# Effects of the *Lr34* and *Lr46* Rust-Resistance Genes on Other Diseases of Wheat

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A thesis submitted to the University of East Anglia

for the degree of Doctor of Philosophy

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September 2014

# ABSTRACT

*Lr34* and *Lr46* are adult plant resistance genes providing durable resistance to biotrophic diseases of wheat like rusts and powdery mildew. In seedlings, these genes increased susceptibility to Septoria tritici blotch (STB), caused by *Zymoseptoria tritici*, in near-isogenic lines (NILs) of spring wheats Lal Bahadur (LB) and Avocet and mutant lines developed from LB, but not in an *Lr34* NIL of Jupateco. A similar effect was observed in adult plants with artificial inoculation in polytunnels.

The role of leaf age in *Lr34* and *Lr46* resistance to mildew and susceptibility to STB was tested. It was hypothesised that enhanced senescence can make leaves more resistant to biotrophs but more susceptible to necrotrophs. In young leaves, LB was less susceptible to STB than *Lr34* or *Lr46* NILs. The opposite pattern was observed in older leaves. There was higher expression of genes associated with senescence and cell death in LB-*Lr34* than in LB, indicating that *Lr34* may enhance senescence. Metabolites associated with senescing leaves accumulated to a higher level in *Z.tritici*-infected LB-*Lr34* NILs than in LB.

In seedlings, *Lr34* and *Lr46* favoured the non-biotrophic fungi *Magnoporthe grisea*, the wheat blast pathogen, and *Ramularia collo-cygni*, the Ramularia leaf spot pathogen of barley. *Lr34* reduced spot blotch (*Cochliobolus sativus*) in field trials but no conclusive results were obtained for tan spot (*Pyrenophora tritici-repentis*) or Fusarium head blight (FHB; *Fusarium graminearum*).

These results presented indicate that there may be significant consequences for the use of *Lr34* and *Lr46* to control rust and mildew in areas where necrotrophic diseases are prevalent, including some which have not previously been economically significant on wheat, and that plant breeding strategies to control multiple diseases simultaneously are required. They also indicate that the potential to breed varieties in which the adverse effects of these genes are mitigated.

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## ACKNOWLEDGEMENTS

I am thankful to MBBSIP for providing the generous funding for my PhD. I would like to express my sincere gratitude to my supervisor Dr. James K.M. Brown and John Innes Centre for giving me the opportunity to pursue a PhD in a world-class facility and their continuous support throughout my research. I really want to thank James for his patience, motivation, enthusiasm, and knowledge. His guidance helped me in all the time of research and writing of this thesis.

I am deeply grateful to Paul Nicholson and Cristobal Uauy for their detailed and constructive comments which helped improve the quality of my work. I want to thank Graham McGrann for all the thought-provoking discussions we had and he really helped me with developing my critical analysis skills. Working with Elizabeth Orton and Laetitia Chartrain was really fun and a great learning experience. They were really helpful in all of times of need and they seemed to make everything better over a cup of tea. I could not have done it without their company and friendship. I also want to thank Margaret Corbitt for her immense help and expertise in all the pathology experiments. Also, thanks to Henry for his friendship and support.

My sincere thanks also goes to the entire staff of JIC for making the valuable resources at JIC available to me. Without their help the project would not have been completed smoothly. During this work I have collaborated with CIMMYT and I want to thank Pawan Singh for hosting me and helping with my work. I am also thankful to my friends who always supported me especially Shruti for being there as a fun-loving friend, a caring sister and one of the best people I know.

I also thank my parents, grandparents and my brother for their constant love, support and encouragement throughout the duration of this project. This thesis is dedicated to you. And last but not the least, I want to thank my dearest Navien for loving me so much and being there for me at every step. Thank you so much for your love, support and dedication even during the most trying of times. You made it possible for me.

