *A. lentis* (Khorramdelazad *et al*., 2018). Differentially expressed genes between resistant and susceptible genotypes were profiled during early stages of *A. lentis* infection. Genes were identified which

had key roles in defense responses. The resistant genotype showed an earlier signalling response to *A. lentis* infection and a high level of expression of defense related genes.

Chapter 3

3.0 Materials and Methods

3.1 Plant material and fungal isolate

RIL population LR-66 was previously developed from a cross between *L. ervoides* accessions L01-827-A and IG 72815 (Gujaria-Verma *et al*., 2014). A total of 94 RILs at the F_9 generation were phenotyped for resistance to *C. lentis* race 0 and race 1 and genotyped for the identification of QTLs (Bhadauria *et al*., 2017a). The resistant RIL LR-66-528 (disease severity 5% $\pm$ 0), the susceptible RIL LR-66-524 (disease severity 72% $\pm$ 1.2), parental accessions IG 72815 (disease severity 32% $\pm$ 1.2) and L01-827A (disease severity 52% $\pm$ 1.2) and the checks *L. culinaris* ssp. *culinaris* cultivars Eston (disease severity 95% $\pm$ 0) and CDC Robin (disease severity 95% $\pm$ 0) were previously planted in three biological replicates in the greenhouse (Kapoor, 2018). Whole plants were inoculated with *C. lentis* isolate CT-30 (race 0) at a concentration of 5×10^4 conidia mL^{-1} using approximately 3 mL of conidial suspension per plant in an experiment with a randomized complete block design with three biological replicates. Five leaves per plant were arbitrarily harvested and fixed immediately in mail tubes containing CMAA fixative (30% chloroform, 60% methanol, 10% acetic acid) for microscopy experiments. The rest of the infected leaves and stems were harvested from each biological replicate and flash-frozen in liquid nitrogen for further molecular experimentation and used the current study. The samples were harvested at 6, 12, 24, 48, 72, 96, 120, 144 hours post inoculation (hpi). Plants with three biological replicates sprayed with water and tween-20 (non-inoculated) collected as mock samples. The biological replicates were blocked over time due to limited space and sampling time constraints (Kapoor, 2018).

A field isolate from a commercial lentil field in Saskatchewan *Colletotrichum lentis* isolate CT-30 (race 0) is was routinely maintained on oatmeal or oatmeal V8 agar plates supplemented with 0.01% chloramphenicol.

3.2 Determination of critical time-points for RNA-Seq analysis through fungal biomass estimation

Earlier microscopy studies revealed few and inconsistent differences among the genotypes (Kapoor, 2018), so quantitative PCR for *in planta* fungal biomass estimation was proposed to supplement histopathology data to identify critical time points for in-depth gene expression studies.

The sample for each biological replicate of LR-66-528 and LR-66-524 from Kapoor (2018) was ground in a mortar with a pestle using liquid nitrogen. From each finely ground sample, 100 mg were transferred into each of two 2 mL capped microcentrifuge tubes, one for fungal biomass determination through qPCR and another for identification of differentially expressed genes in resistant and susceptible genotypes through RNA-seq. These tubes were stored in -80 °C.

For *in planta* fungal biomass determination, genomic DNA was extracted from finely ground samples of both, inoculated and non-inoculated plants using Qiagen DNeasy Plant Mini Kit®. The purity and quantity of DNA was determined using a NanoDrop ND8000 (Thermo Scientific, Wilmington, USA). Samples with an A260/280 ratio larger than 2.0 were discarded and were re-isolated. For qPCR, previously designed primers were used to amplify the housekeeping genes *Elongation factor 1-alpha* of *L. culinaris* (*LcEF1-α*) (Bhadauria *et al*., 2013) and *ACTIN* of *C. lentis* (*ClACT*) (Bhadauria *et al*., 2015) (Table 3.1.). These primers had been validated previously for their specificity using genomic DNA of IG 72815, L01-827A, LR-66-528 and LR-66-524 in a C1000™ Thermal Cycler (Bio-rad Laboratories, Inc., Hercules, CA, USA) and by testing amplification efficiency using qPCR in a QuantStudio™ 3 System (Applied Biosystems Inc., Foster City, CA, USA) for a total of 5 dilutions for three biological replicates of mock samples of both the resistant and susceptible genotypes (J. Halliday, Research Officer, Pulse Crop Pathology Group, University of Saskatchewan).

The relative fungal biomass was quantified *in planta* from samples harvested at 6, 12, 24, 48, 72, 96, 120 and 144 hpi. The genomic DNA of the plant tissues was adjusted to 25 ng μL^{-1}. Each qPCR reaction contained 2 µL DNA template, 5 µL SYBR® Green (catalog no. 4309155, Thermo Scientific), 0.2 µL of each 10 µM forward and reverse primers, and 2.6 µL sterilized water. The qPCR amplifications were performed in a QuantStudio™ 3 System (Applied Biosystems Inc., Foster City, CA, USA) using a fast-run program with default settings. The qPCR data of *ClActin* were normalized using *EF1-α* as a reference gene. The relative fungal biomass was reported relative to the mock (non-inoculated samples) by calculating the log2fold

change following the method of Livak and Schmittgen (2001). The Student's t-test was used to determine the differences among the genotypes at different time-points (p-value < 0.05).

Table 3.1. Forward and reverse primer pairs designed for fungal biomass determination of race 0 isolate CT-30 of *Colletotrichum lentis* in resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides*. Primer ClActin designed from exonic region of the *ACTIN* gene of *C. lentis* (*ClACT*) (Bhadauria *et al*., 2015) used as target gene and primer LcEF-1α designed from *Elongation factor 1-alpha* of *L. culinaris* (*EF1-α*) used as reference gene (Bhadauria *et al*., 2013).

Primer name	**Gene**	**Forward primer**	**Reverse primer**
ClActin (target)	*ClACT*	CACGCTCTACTACGACGGAC	CGAAGACGAAGTTGTCGGGA
LcEF-1α (Reference)	*EF1-α*	TGTCGACTCTGGGAAGTCAA	CTCTTTCCCTTTCAGCCTTG

3.3 Identification of differentially expressed genes

In order to identify differentially expressed genes, total RNA was extracted and purified from three biological replicates per time-point (24, 48, 72 and 96 hpi) of CT-30-inoculated plants of LR-66-528 (resistant) and LR-66-524 (susceptible), as well as from three biological replicates of non-inoculated plants (30 samples in total) using the Qiagen RNeasy Plant Mini Kit (with on-column DNAse treatment). The RNA integrity was determined by denaturing agarose gel electrophoresis (Barril and Nates, 2012) and its quality and quantity were determined using NanoDrop ND8000 (ThermoFischer Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent, USA). Strand-specific RNA libraries (adapted from the NEB Ultra-Directional RNA Library Prep protocol) were generated and were sequenced using the Illumina PE125 HiSeq 2500 (v4 chemistry) at the Michael Smith Genome Sciences Center, BC, Canada (BCGSC). The returned raw fastq reads were filtered in Trimmomatic (version 0.36) (Bolger *et al*., 2014) to remove

adaptors and low quality reads with the parameters TruSeq3-PE-2.fa:2:30:10 which removes the Illumina adapters, leading:3, trailing:3 which removes first and third base pair of each read, slidingwindow:4:15 which scans the read with a 4-base wide sliding window and cuts when the average quality per base drops below 15 and minlen:36 which drops the reads which are less than 36 bases long. Removal of low-quality reads and adaptors was confirmed using FASTQC, the cleaned reads were submitted to the program Spliced Transcripts Alignment to a Reference (STAR version 2.6.1a; default settings) (Dobin *et al*., 2013) for mapping against the *L. culinaris* genome V1.2 (K. Bett, Dept. of Plant Sciences, University of Saskatchewan, unpublished). STAR is an aligner that aligns the reads to a reference genome by creating a genome index. This alignment algorithm was used to map the reads to the *L. culinaris* genome. The read counts per gene were determined using STAR during the mapping process.

3.3.1 Assessment of variability among sequencing samples

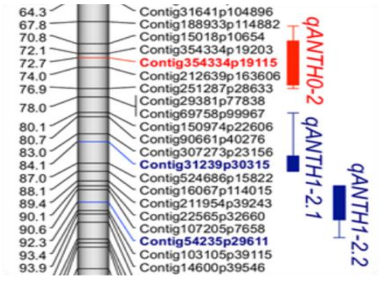
Principal Component Analysis (PCA) implemented in JMP genomics 8.0 was used to obtain the variability of gene expression among the sequencing samples generated through RNA-Seq by reducing the dimensions of the data set. Similarities between the genotypes and time-points were correlated to the distances in the projection of the space defined by the principal components. The gene read counts file was fed as the input file. Principal Component Variance Analysis (PVCA) was also implemented in JMP genomics 8.0 and was used to estimate the proportion of variability accounted for by the variance components of the sequencing samples. Based on the proportion, it was determined which variance component was most and least prominent in the data.

Before identifying the differentially expressed genes between the resistant and the susceptible RILs, comparisons were made between non-inoculated mock and inoculated sample data from 24, 48, 72 and 96 hpi for each RIL to identify the genes that responded to CT-30 infection. The R package Deseq2 was used to compare the mock with samples from different time-points using $P_{adj} < 0.05$ and gene expression fold change > 2. The raw counts file for all genes obtained using the STAR aligner was used as an input file. Subsequently, the identified genes were used to conduct the pair-wise comparisons between LR-66-528 and LR-66-524 at 24, 48, 72 and 96 hpi to obtain DEGs at each time-point, using a threshold $P_{adj} < 0.05$ and gene expression fold change > 2. Common sets of DEGs among different time-points were visualized in a Venn diagram

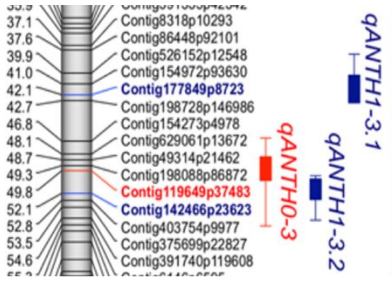
generated with the bioinformatic tool jvenn (Bardou *et al*., 2014). Lists of genes at different time-points were merged to obtain a complete list of DEGs.

3.3.2 Comparison of transcriptome and QTL mapping data

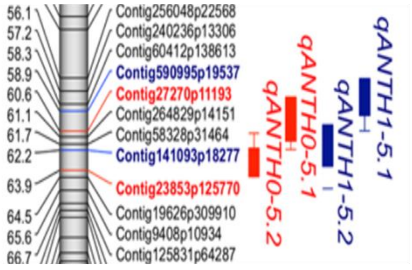
Previously identified QTLs associated with anthracnose resistance (Fig.3.1., Bhadauria *et al*., 2017a) were dissected to identify the chromosomal locations of the genes underlying the QTLs (personnel communication, Z. Cao, PDF, Pulse Crop Pathology Group, University of Saskatchewan) The anthracnose QTLs (2-LOD) intervals mapped on different chromosomes were projected onto the physical map of *L. culinaris* (v1.2, K. Bett, Dept of Plant Sciences, University of Saskatchewan, unpublished) using the software Strudel (v 1.15.08.25) (Bayer *et al*., 2011). The main region on the physical map with high density projections was focussed, whereas minor regions projecting to other chromosome were not taken into consideration in order to restrict the distance. Using the GTF file of the *L. culinaris* genome, the markers spanning the highly dense region were used to identify the genes present in those intervals. DEGs identified by RNA-seq were compared with the genes identified in the high-density regions of the QTLs to identify disease resistance candidates detected by co-localization..



Chromosome 2



Chromosome 3



Chromosome 5

Chromosome 7

Figure 3.1. Quantitative trait loci controlling resistance to *Colletotrichum lentis* on chromosomes 2, 3, 5 and 7 of the linkage map of *Lens ervoides* recombinant inbred line population LR-66 derived from cross between L01-827A and IG 72815. On the right side of each linkage groups are the SNP markers and positions of the SNPs in centimorgans are on the left side. The position (p) of a SNP is indicated by the marker name in the contig of the QTLs conferring resistance to *C. lentis* race 0 isolate CT-30 are shown as red and *C. lentis* race 1 isolate CT-21 as blue. 2-LOD likelihood intervals are represented as vertical lines with caps on bars (adapted from Bhadauria *et al*., 2017a).

3.3.3 Gene expression profile analysis

The initial read counts per gene data procured using the STAR aligner were used to identify the read counts of DEGs. The read counts of DEGs were normalized by transforming to Reads Per Kilobase of exon per Million reads (RPKM). The genes with less than read counts of 10 were removed before normalization. The resulting file was submitted to JMP genomics 8.0 (JMP Genomics®, SAS Institute) for K-mean clustering analysis. To comprehend the biological relevance of the differentially expressed genes, GO enrichment analysis was performed for genes in each of the clusters. The *L. culinaris* gene IDs were transformed to the *M. truncatula* gene IDs because the *L. culinaris* is not annotated. The Entrez IDs of the corresponding genes were used as the input file for the gene enrichment analysis. The file was submitted to http://plantregmap.cbi.pku.edu.cn/go.php for GO term enrichment. *Medicago truncatula* was selected as the input species. The biological process aspect was selected for analysis at threshold False Discovery Rate (FDR) value (< 0.001) calculated by Benjamini and Hochberg (1995) method.

3.4 Validation of gene expression using quantitative PCR

Reproducibility of results from the transcriptomics experiment was assessed by validating the expression of selected DEGs at 24, 48, 72 and 96 hpi through RT-qPCR. For this purpose, samples of the genotypes LR-66-528 and LR-66-524 stored in -80 °C were used from an additional, previously conducted experiment with three biological replicates, which had been

inoculated with isolate CT-30 and sampled at 6, 12, 24, 48, 72, 96, 120 and 144 hpi. This experiment was independent of that used for RNA-Seq analysis (Kapoor, 2018).

Total RNA was isolated from 100 mg of the ground samples using Qiagen RNeasy Plant Mini Kit (with on-column DNAse treatment) from CT-30-inoculated resistant LR-66-528 and susceptible LR-66-524 with three biological replicates collected at 24, 48, 72 and 96 hpi and non-inoculated RILs with three biological replicates (30 samples in total). The RNA integrity was determined by denaturing agarose gel electrophoresis (Barril and Nates, 2012) and its quality and quantity were determined using NanoDrop ND8000 (ThermoFischer Scientific, USA). RNA for all the samples were normalized to 50 ng L^{-1} to synthesize cDNA using High-Capacity cDNA Reverse Transcription kit (Thermo-Fischer Scientific).

From among all DEGs identified at 24, 48, 72 and 96 hpi in the RNA-Seq analysis, 21 DEGs genes were selected for expression validation using RT-qPCR based on their role in disease resistance from the gene annotations of the *L. culinaris* genome. Some of these selected 21 genes were up-regulated in the resistant RIL LR-66-528 and some were up-regulated in susceptible RIL LR-66-524. Out of the 21 selected genes, 8 genes were selected which were expressed at 24 hpi, 6 genes expressed at 48 hpi, 6 genes expressed at 72 hpi and 8 genes expressed at 96 hpi. Out of these, 5 genes were expressed at more than one time-point: Gene Lc23518 expressed at 24 and 96 hpi, Lc35307 expressed at 24 and 48 hpi, Lc34856 expressed at 24, 48 and 72 hpi, Lc20454 was expressed at 24 and 96 hpi and Lc35937 at 48 and 72 hpi. Three additional genes up-regulated to similar levels in both RILs based on RNA-seq (Fold change < 2 and $P_{adj} < 0.05$) were selected for expression validation in samples from 24, 48 and 72 hpi using RT-qPCR. (Table 3.2).

Elongation factor 1-alpha (*EF1-α*) was used as the reference gene. Primers were designed with Primer3 (web version 4.1.0) using default settings from the exonic regions of the selected genes based on the lentil genome (v1.2, K. Bett, Dept of Plant Sciences, University of Saskatchewan, unpublished) (Table 3.2). The amplification efficiency tests were conducted for all designed primers using qPCR in a QuantStudio™3 System (Applied Biosystems Inc., Foster City, CA, USA) using cDNA (50 ng/µl) serially diluted 1:2 (V/V) 4 times in nuclease-free ultra pure water (Invitrogen Life Technologies, Carlsbad, CA, USA) for a total of 5 dilutions for 3 biological replicates of mock samples of the resistant and susceptible RILs. The amplification efficiencies

were calculated based on the slope of the standard curve. The equation used for calculating the efficiency was $E = -1+10^{(-1/slope)}$. The desired amplification efficiency ranged from 90 to 120%.

Each RT-qPCR reaction consisted of 2 µL cDNA template, 5 µL SYBR green, 0.8 µL of each forward and reverse primers and 1.4 µL of nuclease-free water. It was performed in QuantStudio™ 3 System (Applied Biosystems Inc.) using a fast-run program with default settings. The relative expression of the target genes was calculated as $2^{-(CT\ gene\ of\ interest-CT\ reference\ gene)}$ (Livak and Schmittgen, 2001). Student's t-tests were used to determine significant differences in gene expression between resistant and susceptible RILs.

Table 3.2. Gene description and primer sequences designed for gene expression validation by RT-qPCR of genes identified by transcriptome analysis of resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* sampled 24, 48, 72 and 96 h postinoculation (hpi). A total of 21 differentially expressed genes were selected based on their role in disease resistance from gene annotations of *L. culinaris* genome.

Differentially expressed genes				
Gene ID	**hpi**	**Description**	**Forward sequence**	**Reverse sequence**
Lc23464	24	Serine/threonine-protein phosphatase	GGTTCACCGTTTTGCTTCCT	GGACAATCGGACCTCAGTGA
Lc23518	24, 96	LRR receptor-like kinase	CCGGTTTGAAGTTTCTCTCCA	TCATGCTTGTTGATGTGATGAC
Lc29239	24	Two-component response regulator-like APRR7 protein	ACACACTCACACCCACTCAA	AGGTGGTGGGGACTAACATG
Lc13986	24	Adenosylhomocysteinase (AdoHcyase)	CCGAGGCTGACATTTTCGTT	ACCCATCTGTCAGTCTGTGG
Lc34856	24, 48, 72	F-box/LRR-protein	GCTTTCAGTGCCTTTTATTAACG	AATCCCCATGTTCGTCACCC
Lc35937	24, 48, 72	LRR receptor-like kinase	ACGGGAGCTTGGAAGATTGG	GCCAAACCAAAGTCTCCAACC
Lc35307	24, 48	Serine/threonine-protein kinase	TTGCTTCTTCAACGCCTTGT	TGCCCTTGACTCCACAACTC

Lc20454	24, 96	LRR receptor-like kinase	TGCTGGCTACTGGATTGGAA	CTCGGTGCAATATTCGTGGG
Lc38516	48	Receptor-like kinase	CTACACCAAGGTTGCGACATG	GTACAGCAACTCAGGAGCCA
Lc34767	48	TMV resistance protein N	TCTATCATTCTTGTCCTCAGTCG	ATCAGGGCAAAACTTCTCCG
Lc14149	72	LRR and ubiquitin-like domain plant-like protein	CCGGGTCTGCTTGCAATAAA	GCTGCCAGTTTATAACAACACC

Table 3.2. Continued

Gene ID	**hpi**	**Description**	**Forward sequence**	**Reverse sequence**
Lc34004	72	RPM1-interacting protein 4 (RIN4) family protein	ATTGGGATCAGAAGGGGCAA	CGAGGGAGGTGTTGTTGTTG
Lc34550	72	LRR and NB-ARC domain disease resistance protein	GGTTGGCACTTCTCCTCTCT	GGAACAACAGATCCCATGCC
Lc36233	72	Avr9/Cf-9 rapidly elicited protein	CACTCTCATGCTGAATCGCC	TAACAGCCTCCACTTGACGT
Lc09295	96	MYB transcription factor MYB91	CCAAACCACCTGCACCAAAA	TTTCCTCGACCAACTTTGCA
Lc13120	96	Transmembrane protein, putative	TGATGCAAACCTTACTCGCG	GCTCCCAACTCAAAAGCTCC
Lc17524	96	PPR containing plant protein	CCCTTCAAACAAAGCCTCAGT	CAACACAGAAGCAAGACCCA
Lc33978	96	LRR receptor-like kinase	GCATTGTTTCTGGTGGGGTT	ACAGTTCCACAAGCTCCTCT
Lc38860	96	Pathogenesis-related thaumatin family protein	CATGCGTTACTGGAGACTGC	TCGTTCAAGTCCACCGTACA
Lc34187	96	Serine/threonine protein kinase	TGCAGTTTTGGGAAGTGACG	AGGACAGCTACATTACGGT
Non-differentially expressed genes				

Lc16648	24	Pentatricopeptide repeat-containing protein	ACGCAATACAGTCAACACGG	ACTCTTAACACGGTCCCCTG
Lc35494	48	NAD(P)-binding rossmann-fold protein"	TTTGAGTCCCACCCAACCTT	TGGAAGGAAGAAACTGAGAGCA
Lc30103	72	Peroxidase	ATCATTCAACATGTCCAGATGCT	TGCAAACGGATGATGGATGG

Chapter 4

4.0 Results

4.1 Determination of critical time-points for RNA-Seq analysis through fungal biomass estimation

Quantitative PCR was conducted for fungal biomass determination *in planta* expressed as the Log2 fold change of the fungal actin gene *ClACT*. Overall, there was a trend for higher biomass of *C. lentis* in susceptible RIL LR-66-524 as compared to resistant RIL LR-66-528 (Fig. 4.1). There was high variability among biological replicates most likely because the experiment was blocked over time, so the difference in fungal biomass of *C. lentis* (race 0 isolate CT-30) was statistically significant only at 24 hpi (P = 0.0396). In both RILs, fungal biomass proliferated gradually from 6 to 24 hpi and then increased dramatically from 48 to 120 hpi in the susceptible RIL LR-66-524 and 48 to 96 hpi in the resistant RIL LR-66-528.

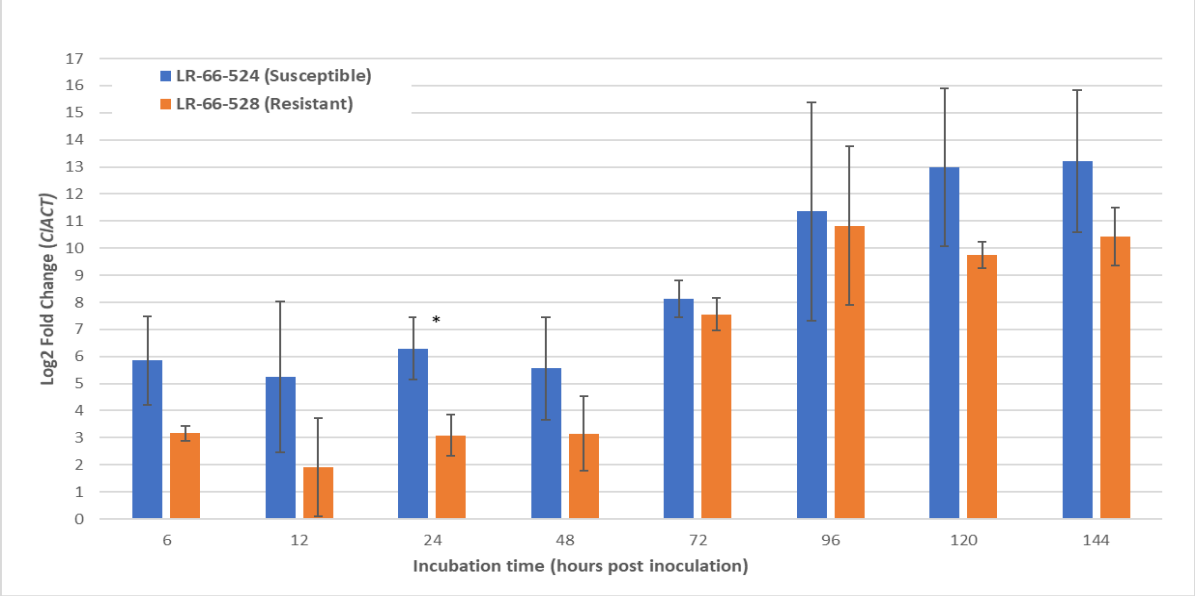


Figure 4.1. Relative fungal biomass of *Colletotrichum lentis* (race 0 isolate CT-30) expressed as the Log2 fold change of *C. lentis ClACT* (determined by qPCR) relative to mock (non-inoculated) samples in resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 at 6, 12, 24, 48, 72, 96, 120 and 144 hours post inoculation. Bar plots are

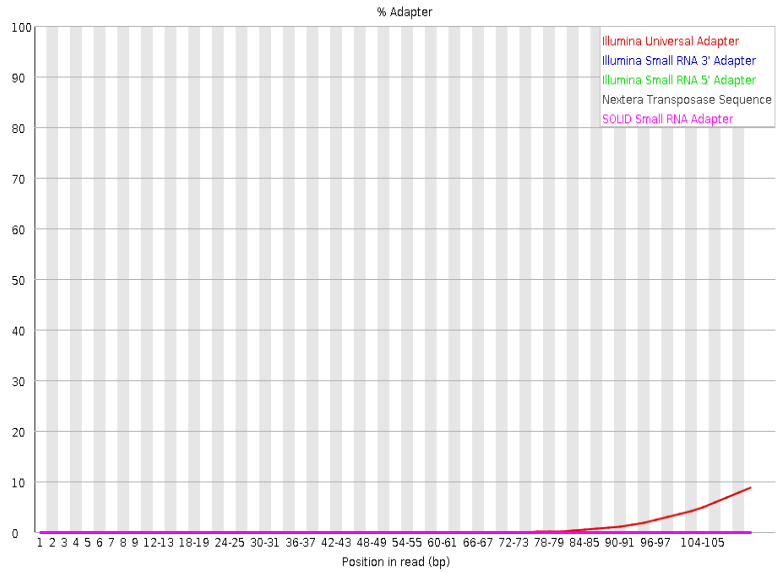
means ± SE (standard error of mean) of 3 biological replicates. Asterisks (*) denote statistical significance at $P < 0.05$.

The trend indicted that the period of 24 hpi to 96 hpi may be critical for exponential biomass increase of fungus so based on these observations, the time-points selected for transcriptome studies were mock (non-inoculated), 24, 48, 72 and 96 hpi for 3 biological replicates of RIL LR-66-528 and RIL LR-66-524.

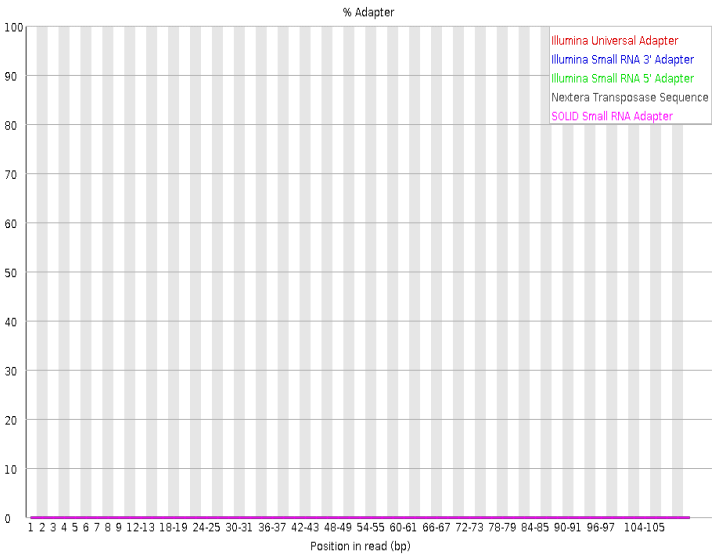
4.2 Identification of differentially expressed genes in resistant and susceptible genotypes and co-localization with QTLs for anthracnose resistance

Libraries were prepared and RNA-Seq was conducted on mock-inoculated and inoculated samples of LR-66-528 and LR-66-524 harvested at 24, 48, 72 and 96 hpi in order to identify DEGs. About 10-14 million high-quality raw reads were generated as paired-end sequences in a FASTQ format with an average length of 125 bp per library. The phred score of the sequences was higher than 30, which indicated that the base call accuracy was > 99%. The paired-end reads were ligated with universal adapter sequences for Illumina sequencing. The adapter sequences, which were removed from the de-multiplexed raw reads, accounted for less than approximately 10% of the sequence length. Quality check of the trimmed sequences showed that the adapters were removed successfully from both ends of the reads (Fig. 4.2).

The STAR aligner output showed that approximately 80-85% of the filtered reads aligned to the annotated *L. culinaris* genome. The read counts file generated during the mapping process had more than 30,000 genes.The gene read counts were used to analyze the variability among the sequencing samples. A three-dimensional Principal Component Analysis plot was generated that separated the samples of the three time-points (Fig. 4.3).



a **Adapter ligated read (524-24hpi-R1-PE2)**



b **Adapter removed read (524-24hpi-R1-PE2)**

Figure 4.2. Quality control graphs of reads generated from RNA-Seq of resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis*. Reads visualized in FastQC software: x-axis represents the position of ligated adapter on read (bp) and y-axis represents the % adapter ligated; a: Universal ligated adapter sequence (red) on a paired-end sequence accounting for 10% of the sequence length from 85 bp to 105 bp of replicate 1 of susceptible RIL LR-66-524 at 24 hpi. b: Trimmed sequence showing removed adapter from the paired-end sequence of replicate 1 of LR-66-524 at 24 hpi using software TRIMMOMATIC.