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# 1. Introduction

Over the years there have been tremendous improvements in plant disease control strategies but there is still a major lack of sustained global food supply due to multiple pathogens and pests. The diseases caused by these pathogens can cause dramatic reduction in crop yield and these effects are more severe in developing countries because of high costs involved with efficient disease management strategies including improved agronomy (reducing pathogen pressure), use of resistant varieties (reducing damage by pathogen) and pesticides (reducing amount of pathogen). Pesticides and chemical treatments are effective to some extent but they have deteriorating effects on the environment and are always not cost-effective. It was estimated that the amount of pesticide actually coming in contact to the target pest is less than 0.3% while the rest is exposed to non-target organisms like humans and animals and may lead to undesirable effects ranging from mild allergies to more severe effects like mutagenesis, carcinogenesis, immune system disorder and nervous system disorders (van der Werf, 1996). Also the emergence of insensitive pathogen strains can render the use of pesticides ineffective (McDowell and Woffenden, 2003, Chen et al., 2013). This is the reason that plant pathologists are

intrigued by the basis of how and why a certain pathogen causes disease in a particular host plant to develop more efficient strategies to counter the pathogen infection and sustain crop productivity.

## 1.1. Plant-pathogen associations

Depending on the feeding lifestyles, plant pathogens can be broadly classified into biotrophs and non-biotrophs. There is a spectrum of non-biotrophy in terms of the length of the latent period and the extent to which the pathogen relies on host metabolism. In this thesis, non-biotrophic pathogens with a long latent phase and considerable dependence on host plants will be called hemibiotrophs and pathogens with a relatively prominent and important necrotrophic phase will be termed necrotrophs.

Biotrophs invade the living tissues and depend upon the plant's metabolism to derive their nutrition. They infect host plants without causing cell death for several days and keep the host tissue alive throughout the association. Many biotrophs deploy a genetic program to maintain viability of their plant hosts during at least part of the infection cycle (Spanu, 2012). Rusts and mildews of cereals are caused by biotrophic fungi which form specialized feeding structures like haustoria, which are determinate branches of intercellular or

intracellular hyphae that terminate in the host cell and are surrounded by invagination of host plasma membrane. Several plant pathogens form haustoria like downy mildews, powdery mildews and rusts and also control host immunity by secretion of effector molecules (Panstruga and Dodds, 2009). The obligate biotrophs cannot be cultured in vitro extensively. The facultative biotrophs can survive on necrotic or dead tissue but need a biotrophic phase to complete their life cycles. An example is the maize smut fungus *Ustilago maydis* (Spanu, 2012).

Hemibiotrophs are characterized by an initial biotrophic phase, during which the host's immune system and cell death are suppressed allowing fungal hyphae to spread throughout the infected plant tissue, followed by a necrotrophic phase during which toxins are secreted by the pathogen to induce host cell death (Koeck et al., 2011). Fungi like *Cladosporium fulvum* and *Pyrenopeziza brassicae* are considered hemibiotrophs as they penetrate the leaf through stomata and leaf cuticle respectively, spread within the cell walls of the host via intercellular hyphae and continue to grow without producing any symptoms. This is followed by a necrotrophic phase when the symptoms first appear (Stotz et al., 2014). Intracellular hyphae are less specialized than haustoria and are not determinate (Al-Khesraji and

Lösel, 1981). They are common in hemibiotrophs like *Colletotrichum lindemuthianum*, a pathogen of bean. Both haustoria and intercellular hyphae have been proposed to have a role in signalling, communication and escaping host recognition (Giraldo and Valent, 2013, Perfect and Green, 2001).

Necrotrophs kill the host cells first and then metabolize their contents to derive nutrition for their growth by deploying toxins and enzymes targeted towards specific substrates in the host tissue (Horbach et al., 2011). Examples are fungi like *Pythium* and *Botrytis*. All true necrotrophic pathogens initially have a short biotrophic phase in which they asymptomatically colonize the host tissues. *Botrytis cinerea* survive as latent symptomless infections as biotrophic endophytes before initiating coordinated host cell death mechanisms (Heller and Tudzynski, 2011).

Non-biotrophic pathogens like *B. cinerea*, *Zymoseptoria tritici*, *Magnoporthe oryzae*, *Fusarium graminearum* and *Fusarium oxysporum* f.sp. *dianthi* have different lifestyles, but they all express a high number of cell wall degrading enzymes and also share a common necrotrophic phase (Kema et al., 2008). The expression of these enzymes can possibly be used as a distinguishing feature of the

necrotrophic phase, in contrast to the biotrophic phase. Genome analysis of barley powdery mildew, *Blumeria graminis* f.sp. *hordei*, revealed that genes coding for enzymes of primary and secondary metabolism, carbohydrate active enzymes, and transporters were absent from the genome (Spanu et al., 2010). This might be the reason for the obligate biotrophic lifestyle of powdery mildews.

### 1.1.1. Trade-offs between plant defences against pathogens with different lifestyles

There is increasing evidence for trade-offs between responses to different diseases and pests (Grant and Jones, 2009, Brown and Rant, 2013). Plant hormones are major components of defence pathways and regulate differential responses to specific types of attackers (Pieterse et al., 2012). When infected with pathogens, plants respond with hormone signals comprising of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) varying in quantity and timing (Pieterse et al., 2009, Thaler et al., 2012). Signalling pathways which are activated by these signalling molecules regulate different defence responses towards different classes of pathogens to limit the pathogen growth (Spoel et al., 2007). Resistance against biotrophs can be achieved by SA-mediated defences while necrotrophs and herbivorous insects are more sensitive to JA/ET-mediated defences (Glazebrook, 2005, Pieterse et

al., 2012). Mutants failing to accumulate SA or insensitive to SA tend to be more susceptible to biotrophic infection. Arabidopsis mutant *npr1* was identified in a SA-nonresponsive screen (Cao et al., 1994, Spoel et al., 2007). This mutant failed to accumulate pathogenesis related (PR) proteins which are activated as a result of SA induced defence-responses. It has been shown that infection with biotrophic *Pseudomonas syringae*, makes plants more susceptible to the necrotrophic pathogen *Alternaria brassicicola* by suppression of the JA signalling pathway (Spoel et al., 2007). This process is partly dependent on the cross-talk modulator NPR1. This trade-off was restricted to tissues adjacent to the site of initial infection and occurred only with the virulent *Pseudomonas* strain. This might be advantageous to the plant for restricting growth of necrotrophic pathogen in tissues undergoing PCD. Interactions between SA and ET, JA and abscisic acid (ABA), and JA and ET have been proposed to be involved in response of plants to herbivores and pathogens with different lifestyles (Koornneef and Pieterse, 2008). ET produced by Arabidopsis in response to caterpillars of the small cabbage *Pieris rapae* induced SA-mediated defences resulting in enhanced resistance against biotrophic pathogen, *Turnip crinkle virus* (De Vos et al., 2006).

## 1.2. Genetic inheritance of resistance and pathogenecity

The concept of genetic inheritance of pathogenecity and resistance developed when it was shown that cereal rust fungus *Puccinia graminis* has different morphologically similar races which differ in their pathogenecity to their hosts by Eriksson in 1894. Flor (1954) showed that for each gene in host for resistance, there was a corresponding gene in the pathogen for avirulence. This concept is known as a gene-for-gene relationship. It was then suggested in 1963 by Vanderplank that there are two types of resistance:

1. Vertical resistance which is controlled by a few "major" genes, is strong but effective only against a few races of the pathogen.

2. Horizontal resistance which is controlled by many "minor" genes, is weaker but effective against all the races of the pathogen.

### 1.2.1. Host resistance

Disease resistance which is controlled by one or more genes in plants is known as host resistance. The pathogen and the host can be incompatible with each other either if the host plant can defend itself against the pathogen, leading to specificity for resistance as in a gene-for-gene system or if there is no recognition between the host and the

pathogen for as in a toxin-receptor system. Types of host resistance can be described as follows:

### *1. R gene resistance, Race-specific, monogenic or Vertical resistance*

If a plant variety is more resistant to one race of a pathogen then the other races, such a resistance is called race-specific resistance. It clearly differentiates between various races of a pathogen as it is effective against some races and ineffective against others. It is usually controlled by one gene (or in some cases by a few genes) and called monogenic or major gene (*R* gene) resistance (McDonald and Linde, 2002). Many *R* genes often cluster in the genome and are encoded by nucleotide-binding–leucine-rich repeat (NB-LRR) proteins (Michelmore et al., 2013). Some major *R* genes are sensitive to environmental conditions such as temperature (Zhu et al., 2010) which may become increasingly important with global warming. In cereals, a number of genes conferring resistance to *Puccinia* spp. have been cloned, with most coding for receptors with NB-LRR domains (Lowe et al., 2011). Many receptor-like kinases (RLKs) control gene for gene resistance to fungal pathogens (Goff and Ramonell, 2007). The ERECTA RLK was identified for resistance to the necrotrophic fungal pathogen, *Plectosphaerella cucumerina* in Arabidopsis (Llorente et al.,

2005). RFO1 is an RLK identified in the resistance response to the fungal pathogen *Fusarium oxysporum* (Diener and Ausubel, 2005). This type of resistance was described as vertical resistance by Vanderplank (1963).