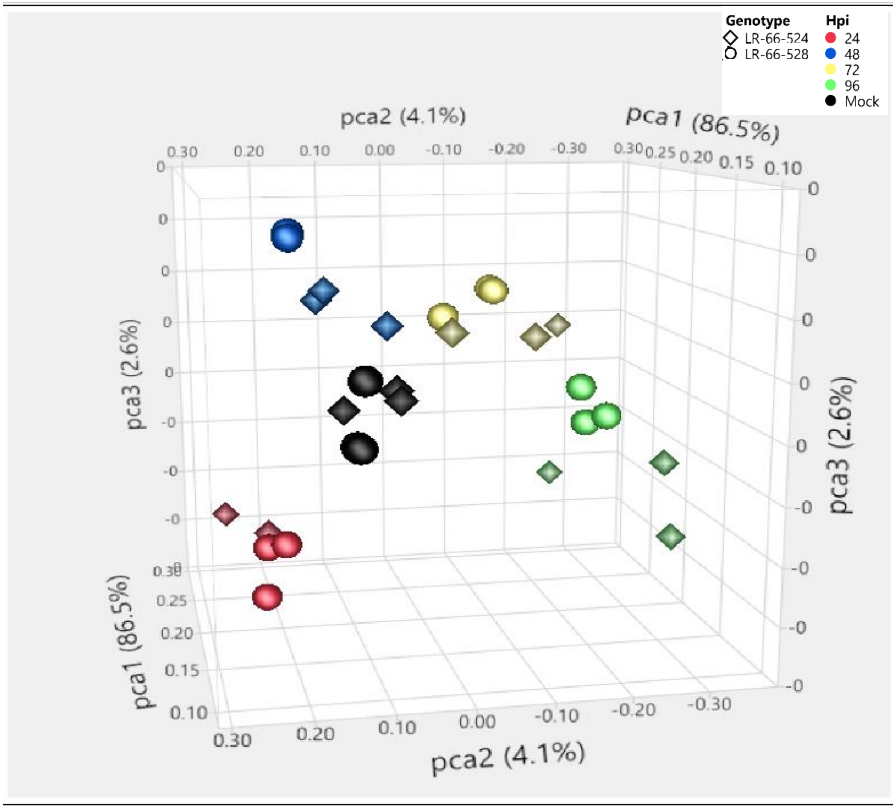


Figure 4.3. Three-dimensional Principal Component Analysis plot representing the variability among sequencing samples (three biological replicates each of resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66) inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* and incubated for 24, 48, 72 and 96 h. Data is represented in x-y-z coordinates with principal component axes (pca1, pca2 and pca3 representing genotypes, biological replicates and time-points.

Differences between the RILs were observed with increasing time after inoculation. At 48 and 96 hpi, the biological replicates of both RILs were far apart compared to biological replicates at 24 and 72 hpi. In order to assign the percentage of variance to RILs, hpi and the interaction of RILs x hpi, Principal Variance Component Analysis was conducted on these samples (Fig. 4.4). Results showed that incubation time, RILs and the interaction of RILs x hpi collectively accounted for 79.9% of the total variance. The largest variance proportion could be attributed to hpi (77.6%), followed by the RILs x hpi interaction (1.7%) and RILs (0.6%). The residual proportion was 20.1%, which remained unexplained.

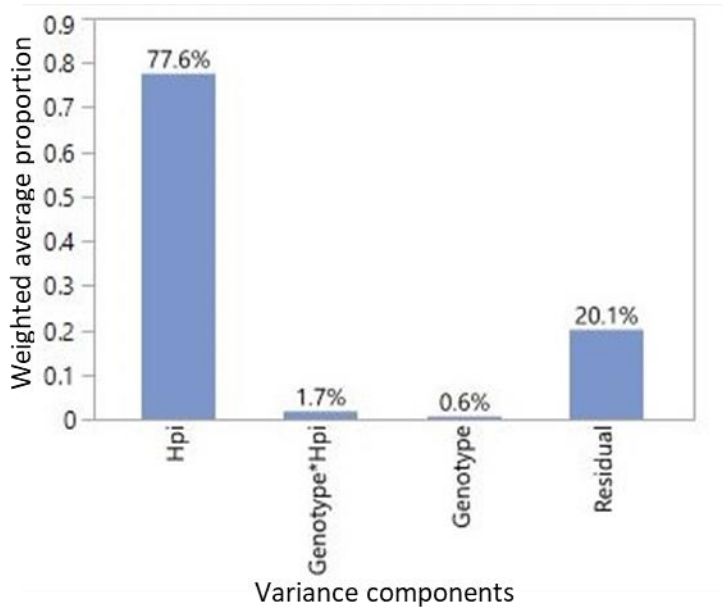


Figure 4.4. Weighted average proportion (%) of variance across the principal components (hpi, genotype x hpi, genotype and residual) contributing to the total variance among the sequencing samples of resistant RIL LR-55-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 (three biological replicates each) inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* and incubated for 24, 48, 72 and 96 h.

The gene expression of mock-inoculated samples was compared to that of samples harvested at 24, 48, 72 and 96 hpi for each RIL which resulted in 3,091 genes with a fold change larger than 2 ($P_{adj} < 0.05$) during the CT-30 infection process. Among these 3,091 genes, 477 genes were differentially expressed in samples of the two RILs harvested at 24, 48, 72 and 96 hpi (fold change > 2, $P_{adj} < 0.05$) (Appendix 1) Visualization as a Venn diagram (Fig. 4.5.) showed that out of 477 DEGs, 80 genes were expressed during the entire period lasting from 24 to 96 hpi. Two genes were expressed exclusively at 24 hpi, 108 genes at 48 hpi, 38 genes at 72 hpi and two genes at 96 hpi. One gene expressed at both 24 and 48 hpi, 20 genes at 48 and 72 hpi, one gene at 48 and 96 hpi and 185 genes at 24 and 96 hpi. There were no genes commonly expressed at 24 and 72 hpi and none at 72 and 96 hpi. One gene was commonly expressed at 24, 48 and 72 hpi. A total of 17 genes were expressed at 24, 72 and 96 hpi and 17 genes expressed at 24, 48 and 96 hpi. Two genes were expressed at 24, 48 and 96 hpi.

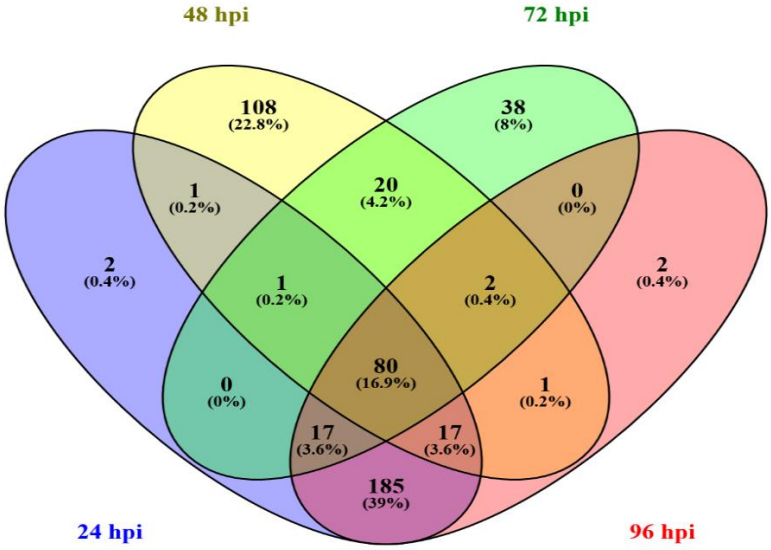


Figure 4.5. Venn diagram showing the number of differentially expressed genes between resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* and incubated for 24, 48, 72 and 96 h, representing the genes expressed exclusively and common among different time-points. Violet color represents genes expressed exclusively at 24 hpi, yellow for genes at 48 hpi, green for genes at 72 hpi and pink for genes at 96 hpi. Genes in overlapping areas are expressed at more than one time-point.

The 477 differentially expressed genes from the RNA-seq analysis were clustered in six expression clusters (Fig. 4.6). Cluster 1 contained 56 genes up-regulated in susceptible RIL LR-66-524 mainly at 48, 72 and 96 hpi and Cluster 2 contained 79 genes that were up-regulated in LR-66-524 mainly at 96 hpi. A total of 91 genes were present in Cluster 3 that were up-regulated in the resistant RIL LR-66-528 mainly at 24, 72 and 96 hpi. Cluster 4 contained 97 genes that were up-regulated in LR-66-524 primarily at 24 and 48 hpi. Cluster 5 was the smallest of all the clusters. It contained 51 genes that were up-regulated in LR-66-528 and down-regulated in LR-

66-524. The last Cluster 6 included 95 genes up-regulated in the resistant RIL primarily at 48 hpi and down-regulated in the susceptible RIL.

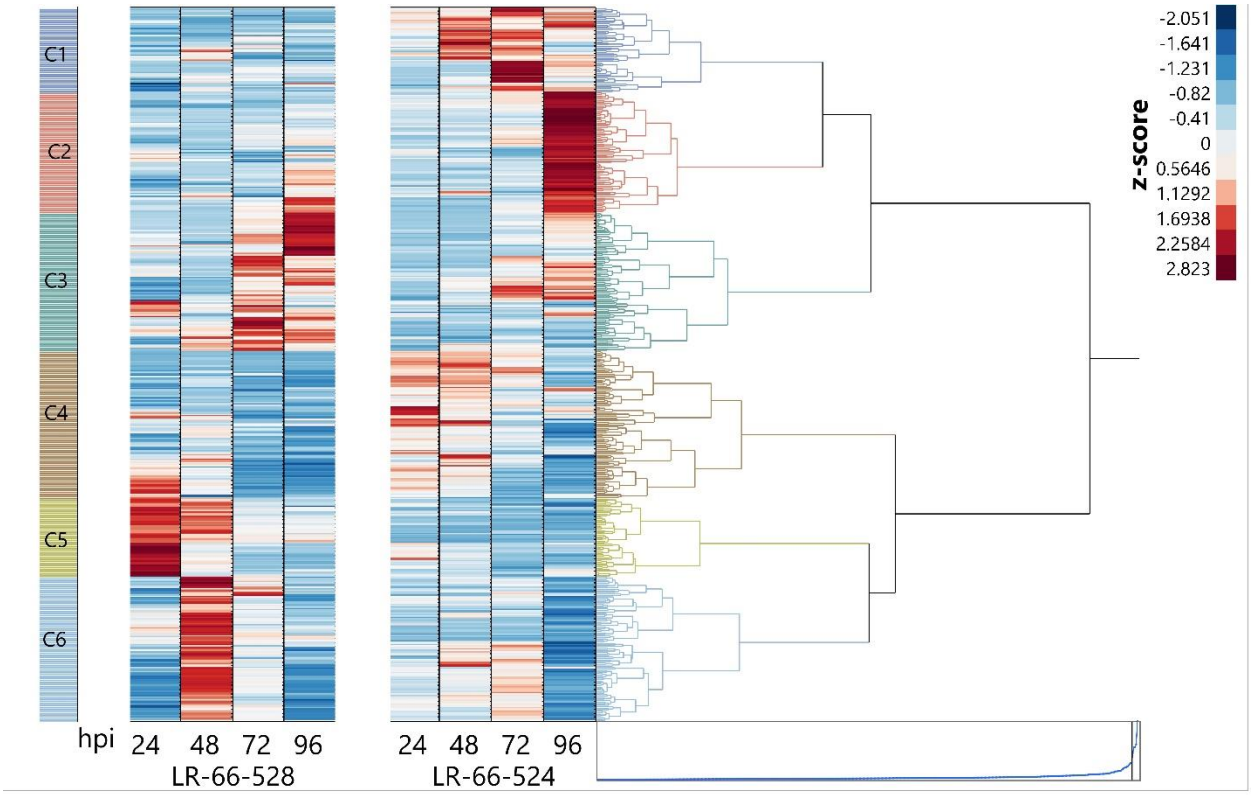


Figure 4.6. Hierarchical cluster analysis of gene expression profiles of differentially expressed genes between resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* and incubated for 24, 48, 72 and 96 h, representing the density of up- and down-regulated genes. C1 to C6 represent different clusters based on the expression of genes. 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 Pearson's correlation with an average distance among clusters.

The results of gene ontology showed that a total of 370 out of 477 DEGs had homology with the *M. truncatula* genome. The GO terms were assigned to the genes in different hierarchical clusters and were involved in different biological processes (Table 4.1). The GO terms for genes in Cluster 1 showed that these were enriched in cell death related processes such as

“programmed cell death”, “regulation of cell death” and cell wall organization or biogenesis such as “cell wall macromolecule metabolic process”. Enriched GO terms for Cluster 2 were involved in responses to stress such as “response to water”, “response to water deprivation”, and cell morphogenesis involved in differentiation such as “pollen tube growth” and “cell tip growth”. GO terms for genes in Cluster 3 were enriched in protein phosphorylation processes such as “peptidyl-tyrosine dephosphorylation” and RNA processing such as “mRNA splicing, via spliceosome”, RNA splicing, via transesterification reactions” and “RNA splicing, via transesterification reactions with bulged adenosine as nucleophile”. GO mapping of genes in Cluster 4 showed that these were enriched for genes with function in cell growth and differentiation processes such as “cellular developmental process”, cellular biogenesis such as “plant-type secondary cell wall biogenesis”, “regulation of cellular component biogenesis”, and metabolic processes such as “hormone metabolic process” and “regulation of hormone levels”. GO terms for genes in Cluster 5 were enriched in carbohydrate and amino acid metabolic processes such as “oligosaccharide biosynthetic process” and “aspartate family amino acid metabolic process”. Go terms for genes in Cluster 6 were enriched with stress-related process such as “response to water”, DNA metabolic process such as “DNA recombination”, regulation of hydrolase activity such as “regulation of GTPase activity”, DNA packaging processes such as “chromatin assembly” and “nucleosome organization”.

Table 4.1. Gene ontology (GO) terms assigned to differentially expressed genes between resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis*. Genes present in different expression clusters enriched with roles in various biological processes with gene counts per GO term at threshold FDR < 0.001

GO ID	GO Term	Count	Percentage per cluster (%)	False discovery rate (FDR)	Cluster
GO:0043069	negative regulation of programmed cell death	2	7.4	0.00017	1
GO:0060548	negative regulation of cell death	2	7.4	0.00026	1
GO:0009626	plant-type hypersensitive response	2	7.4	0.00063	1
GO:0034050	host programmed cell death induced by symbiont	2	7.4	0.00063	1
GO:0043067	regulation of programmed cell death	2	7.4	0.00101	1
GO:0010941	regulation of cell death	2	7.4	0.00159	1
GO:0010410	hemicellulose metabolic process	2	7.4	0.0032	1
GO:0012501	programmed cell death	2	7.4	0.00345	1
GO:0010383	cell wall polysaccharide metabolic process	2	7.4	0.00444	1
GO:0008219	cell death	2	7.4	0.00576	1
GO:0016265	death	2	7.4	0.00576	1
GO:0044036	cell wall macromolecule metabolic process	2	7.4	0.0062	1
GO:0032787	monocarboxylic acid metabolic process	3	11.1	0.00773	1
GO:0009414	response to water deprivation	3	17.6	0.0052	2
GO:0009415	response to water	3	17.6	0.0056	2
GO:0009064	glutamine family amino acid metabolic process	2	11.7	0.0063	2
GO:0009860	pollen tube growth	2	11.7	0.0073	2
GO:0071702	organic substance transport	5	29.4	0.0081	2
GO:0009932	cell tip growth	2	11.7	0.0098	2
GO:0035335	peptidyl-tyrosine dephosphorylation	2	25	0.00083	3
GO:0000398	mRNA splicing, via spliceosome	2	25	0.00524	3
GO:0000375	RNA splicing, via transesterification reactions	2	25	0.00778	3
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	2	25	0.00778	3
GO:0048869	cellular developmental process	6	11.3	2.00E-04	4