### *2. Partial, quantitative, polygenic, or horizontal resistance*

Polygenic resistance is determined by several to many genes with small but additive effects and can have phenotypes ranging from partial to full resistance effective against multiple strains of a pathogen. It is often sensitive to environmental conditions (Michelmore et al., 2013, St.Clair, 2010). This resistance is usually controlled by several genes which may be NB-LRR-encoding genes, receptor-like kinases (RLKs), or unrelated genes with diverse functions such as secretion or cell wall reinforcement (Poland et al., 2009, Schweizer and Stein, 2011, Kou and Wang, 2010). One of these genes alone may be ineffective and only play a minor role in total resistance. The several genes which are involved in the resistance mechanism may control different steps of the physiological and metabolic pathways that provide resistance to the plant.

*3.* ***Tolerance to disease***

Tolerance is the ability of the plant to maintain its productivity even when it is infected with a pathogen. Tolerant plants are susceptible to the pathogen but survive the pathogen infection though the genetics of the process is not well understood (Brown and Handley, 2006). Disease tolerance is commonly seen in plant-virus infections. Rice crops fertilized by silicates have been shown to be tolerant to blast (*Pyricularia oryzae*) as well as many other diseases (Ning et al., 2014).

### 1.2.2. Non-host resistance

Non-host resistance is broad-spectrum resistance of an entire plant species against all isolates of a pathogen that is virulent on other plant species (Heath, 2000, Senthil-Kumar and Mysore, 2013). It is the most common form of disease resistance exhibited by plants. Non-host resistance provides a robust and durable barrier to a variety of pathogens whereas race-specific resistance is non-durable and can be rapidly overcome by pathogen isolates by evolving a matching virulence (Lowe et al., 2011). Non-host resistance is composed of many overlapping pathways and mechanisms which may be the reason for its durability making it difficult for the pathogen to evolve corresponding to all of them (Ham et al., 2007). The processes

controlling non-host resistance are of particular interests to plant breeders and pathologists as non-host resistance has not been shown to break down easily (Senthil-Kumar and Mysore, 2013).

#### 1.2.2.1. Components of non-host resistance

Individual genes that confer non-host resistance have been identified in *Arabidopsis* by mutational analysis. *NHO1* (Non- Host1) against *Pseudomonas syringae* pv. *phaseolicola* and the *PEN1*, *PEN2* and *PEN3* genes that prevent penetration by the barley powdery mildew fungus are examples of such genes (Stein et al., 2006, Sumit et al., 2012). *nho1* mutants lack nonspecific resistance to *Pseudomonas* bacteria as they support the growth of *P. s. tabaci* and *P. fluorescens* bacteria which are non-pathogenic on *Arabidopsis* (Kang et al., 2003, Lu et al., 2001). *PEN1* encodes a soluble N-ethylmalemide sensitive attached receptor (SNARE) protein which transports toxic compounds to the pathogen infection sites (Collins et al., 2003). *PEN2* is associated with plant cell wall architecture and mutations in it cause constitutive alterations in the cell wall (Mellersh and Heath, 2001). It supports the evidence that cell wall and associated structures provide an important barrier to pathogen invasion. *PEN3* encodes an ATP-binding cassette (ABC) protein of the plasma membrane and together with *PEN2*

transports toxic chemicals into the infection sites (Lipka et al., 2010) in response to powdery mildews. A second layer of NHR in Arabidopsis involving defence genes like *EDS1, PAD4* and *SAG101* is activated if first layer is overcome by pathogen (Lipka et al., 2005). Also SA and JA pathways are activated upon infection with biotrophic and necrotrophic pathogens respectively (Glazebrook, 2005). SA and JA pathways are involved in the expression of non-host resistance in Arabidopsis against the soybean rust pathogen *Phakopsora pachyrhizi* as shown in mutants lacking *PEN1*, *PEN2* and *PEN3* (Loehrer et al., 2008).

### 1.2.3. Model of plant immune system

There are two branches of the plant immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to pathogen-associated molecular patterns (PAMPs), such as flagellin (D et al., 2007). The second uses the polymorphic NB-LRR (nucleotide binding- leucine rich repeat domains) protein products encoded by most *R* genes (Gómez-Gómez, 2004, Jones and Dangl, 2006). The plant immune system has been described as a four phased 'zigzag' model (Jones and Dangl, 2006). PAMPs are recognized by PRRs which results in PAMP-triggered immunity (PTI) which stops colonization. Pathogens deploy effectors which interfere with PTI

resulting in effector-triggered susceptibility (ETS). The effectors are specifically recognized by NB-LRR proteins which results in effector-triggered immunity (ETI) causing a hypersensitive cell death response (HR) at the infection site. Pathogens counter ETI either by dispensing with or varying the recognized effector gene, or by gaining effectors that suppress ETI. Natural selection can lead to evolution of new *R* genes corresponding to previously unrecognised pathogen effectors, resulting once again in effective triggering of ETI.

### 1.2.4. Genetics of virulence in pathogens

For activation of plant defence responses, it is necessary that the pathogen is perceived by the host in a timely manner. The pathogen can either penetrate the cell wall or membrane or release effectors that can be detected by the host. Biotrophic and hemibiotrophic pathogens secrete effectors including Avr or avirulence proteins that are recognized by corresponding proteins encoded by *R* genes (Vleeshouwers and Oliver, 2014). In the Guard model (Mackey et al., 2003, Dangl and Jones, 2001), these Avr proteins target the host proteins that control defence responses or affect pathogen virulence and the host plant *R* genes encode putative receptors for Avr proteins and trigger signal transduction pathways which activate several plant defences (McDowell and Woffenden, 2003). AVR3a is secreted from

haustoria into the host cells during initial phases of infection by *Phytopthora infestans* stabilizing the plant E3 ligase CMPG1 to mediate suppression of host cell death at early biotrophic infection phases (Stotz et al., 2014, Vleeshouwers and Oliver, 2014). In *P. syringae* pv. *phaseolicola,* the AvrPphC effector can suppress ETI triggered by the AvrPphF effector in some cultivars of bean (Rohmer et al., 2004). Necrotrophic effectors include a range of molecular compounds, many of which are host-specific or host-selective toxins (HST). *Cochliobolus* and *Alternaria* genera produce small molecular weight effectors including polyketide (PK) and non-ribosomal peptide (NRP) whereas *Stagonospora nodorum, Pyrenophora tritici-repentis, Rhyncosporium. secalis, Botrytis cinerea,* and L. *maculans* produce proteins (Vleeshouwers and Oliver, 2014, Oliver et al., 2012, Friesen et al., 2009). T-toxin and victorin produced by *Cochliobolus* target the mitochondria and ToxA produced by *S. nodorum* targets the chloroplast and generally these necrotrophic effectors induce cell death to provide access to plant nutrients to the pathogen (Vleeshouwers and Oliver, 2014, Hammond-Kosack and Rudd, 2008).

## 1.3. Septoria tritici blotch (STB)

STB is one of the most prevalent and damaging diseases of wheat worldwide and the most important disease in UK. It is caused by the ascomycete fungus *Zymoseptoria tritici* (Stukenbrock et al., 2012) formerly known as *Mycosphaerella graminicola* (anamorph, *Septoria tritici*) (Suffert et al., 2010). It is propagated by both sexual ascospores and asexual pycnidiospores (Kema et al., 1996) which spread by wind and rain-splash respectively.

### 1.3.1. Infection and Symptoms

STB presents itself as leaf spots that appear as small yellowish specks that enlarge and turn yellowish grey and finally dark brown. The spots form irregular blotches with small black pycnidia which can cover entire leaf. Leaf penetration by the fungus occurs within first three days of inoculation. It is followed by the symptomless phase which lasts for 10 to 14 days (Keon et al., 2007, Kema et al., 1996). During this period, fungus establishes itself in the substomatal cavities and the apoplast surrounding the mesophyll cells of the leaves. After that necrotrophic stage begins and symptoms begin to appear. There is host-tissue collapse, fungal growth becomes more rapid. Lastly, there is development of pycnidia with necrotic diseased tissue and release of

pycnidiospores. There is no development of any specialized feeding or penetrating structures (Kema et al., 1996).