	Table 4.1. Continued				
GO ID	**GO Term**	**Count**	**Percentage per cluster (%)**	**False discovery rate (FDR)**	**Cluster**
GO:0030154	cell differentiation	5	9.4	0.00041	4
GO:0060560	developmental growth involved in morphogenesis	4	7.5	0.00046	4
GO:0010817	regulation of hormone levels	3	5.6	0.00149	4
GO:0016049	cell growth	4	7.5	0.00153	4
GO:0048589	developmental growth	4	7.5	0.00183	4
GO:0065008	regulation of biological quality	6	11.3	0.00333	4
GO:0009834	plant-type secondary cell wall biogenesis	2	3.7	0.00343	4
GO:0009826	unidimensional cell growth	3	5.6	0.00403	4
GO:0044087	regulation of cellular component biogenesis	2	3.7	0.00438	4
GO:0022412	cellular process involved in reproduction in multicellular organism	2	3.7	0.00526	4
GO:0040007	growth	4	7.5	0.00531	4
GO:0048468	cell development	3	5.6	0.0063	4
GO:0042445	hormone metabolic process	2	3.7	0.00811	4
GO:0000902	cell morphogenesis	3	5.6	0.00932	4
GO:0009066	aspartate family amino acid metabolic process	2	25	0.0016	5
GO:0009312	oligosaccharide biosynthetic process	2	25	0.0016	5
GO:0009311	oligosaccharide metabolic process	2	25	0.0054	5
GO:0015698	inorganic anion transport	2	25	0.0067	5
GO:0006310	DNA recombination	3	6.5	0.00095	6
GO:0043087	regulation of GTPase activity	2	4.3	0.00431	6
GO:0043547	positive regulation of GTPase activity	2	4.3	0.00431	6
GO:0051336	regulation of hydrolase activity	3	6.5	0.00527	6
GO:0006334	nucleosome assembly	2	4.3	0.00534	6
GO:0051345	positive regulation of hydrolase activity	2	4.3	0.00534	6
GO:0034728	nucleosome organization	2	4.3	0.00552	6
GO:0031497	chromatin assembly	2	4.3	0.00589	6
GO:0006333	chromatin assembly or disassembly	2	4.3	0.00608	6
GO:0009414	response to water deprivation	3	6.5	0.00617	6
GO:0006259	DNA metabolic process	5	10.8	0.00649	6

	Table 4.1. Continued				
GO ID	**GO Term**	**Count**	**Percentage per cluster (%)**	**False discovery rate (FDR)**	**Cluster**
GO:0065004	protein-DNA complex assembly	2	4.3	0.00748	6
GO:0006323	DNA packaging	2	4.3	0.00769	6
GO:0006325	chromatin organization	3	6.5	0.00874	6
GO:0006950	response to stress	11	13.0	0.00897	6

The projection of anthracnose resistance QTLs qANTH0-2, qANTH1-2.1 and qANTH1-2.2 present on linkage group 2 (Bhadauria *et al*., 2017a) of the *L. culinaris* linkage map onto the physical map led to the identification of 3,078 genes. The QTL intervals qANTH0-3, qANTH1-3.1 and qANTH1-3.2 on linkage group 3 contained 605 genes, and those of qANTH0-5.1, qANTH0-5.2, qANTH1-5.1 and qANTH1-5.2 present on linkage group 5 had 1,437 genes. The projection of qANTH0-7 localized on linkage group 7 had a total of 713 genes. Comparison of these genes with DEGs identified by RNA-seq led to the co-localization of a total of 22 DEGs of which 9 DEGs were found on chromosome 2, 10 DEGs on chromosome 5 and 3 DEGs on chromosome 7 (Table 4.2).

Two of these genes, *Lc23518* and *Lc09295*, were included in the gene expression validation through RT-qPCR. *Lc23518* was down-regulated at 24 hpi and up-regulated at 96 hpi in the resistant RIL and was found in the QTL interval qANTH0-5.1 localized on linkage group 5. *Lc09295* was up-regulated at 96 hpi in the resistant RIL and was located in the QTL interval qANTH0-2 on linkage group 2.

Table 4.2. List of *Lens culinaris* genes present in resistance QTLs to race 0 of *Colletotrichum lentis* on linkage groups 2, 5 and 7 of the linkage map developed for *Lens ervoides* recombinant inbred line population LR-66 (Bhadauria *et al*., 2017a) and differentially expressed in infected lentil tissue based on RNA-Seq, their functional annotations and fold change. Positive or negative fold change depicts up- or down-regulated in resistant RIL

Gene ID	hpi	QTL	Gene annotation	Fold change
Lc09011	96	qANTH0-2	Uncharacterized protein	-2.43221
Lc09511	24, 96	qANTH0-2	DEAD-box ATP-dependent RNA helicase, putative	4.053338
Lc09494	24	qANTH0-2	C-repeat binding factor 3	4.328555
Lc05315	24, 96	qANTH0-2	DEAD-box ATP-dependent RNA helicase 27	5.171428
Lc09713	24, 96	qANTH0-2	Acetyl-CoA acetyltransferase, mitochondrial, putative	-1.99133
Lc05920	48	qANTH0-2	Lissencephaly type-1-like homology motif WD40-like	4.329072
Lc06016	24, 96	qANTH0-2	RNA 2'-phosphotransferase, Tpt1/KptA family protein	-1.14748
Lc09295	24, 96	qANTH0-2	MYB transcription factor MYB91	4.609405
Lc10375	24, 48, 96	qANTH0-2	Uncharacterized protein	5.910138
Lc20642	24, 96	qANTH0-5.1/qANTH0-5.2	Putative ribonuclease H protein	-1.65566
Lc23822	24, 48, 96	qANTH0-5.1/qANTH0-5.2	AFG1-family ATPase	4.934666
Lc22530	24, 48, 96	qANTH0-5.1/qANTH0-5.2	PolI-like B DNA polymerase	-6.49014
Lc22194	24, 48, 96	qANTH0-5.1/qANTH0-5.2	Uncharacterized protein	-1.40243

Gene ID	hpi	QTL	Gene annotation	Fold change
Lc22494	24, 96	qANTH0-5.1/qANTH0-5.2	Uncharacterized protein	-1.09764
Lc23870	24, 96	qANTH0-5.1/qANTH0-5.2	Uncharacterized protein	-2.00591
Lc22814	24, 96	qANTH0-5.1/qANTH0-5.2	Global transcription factor group protein	-2.78892
Lc23518	24, 96	qANTH0-5.1/qANTH0-5.2	LRR receptor-like kinase	4.048737
Lc23062	24, 96	qANTH0-5.1/qANTH0-5.2	hypothetical protein	4.283973
Lc20626	24, 96	qANTH0-5.1/qANTH0-5.2	Nuclear ribonuclease Z	-7.17729
Lc30825	24, 48, 96	qANTH0-7	AT hook motif DNA-binding family protein	-5.76491
Lc29268	24, 96	qANTH0-7	MATH domain-containing protein	-1.89147
Lc29421	24, 96	qANTH0-7	Transducin/WD-like repeat-protein	-1.35238

4.3 Validation of gene expression using RT-qPCR

Results of gene expression using RT-qPCR of the selected 21 DEGs based on functional annotations that had a putative role in disease responses showed that expression of 12 DEGs out of 21 which were up-regulated in the resistant RIL LR-66-528 based on RNA-Seq were up-

regulated based on RT-qPCR (Fig. 4.7a -q). The expression of nine genes out of 21 DEGs which were up-regulated in the susceptible RIL LR-66-524 based on RNA-seq were also found to be up-regulated based on RT-qPCR. (Fig. 4.8a-k).

Statistical analysis of the results of RT-qPCR showed that 16 genes out of 21 selected DEGs (76%) had significant differences in gene expression levels between the resistant and susceptible RILs.

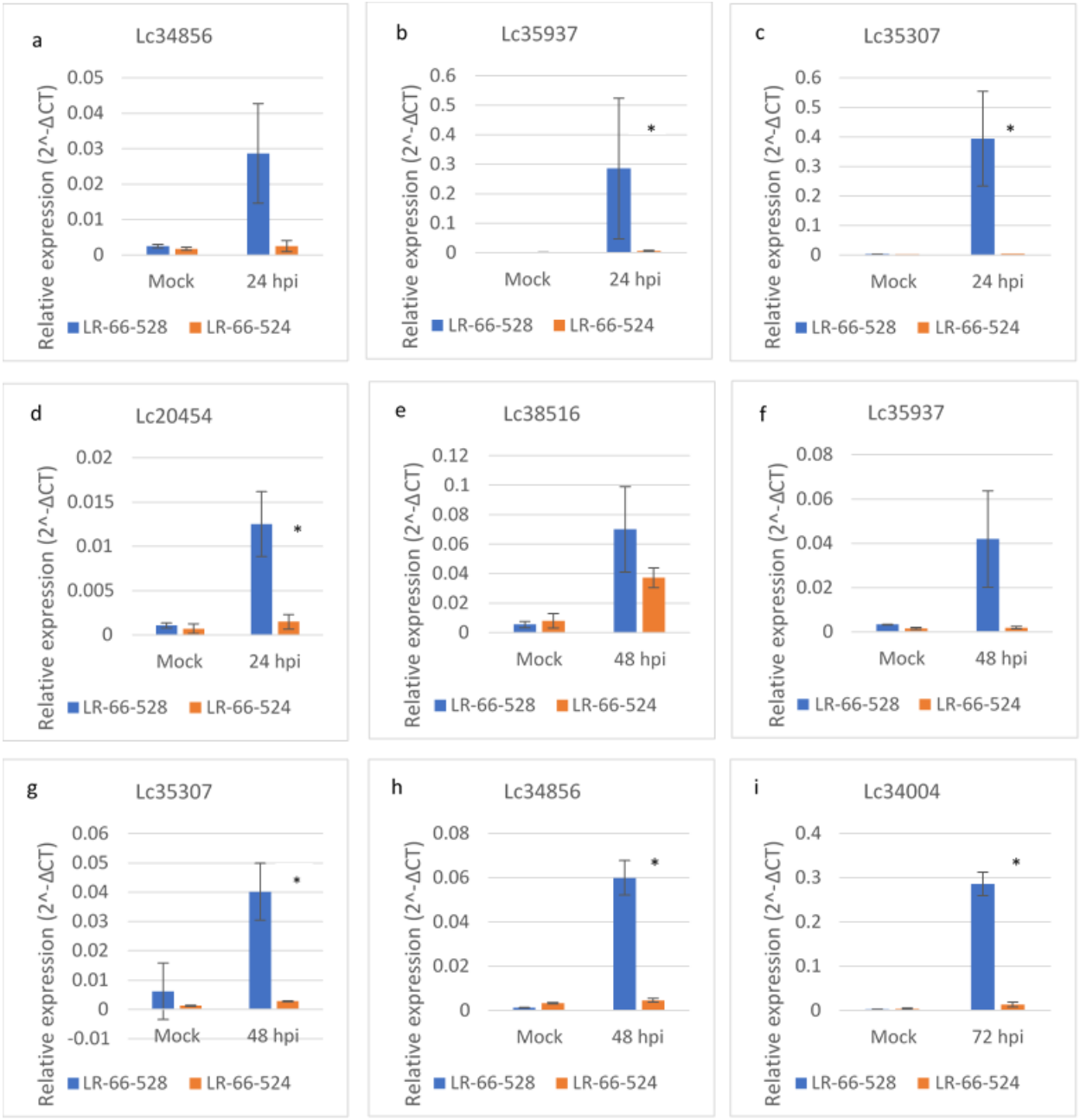


Figure 4.7 a-q. Relative expression profiles of selected genes estimated by RT-qPCR in resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis*. Genes were selected based on functions related to disease resistance. All genes were up-regulated in the resistant RIL LR-66-528 based on RNA-Seq. Error bars represents the standard errors of means of the samples with 3 biological replicates. Asterisks (*) denote statistical significance with P <0.05.