### 1.3.2. Disease management

The disease can be controlled to some extent by use of disease-free seed in the field which is free of the pathogen, 2- to 3-year crop rotations, field-sanitation by deep ploughing and by chemical sprays. Several fungicides can be used for control of *Septoria* diseases. However, the cost of fungicides, their environmental impact, and reduced sensitivity of the pathogen to them requires alternate methods to be developed. Isolates of *Z. tritici* which are insensitive to strobilurin based fungicides were recently discovered in Europe (McCartney et al., 2007). Recent shifts in sensitivity to azoles in *Z. tritici* populations are reducing the effectiveness of this class of fungicides by constant emergence of azole-resistant strains (Cools and Fraaije, 2013). Use of resistant wheat varieties is thus becoming more and more common. Breeding efforts are undergoing to identify wheat cultivars with resistance to STB.

### 1.3.3. Resistance against STB

Both quantitative and qualitative resistance against STB have been identified in wheat germplasm. Several lines have been identified which have specific resistance or susceptibility to specific isolates of *Z.*

*tritici* suggesting a gene-for-gene relationship (Brown et al., 2001, Brading et al., 2002). A total of 18 major resistance genes (Stb1-18) have been mapped in wheat (Orton et al., 2011, Rosa Simon et al., 2010). Two loci have been described on chromosome 7D of wheat namely *QStb.ipk-7D1* and *QStb.ipk-7D2*. The adult resistance locus *QStb.ipk-7D2* lies in the region where *Lr34/Yr18* has been mapped which is an adult-plant disease resistance locus effective against leaf rust, stripe rust and powdery mildews. The seedling resistance locus *QStb.ipk-7D* is found in the region which corresponds to the major resistance genes against STB, *Stb4* and *Stb5* (Rosa Simon et al., 2010). A total of 26 QTLs were identified for STB resistance by QTL analysis using four bi-parental populations, with each individual QTL explaining 3 to 21% of the phenotypic variance (Risser et al., 2011). Quantitative trait loci (QTL) analysis of a wheat population with five *Z. tritici* isolates in the seedling stage identified four QTLs on chromosomes 3AS, 1BS, 6DS and 7DS, and occasionally on 7DL (Ghaffary et al., 2011).

### 1.3.4. Molecular basis of STB infection

Genes similar to those coding for cell wall degrading enzymes have been identified in *Z. tritici*. These genes may play a role in pathogenicity of STB. In *Botrytis cinerea*, endo-1,4-beta-xylanase is

required for pathogenicity. In *Z. tritici*, this enzyme production is highly correlated with necrosis development (Douaiher et al., 2007). Also β-xyloxidase and polygalacturonase production has been correlated with lesion length and lesion frequency respectively. Presence of several such enzymes suggests an active cell wall degradation which is consistent with the observation of cell collapse during the later stages of pathogenesis (Kema et al., 2008). A similar role for such enzymes has been proposed in other pathogens. Alpha-L-arabinofuranosidase was upregulated in both *F. oxysporum* f.sp. *dianthi* and *F. graminearum* infection (Chacón-Martínez et al., 2004). Using a rice cell wall-derived medium, it was shown that there is an overrepresentation of cell wall degrading enzymes in the expressed sequence tag (EST) libraries of *Magnoportha grisea*, suggesting that these genes may likely play a role in fungal growth on cellulosic and hemicellulosic substrates (Ebbole et al., 2004).

It has been demonstrated that successful infection by *Z. tritici* is associated with a programmed cell death response in the host which has similar features like HR-like response which is seen during resistance to biotrophs (Keon et al., 2007). This response might relieve a nutritional starvation condition in the fungus by releasing nutrients in the apoplast. It has also been shown by a marked DNA laddering

response which was initiated when disease symptoms start to develop (Keon et al., 2007). No HR-like cell death response has been observed in incompatible reactions even at high levels of fungal inoculum (Kema et al., 1996, Keon et al., 2007).

It was suggested that there is a communication between the plant and the fungus in the apoplast which involves soluble toxic compounds in compatible reactions. It is possible that the resistance mechanism to STB does not involve defence responses to cell wall degrading enzymes but relies on compounds or toxins that may inhibit establishment of the fungus and its growth (Kema et al., 1996). There is no evidence that *Septoria* feeds on the host tissue during its biotrophic phase as there is no depletion of nutrients from the apoplast. Quantitative PCR measurements showed little increase in fungal biomass before host cell death and it is unclear if *Z.tritici* growth is supported by apoplast-derived nutrients in the latent phase (Keon et al., 2007, Shetty et al., 2007). There is a change in levels of reactive oxygen species and defence gene expression during the latent phase of the fungus during both compatible and incompatible reactions but it is not known whether they have a role in containing the fungus or killing it or neither of these (Keon et al., 2007). A large number of fungal genes expressed during pathogenesis have been discovered which