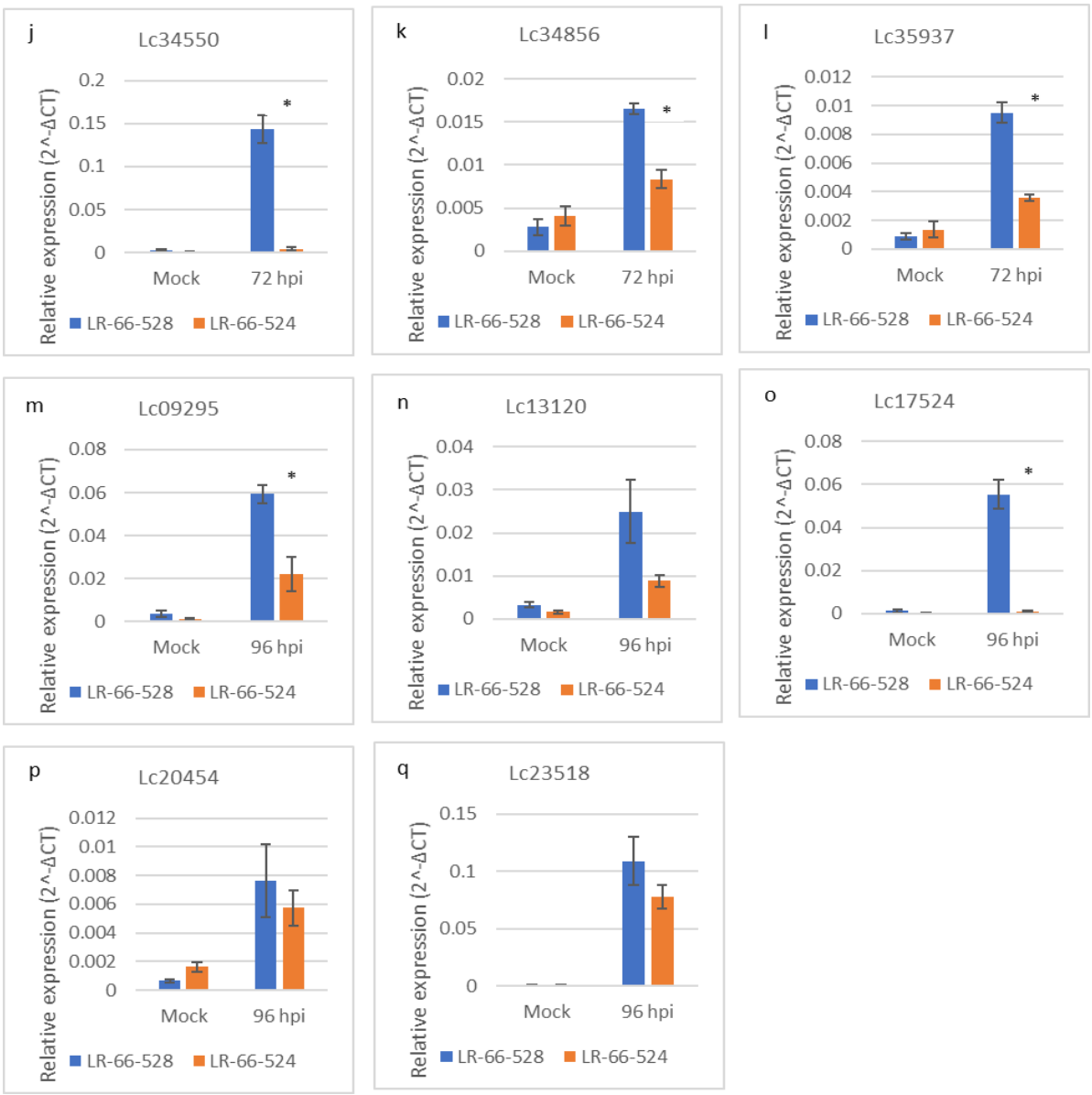


Figure 4.7 a-q. Continued

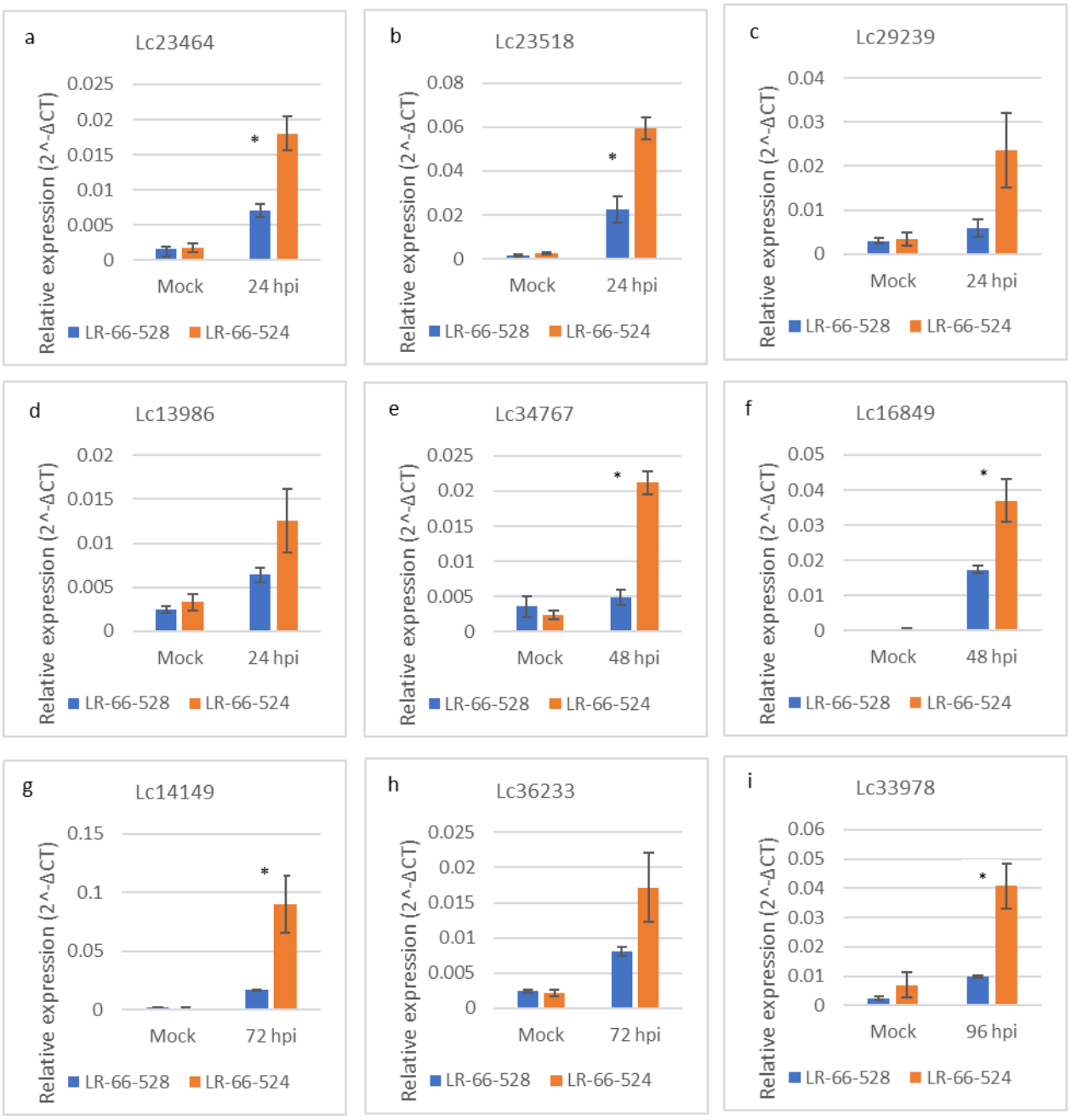


Figure 4.8 a-k. Relative expression profiles of selected genes estimated by RT-qPCR in resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis*. Genes were selected based on functions related to known disease resistance. All genes were up-regulated in the susceptible RIL LR-66-524 based on RNA-Seq. Error bars represents the standard errors of means of the samples with 3 biological replicates. Asterisks (*) denote statistical significance with p-value: <0.05.

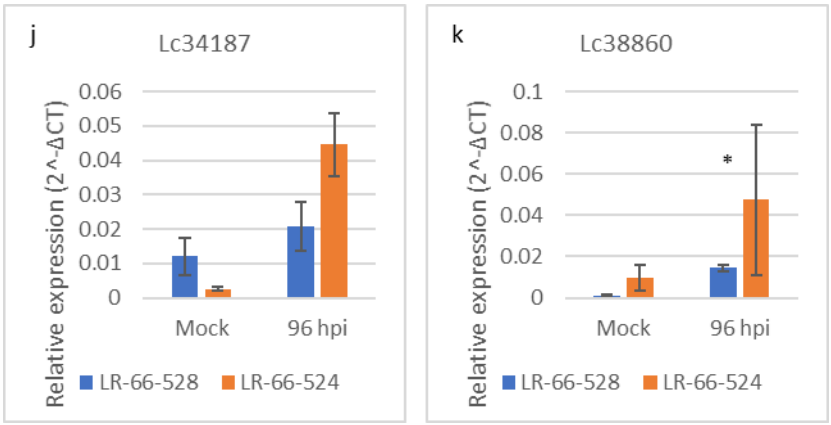


Figure 4.8 a-k. Continued

The expression validation of three non-DEGs (Lc16648, Lc30103 and Lc35495) showed no differences in expression level between the resistant and susceptible RIL (Fig. 4.9a-c).

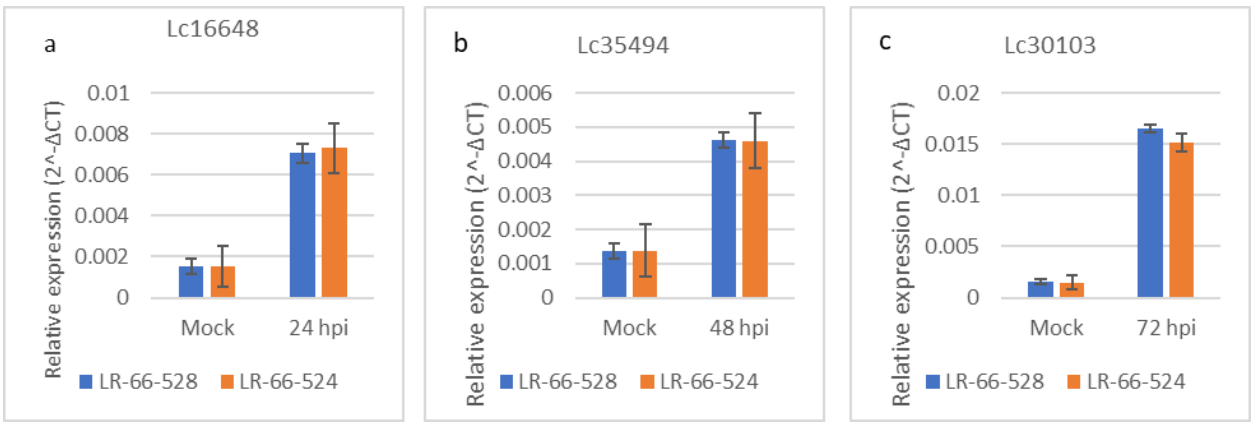


Figure 4.9a-c Relative gene expression profiles estimated by RT-qPCR of genes with similar expression levels in resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis*. Genes were selected based on their upregulation in both RILs (fold change < 2, P_{adj} > 0.05). Error bars represents the standard errors of means of the samples with 3 biological replicates.

Chapter 5

5.0 Discussion

The objective of the current research was to contribute to an overall effort to identify anthracnose genes associated withresistance in *L. ervoides*. This research is crucial as lentil is one of the major crops contributing to the economy of Saskatchewan. Efforts into the development of crop management strategies and breeding for agronomic traits of lentil cultivars have increased in Saskatchewan in the past three decades. However, these breeding efforts resulted in the narrowing of the genetic base of the crop. As a consequence, lentil may have become more prone to both biotic and abiotic stresses. One of the major biotic stresses is the fungal disease anthracnose, which is currently the most important foliar disease of lentil in Canada and the USA. To control this disease, a widely preferred method is to introduce disease resistance through transfer of anthracnose genes associated with resistance into high yielding varieties. Both, genetic diversity and genetic gain can be increased by introgression of genes from wild lentil germplasm into cultivated lentil (Vandenberg *et al*., 2002). In the breeding programs, durability of resistance is a major objective as erosion of a newly released source of resistance can undermine the costs and efforts involved (Sari *et al*., 2018). Therefore, combining several resistance genes in a gene pyramiding program may contribute to durable resistance by increasing the time required for pathogens to evolve virulence factors. This may decrease the risk of resistance break-down (Pilet-Nayel *et al*., 2017). In order to manage the diseases, there is a need to implement molecular approaches such as the mapping of quantitative trait loci (QTL), marker-assisted selection, genomics and transcriptomics in the current lentil breeding programs (Kumar *et al*., 2015). In recent years, the advent of next-generation sequencing (NGS) technologies has accelerated the discovery of large number of disease genes (Pilet-Nayel *et al*., 2017).

In the current study, differentially expressed genes were profiled in selected RILs of the bi-parental intraspecific *L. ervoides* RIL population LR-66 infected with race 0 isolate CT-30 of *C. lentis* to uncover the genetic basis of lentil resistance to the pathogen. *Lens ervoides* exhibits superior anthracnose resistance in much higher frequency than other species. Facilitated by embryo rescue technique that overcomes interspecific reproductive barriers between *L. ervoides* and *L. culinaris* (Fiala *et al*., 2009), interest in introgressing useful genes from *L. ervoides* to

elite cultivars has increased as resistance to the dominating and aggressive race 0 of the anthracnose pathogen has been identified in this species (Tullu *et al*., 2006). The present transcriptome study of the resistant and susceptible RILs infected by CT-30 isolate of race 0 of *C. lentis* led to the identification of some candidate genes associated with resistance, which can be further evaluated for their role in resistance to anthracnose.

To date, information on the molecular basis underlying anthracnose resistance in *L. ervoides* has been limited to the identification of five QTLs associated with anthracnose resistance on a SNP-based linkage map of an F_9 recombinant inbred line (Bhadauria *et al*., 2017a). The direct use of these QTLs in marker-assisted selection is limited and identification of candidate genes associated with resistance is difficult because the QTL intervals (2-LOD) are relatively large and contain hundreds of genes (Bhadauria *et al*., 2017a).

In recent years, transcriptome studies have been significantly improved through development of high-throughput next-generation sequencing techniques that generate large amounts of data suitable for in-depth quantification of genome-wide gene expression across treatments, incubation time and genotypes (Han *et al*., 2015). Several RNA-seq studies have been conducted on lentil to profile transcriptomes and develop molecular markers for various biotic and abiotic stresses (Sari *et al*., 2017; Khorramdelazad *et al*., 2018; Sudeesh *et al*., 2016; Singh *et al*., 2017; Verma *et al*., 2013). Most of these studies followed a *de novo* assembly approach. In the current research, the *L. culinaris* genome, recently constructed by Bett *et al*. (2016) was used as the reference genome. Using the reference genome assembly approach, the risks of alignment errors and chimerism error are reduced and downstream analyses of data are improved.

RNA-Seq is a stage-specific technique because RNA transcription is highly dynamic, so identification of the appropriate sampling time is very important to capture the transcriptome for a study. Therefore, the first objective of the current research was to identify the critical time-points where the resistant and susceptible RILs trigger a differential resistance response. A thorough understanding of how *C. lentis* interacts with both, the resistant and the susceptible RIL is essential to identify appropriate times during the infection process for the profiling of gene expression. The quantitative assessments of fungal development using qPCR is highly promising. This technique has been used in several studies for fungal biomass determination in a range of hosts (Horevaj *et al*., 2011; Tellenbach *et al*., 2010; Weβling *et al*., 2012). In

comparison to the traditional histopathological method, this method is high-throughput as it is possible to multiplex hundreds of samples together in a single run. In addition, as DNA extraction can be easily achieved by homogenizing large amounts of diseased tissue, qPCR provides an accurate assessment of disease status of whole plants. This method was used in the current study to estimate the fungal biomass. Differences in the trend of biomass increases of *C. lentis* between resistant and susceptible RIL were observed with fungal proliferation starting from 6 hpi. There was significantly more biomass in susceptible RIL than in the resistant RIL at 24 hpi, which correlates with results of Kapoor (2018) who found that at 24 hpi infection vesicle/ primary hyphae were identified in LR-66-524, but not in LR-66-528. This indicates that penetration of *C. lentis* isolate CT-30 may have been delayed in LR-66-528 compared to LR-66-524. In addition, a larger number of appressoria formed from germinated conidia in LR-66-524 as compared to LR-66-528 (Kapoor, 2018). Cell death was observed in a few instances in the resistant RIL, but this was not consistent and it was not the case for LR-66-524. At 120 and 144 hpi, setae and acervuli were visible in both genotypes.

These observations were similar to those in the *A. thaliana- C. higginsianum* host-pathogen system (Birker *et al*., 2009). More than 50% of appressoria of *C. higginsianum* initiated successful penetrations into the susceptible *A. thaliana* accession Ler-0 through the leaf surface to form primary hyphae compared to 10% of appressoria-mediated infection on the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara. An important indicator of a disease reaction after inoculations in a lentil genotype is how early *C. lentis* develops IV/PHs as these infection structures determine the speed of subsequent infection stages by the pathogen. The timing of the development of IVs and PHs was correlated with resistance and susceptibility to anthracnose of LR-66-528 and LR-66-524, respectively (Bhadauria *et al*., 2017a; Kapoor, 2018).

It was observed here that fungal biomass increased dramatically after 48 hpi in both lentil genotypes. Previously, Chongo *et al*. (2002) proposed that *C. lentis* switched from the biotrophic to the necrotrophic phase after 48 hpi. Similarly, Bhadauria *et al*. (2013) determined that the biotrophy-necrotrophy switch occurred between 48-56 hpi. Exponential growth of *C. lentis* was observed from 24 to 96 hpi using qPCR, which suggested that disease responses between resistant and susceptible RILs to *C. lentis* infection could be triggered during this phase.

The second objective of the current research was to identify differentially expressed genes in resistant and susceptible RILs and discover those co-localizing with QTLs for anthracnose resistance. Use of RNA-Seq allowed a more comprehensive picture of the *L. ervoides* transcriptome to identify candidate genes associated with resistance. A total of 477 genes were found to be differentially expressed upon pathogen infection between resistant LR-66-528 and susceptible LR-66-524, which is lower compared to previous transcriptomic studies on other lentil diseases. The sequencing reads were mapped to the *L.culinaris* genome; therefore, differences between *L. ervoides* and *L.culinaris* could also have resulted in some genes being missed that are not present in the L. culinaris genome. Khorramdelazad *et al*. (2018) found a total of 2,617 DEGs between resistant and susceptible lentil genotypes upon infection by *A. lentis*. Both LR-66-528 and LR-66-524 are progenies of the intra-specific *L. ervoides* population LR-66 which may explain the large number of common disease-responsive genes.

Separation of sequencing samples collected at 48 and 96 hpi between the RILs by PCA was evident. This reflected that transcriptome responses of LR-66-528 and LR-66-524 were different after 24 hpi, and more so at 48 and 96 hpi. The sequencing samples of both genotypes from 24 hpi were positioned far from those collected at 48, 72 and 96 hpi in the PCA plot. This could be correlated with the progression from biotrophy to necrotrophy. Based on the results of PVCA it was evident that the highest proportion of the variance (78%) could be attributed to incubation time. This is a reflection of the fact that variation increased with increasing time-points as the pathogen switched from biotroph to necrotroph which may lead to variation in gene expression. The percentage of variability attributed to genotypes was the lowest (0.6%) among all principal components. This may be due to the fact that the resistant and susceptible RILs were from an intraspecific population. These results confirmed that differences between the RILs increased with increasing time-points and that incubation time was the component influencing variability most.

The common sets of DEGs across the time-points was visualized by a Venn diagram. Out of a total of 477 DEGs, 108 DEGs were expressed exclusively at 48 hpi. The biotrophy-necrotrophy switch occurs at approximately 48 hpi. Histopathological studies had revealed that the susceptible RIL LR-66-524 had a higher proportion of IVs/PHs compared to LR-66-528 at 48 hpi (Kapoor, 2018), and the development of these structures was previously associated with anthracnose resistance and susceptibility in *L. ervoides* by Bhadauria *et al*. (2017a). The large

number of DEGs exclusively expressed at 48 hpi (108 compared to 2 to 38 at other time points) may indicate that this early stage in disease development is critical for resistance (or susceptibility).

Two DEGs, Lc33118 and Lc27093 were expressed exclusively at 24 hpi and were up-regulated in the resistant RIL. Functional annotations of these genes determined that they have a role in disease resistance. Lc33118 encodes a cytokinin receptor histidine kinase, which was found to have role in biotic stresses (Ramsong *et al*., 2012). Cytokinin receptor AHK5 was found to be involved in providing resistance to bacterial and fungal infections (Pham *et al*., 2012). Lc27093 encodes a cysteine rich secretory protein. Sm1 is a cysteine rich secretory protein that was secreted by *Trichoderma virens* strain GV29-8. It elicited defense responses in cotton cotyledons and protected them against *Colletotrichum* sp. infection (Djonović *et al*., 2006). Another cysteine-rich protein, HYTL01, and its encoding gene were described from *T. longibrachiatum* strain MK1 (Ruocco *et al*., 2015). It causes defense responses against *B. cinerea* in tomato.

A total of 38 DEGs were exclusively expressed at 72 hpi. Out of these, two DEGs, Lc29695 and Lc33733 were up-regulated in the resistant RIL LR-66-528 and were found to have a role in disease resistance. Lc29695 encodes a disease resistance protein (TIR-NBS-LRR) and Lc33733 encodes a PPR containing plant protein. In rice, PPRs were found to have a resistance role against infection by the rice blast pathogen (Chen *et al*., 2018). At 96 hpi, two DEGs, Lc04266 and Lc34740, were exclusively expressed at this time-point and were up-regulated in LR-66-528. Lc34740 was found to have role in disease resistance, encoding an LRR receptor-like kinase protein, which have been known to play important roles in recognizing PAMPs and in regulating plant immune responses against invasive fungi. *TaLRRK-6D* is a gene that encodes domains of RLKs and contributes to resistance to fusarium head blight in wheat (Thapa *et al*., 2018). It was found that the majority of DEGs exclusively expressed at specific time-points had roles in disease resistance and were up-regulated in the resistant RIL.

Clustering of DEGs revealed genes in six different expression clusters indicating their role in different processes. Genes in Cluster 1 were enriched for cell-death associated functions and were primarily up-regulated in the susceptible RIL, which indicated that the plant's hypersensitive response (HR) and programmed cell death (PCD) cause leaf necrosis and eventually leaf dehiscence (Frederickson *et al*., 2014; Dietz *et al*., 2016). Chowdhury *et al.*

(2017) observed that reactive oxygen species (ROS) signaling, which promotes PCD was downregulated to confer enhanced resistance to the necrotrophic phase of the hemibiotrophic pathogen *Macrophomina phaseolina*. This adaptation enabled the pathogen to take full advantage of defense-related cell death during the necrotrophic stage, which led to an increase in pathogen infection and disease progression rendering a plant susceptible to the disease. It was hypothesized here that cell death was promoted in LR-66-524 during CT-30 infection leading to leaf necrosis, which resulted in increased susceptibility to the pathogen.

Genes in Cluster 2 were enriched for those involved in water deprivation and were up-regulated in the susceptible RIL. A wide range of foliar pathogens have been investigated that disrupt stomata and cause impairment of stomatal opening by releasing toxins which leads to water deprivation (Grimmer *et al*., 2012). The necrotroph *S. sclerotiorum* produces oxalate, which causes the stomata of field beans to remain open resulting in water loss (Guimarães and Stotz, 2004). It can be hypothesized that *C. lentis* releases toxins in LR-66-524 during the necrotrophic phase resulting in water deprivation through a decrease in water uptake by the plants, culminated eventually into plant death.

Cluster 3 genes had functions in RNA splicing and were primarily up-regulated in resistant LR-66-528. The presence of multiple transcript variants through R gene splicing was reported for *RCT1*, which confers resistance to *C. trifolii* causing anthracnose of *M. truncatula*, but their effect on the functionality of *RCT1* remain unclear (Yang *et al*., 2008). Genes highly expressed in LR-66-528 may be involved in alternate splicing of the genes which are involved in resistance thus inducing multiple transcript variants that participate in resistance reaction in LR-66-528. In Cluster 4, a large number of enriched genes were related to cell growth and development processes such as “cell growth”, “cell wall biogenesis” and “regulation of cellular component biogenesis”. These genes were up-regulated primarily in the susceptible RIL. Impairment or over-expression of cell wall-related genes were found to have significant impact on disease resistance and led to susceptible reaction (Bellincampi *et al*., 2014; Malinovsky *et al*., 2014; Miedes *et al*., 2014; Kesten *et al*., 2017). If a cell wall modification is arrested by the host, a resistant phenotype can be expected (Malinovsky *et al*., 2014). Several examples of cell wall modifications in *A. thaliana* were found to be associated with susceptibility to pathogens. Penetration frequency and *C. higginsianum* hyphae establishment were enhanced in leaves of a starch-deficient *A. thaliana phosphoglucomutase* (*pgm*) mutant. Complex alterations were

observed in cell wall monosaccharide composition of *pgm*. These mutants were susceptible to *C. higginsianum* infection (Engelsdorf *et al*., 2017).

Genes in Cluster 5 were primarily enriched for genes with function in primary metabolic and transportation processes. These genes were primarily up-regulated in the resistant RIL LR-66-528. It was reported that carbohydrates and transporters have important roles in the immune system against hemibiotrophs (Stukkens *et al*., 2005; Berger *et al*., 2007; Lecompte *et al*., 2017). High levels of sucrose triggered an accumulation of a variety of flavonoids which increased resistance against *Fusarium oxysporum* in yellow lupin (Morkunas *et al*., 2011). Enhanced disease resistance to the necrotrophic pathogens *B. cinerea* and *Plectosphaerella cucumerina* in *Arabidopsis* was correlated with up-regulation of *NpPDR1* and *AtBCG36*, which are two plant ATP binding cassette transporters (Stukkens *et al*., 2005; Stein *et al*., 2006). Primary metabolism acts as signals regulating various aspects of plant defense (Lim *et al*., 2017). Upregulation of primary metabolism modulates signal transduction cascades and enhances plant defense responses (Rojas *et al*., 2014).

Cluster 6 genes had functions in processes such as "regulation of GTPase activity", and again were primarily up-regulated in the resistant RIL LR-66-528. GTPase functions as molecular switch downstream of immune receptors, triggering immune responses and therefore leads to enhanced disease resistance (Kawano *et al*., 2014). The Rac/Rop family of small GTPases in rice (OsRac1) positively regulates *Pi-a* mediated defense response, where *Pi-a* is a resistance gene to the rice blast fungus (Chen *et al*., 2011). Moreover, Cluster 6 genes had functions in processes of DNA assembly such as "DNA packaging", "Chromatin assembly or disassembly" and "Chromosomal organization". Walley *et al*. (2008) reported that chromatin remodeling had a role in disease resistance in *Arabidopsis*. A chromatin remodeling protein SPLAYED (SYD) binds stress responsive promoters and loss of its activity resulted in increased susceptibility to *B. cinerea* in *Arabidopsis.* Bhadauria *et al*. (2013) studied expressed sequence tags of *C. lentis*-infected lentil leaves identified 13 discrete unigenes encoding G-proteins including small GTPases. GTPases are potentially involved in signal transducing activity in plants. Therefore, GTPases could be involved in signal transduction between lentil and *C. lentis* during the hemibiotrophic infection process. These expression clusters and GO enrichment of DEGs helped to narrow down the DEGs and to group the genes involved in disease resistance.

Another approach to narrow down the candidate genes associated with resistance was to identify the co-localization of the identified DEGs between the resistant and susceptible RILs with the QTLs for anthracnose resistance. A total of five QTLs significantly associated with resistance to *C. lentis* race 0 were previously identified in a SNP-based linkage map of the *L. ervoides* RIL population LR-66 (Bhadauria *et al*., 2017a). The 477 DEGs were compared to the QTL intervals (2-LOD), which led to the identification of a total of 22 genes that were up- or down-regulated in the resistant RIL LR-66-528 at 24, 48, 72 and 96 hpi. Functional annotations of some of these genes showed that they have roles in disease resistance. Lc05911 was found to be up-regulated in LR-66-528 at 24 and 96 hpi and was present in QTL interval qANTH0-2 on linkage group 2. This gene is a DEAD-box ATP-dependent RNA helicase and in *Arabidopsis*, *OsBIRH1* encoding a DEAD-box RNA helicase protein. Expression of *OsBIRH1* was activated in rice seedling after treatment with defense-related signal chemicals such as salicylic acid and jasmonic acid. Transgenic *Arabidopsis* mutants that overexpress the *OsBIRH1* gene were generated and revealed enhanced disease resistance against *Alternaria brassicola* (necrotroph) and *Pseudomonas syringae* (biotroph) (Li *et al*., 2008). Lc05911 was expressed during the biotrophic and the necrotrophic phase of *C. lentis* infection and may be involved in resistance against *C. lentis*. Another gene, Lc23518, was found to be up-regulated in LR-66-528 at 96 hpi and was present in QTL interval qANTH0-5.1 on linkage group 5. This gene is an LRR receptor-like kinase, genes of which form one of the largest family of plant receptors and play an important role in disease resistance in a large number of plants.

The third objective of this research was to validate the expression of identified genes associated with resistance. The RNA-Seq approach used in this project led to the identification of a large number of DEGs between resistant and susceptible RILs of the LR-66 population. Gene annotation analysis and downstream analysis of RNA-Seq involving GO enrichment led to the identification of disease resistance-related genes. A total of 21 DEGs from the combined list of DEGs at all four time-points were selected for gene expression validation using RT-qPCR. Out of these 21 DEGs, Lc23464, Lc35307 and Lc24187 encode Serine/Threonine protein kinases. Serine/Threonine protein kinases are a group of proteins implicated in stress and defense processes of host plant including those involved in translocation. *NEK6* encodes a Serine/Threonine protein kinase that is involved in response to stresses in *A. thaliana* (Zhang *et al*., 2011). Lc23518, Lc34856, Lc35937, Lc20454, Lc14149, Lc34550 and Lc33978 encodes

LRR receptor-like kinase. Lc13986 was up-regulated in the resistant RIL that encodes adenosylhomocysteinase, which is a key enzyme that maintains cellular methylation potential in all organisms. The tomato genome contains three adenosylhomocysteinase genes that encode adenosylhomocysteinase proteins. Co-silencing of these genes led to up-regulation in expression of defense-related genes in *Pseudomonas syringae* and PAMP-triggered immunity marker genes (Li *et al*., 2015). Lc34767 encodes TMV resistance protein N. Tobacco *N* gene confers resistance to TMV. It belongs to TIR-NB-LRR class of resistance genes (Whitham *et al*., 1994). Lc34004 is RPM1-interacting protein 4 (RIN4) family protein. RIN4 specifies resistance to pathogens expressing the effector proteins AvrB and/or AvrRpm1 in *A. thaliana* by phosphorylating itself (Mackey *et al*., 2003). Lc38860 encodes pathogenesis-related thaumatin family protein. In wheat *TaLr35PR5* is a gene encoding a protein exhibiting amino acid and structural similarity to protein thaumatin. This gene is involved in Lr35-mediated adult wheat resistance to leaf rust (Zhang *et al*., 2018). The functional annotations of these genes showed that these were involved in disease resistance and were up- or down- regulated in resistant and susceptible RILs.

5.1 Conclusions

The pathogen-responsive *L. ervoides* transcriptome was dissected after infection by a race 0 isolate CT-30 of *C. lentis* and after determination of critical time-points during infection on the most resistant (LR-66-528) and the most susceptible (LR-66-524) RIL based on quantification of fungal biomass through qPCR. Based on the results of qPCR, the critical time-points selected for RNA sequencing were 24, 48, 72 and 96 hpi. Among 3,091 disease responsive genes, a total of 477 genes were differentially expressed between the RILs. DEGs were functionally annotated to identify those with known functions in disease resistance such as LRR and NB-ARC domain disease resistance protein and wall associated Ser/Thr kinase. The expression of 21 of these genes was validated using RT-qPCR showed up-or down regulation as in the RNA-seq data. Comparison of DEGs with QTL regions for anthracnose resistance revealed that nine DEGs were located in QTL 2 region, 10 in QTL in 5 region and three in QTL 7 region. The reason for this low number could be that the time frame for the transcriptome study was 24 to 96 hpi, whereas the QTL mapping was conducted at 144 hpi. This could indicate that largely different sets of genes were expressed upto 96 hpi, as identified through RNA-Seq, and at 144 hpi, as identified through QTL mapping, with little overlap (22 genes).

This in-depth study of the transcriptome of *L. ervoides* has revealed a catalogue of resistance gene candidates. Out of these genes, 22 DEGs were found to be co-localized with the QTLs conferring resistance to anthracnose. Two genes Lc23518 and Lc09295 were found through both molecular approaches, QTLs and RNA-Seq and their expressions were also validated using RT-qPCR. Lc23518 encodes an LRR receptor-like kinase protein that was up-regulated in the susceptible RIL LR-66-524 at 24 hpi and in the resistant RIL LR-66-528 at 96 hpi. It was also found in the QTL interval qANTH0-5.1/qANTH0-5.2. Lc09295 encodes MYB transcription factor MYB91 that was up-regulated in the resistant RIL at 96 hpi and was located in the QTL interval qANTH0-2. These genes can be further evaluated for their suitability as gene-specific markers to trace resistance from *L. ervoides* in interspecific breeding lines in lentil breeding program.

The proposed hypothesis for this research was that some of the differentially expressed genes in resistant and susceptible RILs of *L. ervoides* RIL population LR-66 identified through RNA-Seq co-localize with QTLs for anthracnose resistance. It is clear from the findings of this research that the proposed hypothesis can be accepted. Although some DEGs were located under QTL intervals, many were not.

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Appendix 1: List of 446 differentially expressed genes (fold change >2, $P_{adj} < 0.05$) between resistant RIL LR-66-528 and susceptible RIL LR-66-524 of *Lens ervoides* RIL population LR-66 inoculated with race 0 isolate CT-30 of *Colletotrichum lentis* incubated for 24, 48, 72 and 96 h.

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc17060	✓	✓	✓	✓	Chlorophyll a-b binding protein AB80, chloroplastic (LHCII type I CAB-AB80) (LHCP) (Precursor)
Lc33978	✓	✓	✓	✓	LRR receptor-like kinase
Lc32568	✓	✓	✓	✓	Protein DETOXIFICATION
Lc37659	✓	✓	✓	✓	Uncharacterized protein
Lc35892	✓	✓	✓	✓	UDP-glucosyltransferase family protein
Lc34550	✓	✓	✓	✓	LRR and NB-ARC domain disease resistance protein
Lc33365	✓	✓	✓	✓	Glycoside hydrolase family 5 protein
Lc23935	✓	✓	✓	✓	26S proteasome regulatory particle triple-A ATPase protein
Lc16746	✓	✓	✓	✓	hypothetical protein
Lc35064	✓	✓	✓	✓	SAP domain protein
Lc04983	✓	✓	✓	✓	Uncharacterized protein
Lc36840	✓	✓	✓	✓	Uncharacterized protein
Lc26443	✓	✓	✓	✓	PRA1 family protein
Lc34429	✓	✓	✓	✓	Uncharacterized protein
Lc32002	✓	✓	✓	✓	hypothetical protein
Lc35505	✓	✓	✓	✓	DUF1677 family protein
Lc05485	✓	✓	✓	✓	Uncharacterized protein
Lc37593	✓	✓	✓	✓	hypothetical protein
Lc16370	✓	✓	✓	✓	PPR containing plant-like protein
Lc38725	✓	✓	✓	✓	MLO-like protein
Lc38935	✓	✓	✓	✓	Sugar transport protein 14
Lc34344	✓	✓	✓	✓	Annexin
Lc08703	✓	✓	✓	✓	PLAC8 family protein
Lc02360	✓	✓	✓	✓	Adaptin ear-binding coat-associated protein 1
Lc20385	✓	✓	✓	✓	NADH-ubiquinone reductase complex 1 MLRQ subunit
Lc36465	✓	✓	✓	✓	Pmr5/Cas1p GDSL/SGNH-like acyl-esterase family protein
Lc23991	✓	✓	✓	✓	Protein DETOXIFICATION
Lc32791	✓	✓	✓	✓	DUF247 domain protein
Lc38165	✓	✓	✓	✓	Epoxide hydrolase
Lc38936	✓	✓	✓	✓	Uncharacterized protein
Lc32631	✓	✓	✓	✓	Ninja-family protein AFP1
Lc36633	✓	✓	✓	✓	Cytochrome P450 family 71 protein
Lc38454	✓	✓	✓	✓	DNA-binding and zinc-finger protein
Lc34602	✓	✓	✓	✓	Putative uncharacterized protein
Lc25773	✓	✓	✓	✓	Purple acid phosphatase
Lc35757	✓	✓	✓	✓	ADP-ribosylation factor GTPase-activating protein AGD10
Lc33247	✓	✓	✓	✓	Uncharacterized protein

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc34409	✓	✓	✓	✓	Transmembrane protein, putative
Lc38786	✓	✓	✓	✓	Glycosyltransferase family 90 protein
Lc36288	✓	✓	✓	✓	4-hydroxy-tetrahydrodipicolinate synthase
Lc02657	✓	✓	✓	✓	Beta-1,2-N-acetylglucosaminyltransferase II
Lc18960	✓	✓	✓	✓	Uncharacterized protein
Lc04546	✓	✓	✓	✓	Metal tolerance-like protein
Lc37907	✓	✓	✓	✓	Pollen Ole e I family allergen
Lc22559	✓	✓	✓	✓	Lipid transfer protein
Lc02116	✓	✓	✓	✓	Blue copper-like protein
Lc33215	✓	✓	✓	✓	Peroxidase
Lc14831	✓	✓	✓	✓	Uncharacterized protein
Lc35690	✓	✓	✓	✓	Class I glutamine amidotransferase
Lc35648	✓	✓	✓	✓	PB1 domain protein
Lc29268	✓	✓	✓	✓	Glycerol-3-phosphate acyltransferase
Lc30776	✓	✓	✓	✓	Uncharacterized protein
Lc31239	✓	✓	✓	✓	hypothetical protein
Lc38912	✓	✓	✓	✓	Kinesin-like protein
Lc22530	✓	✓	✓	✓	PolI-like B DNA polymerase
Lc01338	✓	✓	✓	✓	CBS domain protein/transporter associated domain protein
Lc35859	✓	✓	✓	✓	Glycoside hydrolase family 79 amino-terminal domain protein
Lc35222	✓	✓	✓	✓	Receptor-like kinase
Lc33972	✓	✓	✓	✓	Ankyrin repeat protein
Lc12942	✓	✓	✓	✓	HXXXD-type acyl-transferase family protein
Lc30825	✓	✓	✓	✓	Uncharacterized protein
Lc38885	✓	✓	✓	✓	Uncharacterized protein
Lc37235	✓	✓	✓	✓	Uncharacterized protein
Lc34043	✓	✓	✓	✓	CPN60B
Lc24924	✓	✓	✓	✓	Nodulin MtN21/EamA-like transporter family protein
Lc10813	✓	✓	✓	✓	Salicylic acid carboxyl methyltransferase
Lc04144	✓	✓	✓	✓	Heavy metal-associated domain protein
Lc34004	✓	✓	✓	✓	RPM1-interacting protein 4 (RIN4) family protein
Lc12155	✓	✓	✓	✓	hypothetical protein
Lc29580	✓	✓	✓	✓	Carboxy-terminal domain phosphatase-like protein
Lc26355	✓	✓	✓	✓	Shikimate/quinate hydroxycinnamoyltransferase
Lc32840	✓	✓	✓	✓	Uncharacterized protein
Lc33364	✓	✓	✓	✓	NB-ARC domain disease resistance protein, putative
Lc31238	✓	✓	✓	✓	Spermatogenesis-associated-like protein
Lc36811	✓	✓	✓	✓	Uncharacterized protein
Lc10441	✓	✓	✓	✓	Cysteine-rich receptor-kinase-like protein
Lc35937	✓	✓	✓	✓	LRR receptor-like kinase
Lc20021	✓	✓	✓	✓	hypothetical protein
Lc31240	✓	✓	✓	✓	Cationic amino acid transporter

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc32790	✓	✓	✓	✓	DUF247 domain protein
Lc33118	✓				Cytokinin receptor histidine kinase
Lc37093	✓				CAP, cysteine-rich secretory protein, antigen 5
Lc06927		✓			Uncharacterized protein
Lc28167		✓			Sister chromatid cohesion 1 protein
Lc37523		✓			ATP-dependent 6-phosphofructokinase
Lc36386		✓			SAUR-like auxin-responsive family protein
Lc27797		✓			Uncharacterized protein
Lc34862		✓			Acyl-coenzyme A oxidase
Lc10472		✓			Myb/SANT-like DNA-binding domain protein
Lc08245		✓			Oligopeptide transporter OPT family protein
Lc23658		✓			Late embryogenesis abundant protein
Lc32941		✓			Plant/F3O9-12 protein
Lc36912		✓			Tyrosyl-DNA phosphodiesterase
Lc06934		✓			Lactosylceramide 4-alpha-galactosyltransferase-like protein
Lc33279		✓			Flavanone-3-hydroxylase
Lc05673		✓			Phospholipase A1
Lc28441		✓			Short-chain dehydrogenase/reductase
Lc18550		✓			Transmembrane protein, putative
Lc32943		✓			Strictosidine synthase family protein, putative
Lc38820		✓			Glycosyltransferase
Lc32280		✓			Pmr5/Cas1p GDSL/SGNH-like acyl-esterase family protein
Lc33619		✓			Cytochrome P450 family protein
Lc32427		✓			Uncharacterized protein
Lc31096		✓			Nucleolar protein,Nop52 protein
Lc37492		✓			Uncharacterized protein
Lc32813		✓			Peptidyl-prolyl cis-trans isomerase
Lc23194		✓			Indole-3-acetic acid-amido synthetase
Lc36669		✓			Ferritin
Lc04379		✓			Uncharacterized protein
Lc25247		✓			Hexosyltransferase
Lc37934		✓			ABC transporter B family protein
Lc32924		✓			F-box protein
Lc37852		✓			Ripening related protein family
Lc36873		✓			Uncharacterized protein
Lc05865		✓			DNA-binding WRKY VQ
Lc33970		✓			Uncharacterized protein
Lc18398		✓			SCAR2, putative
Lc33193		✓			Cytochrome P450 family protein
Lc05920		✓			Lissencephaly type-1-like homology motif WD40-like
Lc37246		✓			Plant gibberellin 2-oxidase
Lc08692		✓			Cellulose synthase

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc35250		✓			Uncharacterized protein
Lc37474		✓			Dicarboxylate carrier protein
Lc37786		✓			Avr9/Cf-9 rapidly elicited protein
Lc37668		✓			Jasmonate zim-domain protein
Lc38654		✓			Uncharacterized protein
Lc19176		✓			Putative uncharacterized protein
Lc35998		✓			MACPF domain protein
Lc34477		✓			Endochitinase (3.2.1.14) (Precursor)
Lc10214		✓			Uncharacterized protein
Lc34537		✓			Glycerophosphoryl diester phosphodiesterase family protein
Lc33511		✓			Cytochrome P450 family 71 protein
Lc38647		✓			Cytochrome P450 family 71 protein
Lc37461		✓			Glycosyltransferase
Lc29581		✓			Non-specific serine/threonine protein kinase
Lc33372		✓			Pheromone receptor-like protein
Lc04350		✓			Malectin/receptor-like kinase family protein
Lc37819		✓			Uncharacterized protein
Lc33948		✓			Ribonuclease III domain protein
Lc36939		✓			DNA-binding domain protein
Lc33114		✓			Cytochrome P450 family 71 protein
Lc22557		✓			Lipid transfer protein
Lc38095		✓			Uncharacterized protein
Lc10941		✓			UPF0301 protein
Lc23049		✓			Transferase family protein
Lc23661		✓			Chloride channel protein
Lc19260		✓			Uncharacterized protein
Lc33187		✓			Phytochrome-associated protein phosphatase type 2C
Lc35282		✓			Trehalose-6-phosphate synthase domain protein
Lc22088		✓			Methyltransferase domain protein, putative
Lc32854		✓			Proline dehydrogenase
Lc35913		✓			DUF1262 family protein
Lc32588		✓			Disease resistance-responsive, dirigent domain protein
Lc38594		✓			Kinase interacting (KIP1-like) family protein
Lc36370		✓			Cysteine-rich RLK (Receptor-like kinase) protein
Lc23540		✓			Palmitoyl protein thioesterase family protein
Lc37688		✓			Pathogenesis-related protein bet V I family protein
Lc34688		✓			Putative respiratory burst oxidase-like protein C
Lc36585		✓			Tyrosine phosphatase family protein
Lc38876		✓			Eukaryotic aspartyl protease family protein
Lc38224		✓			Double-stranded RNA-binding motif protein
Lc22781		✓			Phenylcoumaran benzylic ether reductase-like protein
Lc36831		✓			Cellulase (Glycosyl hydrolase family 5)

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc33932		✓			Trehalose 6-phosphate phosphatase
Lc25751		✓			Glutathione synthetase
Lc37486		✓			Putative senescence-associated protein
Lc38360		✓			DUF4228 domain protein
Lc38700		✓			Anthocyanin 5-aromatic acyltransferase
Lc34341		✓			Potassium transporter
Lc32812		✓			Fasciclin-like arabinogalactan protein
Lc37001		✓			ABC transporter B family protein
Lc08903		✓			Acetyltransferase NSI-like protein
Lc35257		✓			F-box plant-like protein, putative
Lc36668		✓			Salicylic acid carboxyl methyltransferase
Lc38077		✓			NAD(P)-binding rossmann-fold protein
Lc33132		✓			Cytochrome P450 family 71 protein
Lc27796		✓			Uncharacterized protein
Lc35476		✓			Uncharacterized protein
Lc37957		✓			Sirohydrochlorin ferrochelatase
Lc37722		✓			Uncharacterized protein
Lc23621		✓			UPF0183 plant-like protein
Lc34117		✓			Uncharacterized protein
Lc36029		✓			Coatomer subunit beta-like protein
Lc32778		✓			Equilibrative nucleoside transporter 6
Lc36226		✓			RNA-binding domain CCCH-type zinc finger protein
Lc28694		✓			Lipoxygenase
Lc22851		✓			Indole-3-acetic acid-amido synthetase
Lc33832		✓			Cytochrome P450 family 71 protein
Lc36384		✓			DUF1005 family protein
Lc14195		✓			Thylakoid lumenal 29.8 kDa protein
Lc38780			✓		Uncharacterized protein
Lc10857			✓		Glycoside hydrolase family 5 protein
Lc38495			✓		MLP-like protein 423
Lc35337			✓		Rhomboid-like protein
Lc33733			✓		PPR containing plant protein
Lc35594			✓		DUF3128 family protein
Lc38037			✓		Nudix hydrolase-like protein
Lc38828			✓		PLAT-plant-stress protein
Lc38046			✓		Transmembrane protein, putative
Lc37768			✓		Uncharacterized protein
Lc35298			✓		Phosphatidylinositol 3-and 4-kinase family protein
Lc35279			✓		Cytochrome c oxidase subunit 2
Lc35272			✓		Uncharacterized protein
Lc38633			✓		Uncharacterized protein
Lc32448			✓		Calcium-binding EF-hand protein

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc34254			✓		Uncharacterized protein
Lc28660			✓		D-arabinono-1,4-lactone oxidase-like protein
Lc35310			✓		Uncharacterized protein
Lc29695			✓		Disease resistance protein (TIR-NBS-LRR class), putative
Lc35227			✓		Putative uncharacterized protein nad1
Lc29070			✓		hypothetical protein
Lc28984			✓		Calcium-binding EF hand-like protein
Lc34156			✓		Armadillo repeat only 1 protein
Lc36914			✓		Benzyl alcohol O-benzoyltransferase
Lc34677			✓		Cell wall-associated hydrolase
Lc35183			✓		SPFH/band 7/PHB domain membrane-associated family protein
Lc24726			✓		Type I inositol 1,4,5-trisphosphate 5-phosphatase CVP2
Lc38573			✓		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
Lc15396			✓		Small heat shock protein
Lc32456			✓		Annexin
Lc37088			✓		Zinc finger protein LSD1
Lc10430			✓		RAB GTPase-like protein B1C
Lc38345			✓		Arabinogalactan peptide-like protein
Lc37285			✓		DUF4228 domain protein
Lc33017			✓		hypothetical protein
Lc09494			✓		C-repeat binding factor 3
Lc33950			✓		Pleiotropic drug resistance protein 3
Lc30727			✓		Aminomethyltransferase, mitochondrial (2.1.2.10) (Glycine cleavage system T protein) (GCVT) (Precursor)
Lc34706	✓		✓	✓	Cytochrome P450 family 71 protein
Lc22114	✓		✓	✓	Glutathione S-transferase, amino-terminal domain protein
Lc38126	✓		✓	✓	Uncharacterized protein
Lc35539	✓		✓	✓	Seed maturation protein PM39, putative
Lc34430	✓		✓	✓	Uncharacterized protein
Lc35492	✓		✓	✓	Peptide/nitrate transporter
Lc36335	✓		✓	✓	Transmembrane protein, putative
Lc37187	✓		✓	✓	NADPH:quinone oxidoreductase-like protein
Lc37234	✓		✓	✓	LAG1 longevity assurance-like protein
Lc38503	✓		✓	✓	Uncharacterized protein
Lc34822	✓		✓	✓	TLD-domain nucleolar protein
Lc36584	✓		✓	✓	LRR receptor-like kinase family protein
Lc36336	✓		✓	✓	Transmembrane protein, putative
Lc37583	✓		✓	✓	Uncharacterized protein
Lc32780	✓		✓	✓	Uncharacterized protein
Lc28575	✓		✓	✓	Polynucleotidyl transferase, Ribonuclease H fold
Lc38849	✓		✓	✓	Uncharacterized protein
Lc34025		✓		✓	Uncharacterized protein
Lc34077	✓			✓	Amine oxidase

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc38845	✓			✓	Tornado protein
Lc34187	✓			✓	Serine/threonine-protein kinase
Lc37067	✓			✓	Cyclin
Lc10944	✓			✓	B-block-binding subunit of tfiiic protein, putative
Lc30670	✓			✓	DNA-directed RNA polymerase
Lc27445	✓			✓	HEAT repeat 7A-like protein
Lc25026	✓			✓	Glycosyltransferase
Lc09713	✓			✓	Acetyl-CoA acetyltransferase, mitochondrial, putative
Lc02985	✓			✓	Transmembrane protein, putative
Lc35633	✓			✓	50S ribosomal protein L24, chloroplastic (CL24) (Precursor)
Lc33555	✓			✓	Cationic amino acid transporter 2 isoform 1
Lc34056	✓			✓	Plant/F18B3-190 protein, putative
Lc03176	✓			✓	3-ketoacyl-CoA synthase
Lc12538	✓			✓	Lipid transfer protein
Lc32580	✓			✓	Tonoplast dicarboxylate transporter-like protein
Lc34466	✓			✓	HXXXD-type acyl-transferase family protein
Lc34176	✓			✓	TPR domain kinase
Lc20626	✓			✓	Nuclear ribonuclease Z
Lc29289	✓			✓	Uncharacterized protein
Lc27166	✓			✓	Fasciclin-like arabinogalactan protein
Lc12418	✓			✓	Lipid transfer protein
Lc35232	✓			✓	Transmembrane amino acid transporter family protein
Lc23931	✓			✓	hypothetical protein
Lc25526	✓			✓	Peptide/nitrate transporter
Lc29754	✓			✓	PPR containing plant protein
Lc29421	✓			✓	Uncharacterized protein
Lc06030	✓			✓	DnaJ-class molecular chaperone
Lc36464	✓			✓	Asparagine synthetase
Lc34980	✓			✓	Transducin/WD40 repeat protein
Lc38704	✓			✓	Polyamine oxidase-like protein
Lc36389	✓			✓	DUF1767 domain protein
Lc24065	✓			✓	Major intrinsic protein (MIP) family transporter
Lc38206	✓			✓	Histone H4
Lc36588	✓			✓	Cation/H+ exchanger 3
Lc01244	✓			✓	Profilin
Lc22561	✓			✓	Alba DNA/RNA-binding protein
Lc36209	✓			✓	GRAS family transcription factor
Lc09011	✓			✓	Uncharacterized protein
Lc17524	✓			✓	PPR containing plant protein
Lc38658	✓			✓	Heavy metal-associated domain protein
Lc36108	✓			✓	Polysaccharide biosynthesis protein
Lc36252	✓			✓	PPR containing plant-like protein

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc38811	✓			✓	Four ACT domain ACT domain protein which protein
Lc37640	✓			✓	Cytochrome P450 family brassinosteroid oxidase
Lc35470	✓			✓	Tonoplast dicarboxylate transporter-like protein
Lc35352	✓			✓	MAP kinase phosphatase
Lc34570	✓			✓	Glycoside hydrolase family 17 protein
Lc23870	✓			✓	Uncharacterized protein
Lc13560	✓			✓	Thylakoid lumenal 17.9 kDa protein
Lc26114	✓			✓	Uncharacterized protein
Lc02996	✓			✓	Transmembrane protein, putative
Lc37040	✓			✓	Uncharacterized protein
Lc13007	✓			✓	Putative cytoplasmic aconitate hydratase
Lc37551	✓			✓	NAC transcription factor-like protein
Lc27939	✓			✓	Alpha amylase domain protein
Lc00587	✓			✓	SART-1 family protein
Lc33837	✓			✓	Pectinacetylesterase family protein
Lc30103	✓			✓	Peroxidase
Lc36938	✓			✓	Ribonucleoside-diphosphate reductase
Lc15780	✓			✓	PPR containing plant-like protein
Lc34188	✓			✓	hypothetical protein
Lc31083	✓			✓	Disease resistance protein (TIR-NBS-LRR class)
Lc37638	✓			✓	E3 ubiquitin-protein ligase RGLG2-like protein
Lc26307	✓			✓	ABA response element-binding factor
Lc36702	✓			✓	Chalcone-flavanone isomerase family protein
Lc36996	✓			✓	Histone H4
Lc33968	✓			✓	Malectin/receptor-like kinase family protein
Lc34436	✓			✓	Trichome birefringence-like protein
Lc37967	✓			✓	Serine/threonine-protein kinase Aurora-1
Lc16112	✓			✓	Carnitine/acylcarnitine carrier CACL-like protein
Lc30408	✓			✓	Phosphatase 2C family protein
Lc24262	✓			✓	GDSL-like lipase/acylhydrolase
Lc03686	✓			✓	Nucleolar complex-like protein
Lc37296	✓			✓	Protein kinase Peptidoglycan-binding LysM
Lc36997	✓			✓	Histone H4
Lc38506	✓			✓	Folate transporter/carrier-like protein
Lc14565	✓			✓	Kinase AFC1
Lc20372	✓			✓	Transcription factor
Lc35355	✓			✓	LURP-one-like protein
Lc27805	✓			✓	Endonuclease/exonuclease/phosphatase family protein
Lc17967	✓			✓	DnaJ heat shock family protein
Lc18409	✓			✓	Histone H3
Lc38205	✓			✓	Histone H4
Lc36804	✓			✓	Uncharacterized protein

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc33278	✓			✓	Auxin-responsive family protein
Lc27804	✓			✓	Endonuclease/exonuclease/phosphatase family protein
Lc36009	✓			✓	Uncharacterized protein
Lc33285	✓			✓	Histone H4
Lc10651	✓			✓	DNA (Cytosine-5)-methyltransferase
Lc13406	✓			✓	Lung seven transmembrane receptor family protein
Lc23518	✓			✓	LRR receptor-like kinase
Lc38959	✓			✓	Sec14p-like phosphatidylinositol transfer family protein
Lc36664	✓			✓	Uncharacterized protein
Lc34411	✓			✓	Uncharacterized protein
Lc33909	✓			✓	Sulfotransferase
Lc14917	✓			✓	Putative uncharacterized protein
Lc38119	✓			✓	Pectinesterase
Lc33844	✓			✓	Purple acid phosphatase
Lc08272	✓			✓	Histidine phosphatase family (Branch 1) protein
Lc33559	✓			✓	ABC transporter A family protein
Lc26967	✓			✓	HAD-family hydrolase IIA
Lc37230	✓			✓	PPR containing plant-like protein
Lc22494	✓			✓	Uncharacterized protein
Lc35096	✓			✓	Carbohydrate-binding X8 domain protein
Lc15488	✓			✓	Zinc finger protein ZAT9
Lc36883	✓			✓	50S ribosomal protein L28
Lc04222	✓			✓	RecQ family ATP-dependent DNA helicase
Lc32512	✓			✓	RALF
Lc32435	✓			✓	Uncharacterized protein
Lc33513	✓			✓	CwfJ carboxy-terminal 1-like protein
Lc10265	✓			✓	Serine hydroxymethyltransferase
Lc27515	✓			✓	O-acyltransferase WSD1-like protein
Lc37166	✓			✓	Arginine N-methyltransferase
Lc38044	✓			✓	Putative uncharacterized protein
Lc17214	✓			✓	GDSL-like lipase/acylhydrolase
Lc24960	✓			✓	DUF1666 family protein
Lc27690	✓			✓	Uncharacterized protein
Lc20642	✓			✓	Putative ribonuclease H protein
Lc06016	✓			✓	RNA 2'-phosphotransferase, Tpt1/KptA family protein
Lc32003	✓			✓	LRR receptor-like kinase
Lc38306	✓			✓	Receptor-like Serine/Threonine-kinase NCRK protein
Lc00901	✓			✓	Uncharacterized protein
Lc34755	✓			✓	RING-H2 finger ATL48-like protein
Lc23062	✓			✓	hypothetical protein
Lc09511	✓			✓	DEAD-box ATP-dependent RNA helicase, putative
Lc37993	✓			✓	Basic 7S globulin

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc35996	✓			✓	Transmembrane protein, putative
Lc22814	✓			✓	Global transcription factor group protein
Lc32814	✓			✓	Heavy metal transport/detoxification superfamily protein
Lc32624	✓			✓	Kunitz proteinase inhibitor 5
Lc28370	✓			✓	Patatin
Lc35586	✓			✓	F-box/RNI/FBD-like domain protein
Lc09278	✓			✓	Uncharacterized protein
Lc36845	✓			✓	Cyclin dependent kinase inhibitor
Lc34530	✓			✓	Histone H4
Lc36116	✓			✓	Long-chain-alcohol oxidase
Lc37084	✓			✓	LRR receptor-like kinase
Lc35875	✓			✓	NAD(P)-binding Rossmann-fold superfamily protein
Lc17665	✓			✓	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta
Lc38416	✓			✓	Uncharacterized protein
Lc32940	✓			✓	Polygalacturonase non-catalytic protein
Lc37154	✓			✓	DNA-3-methyladenine glycosylase I
Lc36682	✓			✓	DUF4378 domain protein
Lc37475	✓			✓	Cytochrome P450 family protein
Lc31458	✓			✓	Acyl-CoA thioesterase
Lc27091	✓			✓	Cinnamoyl-CoA reductase
Lc38340	✓			✓	UDP-glucosyltransferase family protein
Lc35162	✓			✓	Tyrosine kinase family protein
Lc34666	✓			✓	Protein Ycf2
Lc25996	✓			✓	Acid phosphatase/vanadium-dependent haloperoxidase
Lc15608	✓			✓	Uncharacterized protein
Lc37655	✓			✓	Plant intracellular ras group-related LRR protein
Lc14724	✓			✓	hypothetical protein
Lc37038	✓			✓	Uncharacterized protein
Lc35446	✓			✓	PPR containing plant-like protein
Lc37859	✓			✓	Telomerase activating protein Est1
Lc36738	✓			✓	GDSL esterase/lipase plant-like protein
Lc03854	✓			✓	Aminoaldehyde dehydrogenase
Lc36742	✓			✓	Extensin-like region protein
Lc36420	✓			✓	GMP synthase [glutamine-hydrolyzing] protein
Lc02092	✓			✓	Probable U6 snRNA-associated Sm-like protein LSm4 (Glycine-rich protein 10) (GRP 10)
Lc34721	✓			✓	Chromosome condensation regulator RCC1 repeat protein
Lc09295	✓			✓	MYB transcription factor MYB91
Lc37598	✓			✓	Plastocyanin-like domain-containing protein
Lc01098	✓			✓	ENTH/VHS/GAT family protein
Lc37082	✓			✓	Hydroxyproline-rich glycoprotein family protein, putative
Lc19579	✓			✓	Glucan endo-1,3-beta-glucosidase-like protein
Lc37037	✓			✓	MtN19-like protein

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc38919	✓			✓	Transmembrane protein, putative
Lc10031	✓			✓	hypothetical protein
Lc36924	✓			✓	Replication protein A 70 kDa protein
Lc38415	✓			✓	Lipid transfer protein
Lc11920	✓			✓	AP2 domain class transcription factor
Lc36540	✓			✓	Potassium transporter
Lc29697	✓			✓	Disease resistance protein (TIR-NBS-LRR class), putative
Lc37923	✓			✓	LRR receptor-like kinase
Lc34336	✓			✓	Histone H3
Lc34844	✓			✓	60S ribosomal protein L10-2
Lc35901	✓			✓	Uncharacterized protein
Lc36706	✓			✓	hypothetical protein
Lc17713	✓			✓	Histone H2B
Lc32402	✓			✓	Enoyl-(Acyl carrier) reductase
Lc35631	✓			✓	S-adenosylmethionine carrier protein
Lc32992	✓			✓	G protein coupled receptor
Lc10375	✓	✓		✓	Uncharacterized protein
Lc38911	✓	✓		✓	Kinesin-like protein
Lc23822	✓	✓		✓	AFG1-family ATPase
Lc38129	✓	✓		✓	GATA type zinc finger transcription factor family protein
Lc19306	✓	✓		✓	Puromycin-sensitive aminopeptidase-like protein
Lc34576	✓	✓		✓	Lysine-ketoglutarate reductase/saccharopine dehydrogenase
Lc29690	✓	✓		✓	Ubiquitin-protein ligase
Lc22194	✓	✓		✓	Uncharacterized protein
Lc34561	✓	✓		✓	Uncharacterized protein
Lc02611	✓	✓		✓	Plastid lipid-associated protein
Lc30257	✓	✓		✓	Uncharacterized protein
Lc37809	✓	✓		✓	Major intrinsic protein
Lc15046	✓	✓		✓	Uncharacterized protein
Lc37374	✓	✓		✓	UDP-glucosyltransferase family protein
Lc04504	✓	✓		✓	BZIP transcription factor
Lc36194	✓	✓		✓	Nucleotide-diphospho-sugar transferase domain protein
Lc01416	✓	✓		✓	Import inner membrane translocase subunit TIM21, putative
Lc04266				✓	Pollen-specific SF21-like protein
Lc34740				✓	LRR receptor-like kinase family protein
Lc35307	✓	✓			Serine/threonine-protein kinase
Lc34856	✓	✓	✓		F-box/LRR protein
Lc34767		✓	✓	✓	TMV resistance protein N
Lc32033		✓	✓	✓	Beta-hydroxyacyl-ACP dehydratase
Lc33881		✓	✓		TMV resistance protein N
Lc34728		✓	✓		Cytochrome P450 family 71 protein
Lc33010		✓	✓		Anthocyanin 5-aromatic acyltransferase

Gene ID	24 hpi	48 hpi	72 hpi	96 hpi	Gene annotation
Lc33810		✓	✓		hypothetical protein
Lc37739		✓	✓		Folate-sensitive fragile site protein FRA10AC1
Lc33368		✓	✓		Putative uncharacterized protein
Lc33834		✓	✓		Uncharacterized protein
Lc20090		✓	✓		Phosphoenolpyruvate carboxylase
Lc32099		✓	✓		1-phosphatidylinositol-3-phosphate 5-kinase
Lc37289		✓	✓		Uncharacterized protein
Lc16849		✓	✓		Pathogenic type III effector avirulence factor Avr AvrRpt-cleavage: cleavage site protein
Lc05394		✓	✓		Heavy metal P-type ATPase
Lc34396		✓	✓		Lipid transfer protein
Lc37115		✓	✓		hypothetical protein
Lc02095		✓	✓		Uncharacterized protein
Lc33129		✓	✓		Fasciclin domain protein
Lc37981		✓	✓		Uncharacterized protein
Lc33537		✓	✓		Plant-specific B3-DNA-binding domain protein
Lc09229		✓	✓		Uncharacterized protein
Lc25227		✓	✓		LITAF-domain-containing protein
Lc36598	✓				Uncharacterized protein
Lc37827	✓				Cysteine-rich RLK (Receptor-like kinase) protein
Lc05315	✓				DEAD-box ATP-dependent RNA helicase 27