include genes for cell wall degrading enzymes, genes involved in signal transduction, ATP-binding cassettes and transporter genes which might be involved in protection against antifungal compounds or secretion of toxins or pathogenicity factors (Kema et al., 2008). This fungus expresses a large number of genes which are involved in oxidative stress like catalases, superoxide dismutases, peroxidises and heat shock proteins (Keon et al., 2007). These genes might have a possible role in sustaining the fungal growth during the strong HR-response during the compatible reaction (Shetty et al., 2007).

During the compatible reactions, the strong HR-like cell-death response is preceded by strong transcriptional activation of wheat *AtMPK3* homologue, *TaMPK3* (Hammond-Kosack and Rudd, 2008, Rudd et al., 2008). It is proposed that the fungus triggers the disease signalling pathways of the host which are usually deployed against the biotrophs resulting into an oxidative burst which leads to loss of cell membrane integrity. This releases nutrients into the apoplast where they are available to the fungus. It has been suggested that *Z. tritici* produces effector or toxin molecules that trigger the host MAPK and subsequent PCD pathways (Rudd et al., 2008). This might also suggest that a compatible reaction with *Septoria* can create conditions for

resistance to a biotrophic pathogen like *Blumeria graminis*, the powdery mildew fungus.

## 1.4. Powdery mildews (PM)

Powdery mildew (PM) is an important foliar disease of wheat in many parts of the world including China, North and South America and Europe (Li et al., 2013). It is caused by the ascomycete fungus *Blumeria graminis* (syn. *Erysiphe graminis*) f. sp. *tritici* (Lillemo et al., 2006). The disease is quite severe in temperate conditions and is worsened by intensive cultivation techniques like use of semi dwarf varieties and high yielding cultivars with a lot of nitrogen fertilizer input (Lillemo et al., 2008). Late infection of the leaves can lead to heavy losses in the grain yield as the fungus can divert nutrients towards the site where it has colonized (Keller et al., 1999).

### 1.4.1. Infection and Symptoms

PM appears as growths of whitish grey spots of powdery appearance on young plants and leaves. It is most common on the upper side of the leaves. *B. graminis* f. sp. *tritici* (*Bgt*) is an obligate biotroph which obtains nutrients by haustoria formed within epidermal cells. The mycelium produces short conidiophores which produce ovoid conidia that can be carried by the wind (Agrios, 2005).

When a conidium lands on the host tissue, it develops a primary germ tube and then another germ tube which swells at the tip to form an appressorium which is the penetration organ. After the fungus penetrates the cell wall, it forms a haustorium which invaginates the plasma membrane of the host. This intimate association between the host and pathogen helps to provide host nutrients to the fungus while keeping the cell intact (Hückelhoven, 2005). Powdery mildew fungi induce a green island effect on the infected leaves where the leaf tissue which surrounds the fungal colony remains green while the rest of the leaf becomes chlorotic. This suppresses the host tissue death at the site of infection which is necessary for PM growth.

### 1.4.2. Disease management

The chemical control of PM is difficult and may not be cost effective. Some systemic fungicides give some degree of control but many isolates have developed insensitivity to these fungicides (Agrios, 2005). Host plant resistance is thus seen as an economical and environmental friendly alternative to control PM (Ma et al., 2011).

### 1.4.3. Resistance against PM

Both qualitative and quantitative resistance to PM have been described in wheat. The *Pm* genes (*R* genes) control the qualitative or race-specific resistance and they belong to the gene-for-gene category.

There are more than 40 resistance alleles mapped to 43 loci (*Pm1-43*) (Ma et al., 2011). Most of these genes encode proteins which are involved in host-pathogen recognition (Niu J., 2009). *Pm3b* is a dominant powdery mildew *R* gene. It was mapped on chromosome 1A of wheat and is a member of gene cluster coding for CC-NBS-LRR (coiled-coil nucleotide-binding site leucine-rich-repeat) proteins. It is derived from the susceptible allele *Pm3CS* which is widespread in various wheat line (Yahiaoui et al., 2004). Most *Pm* genes confer complete resistance to powdery mildew controlled by a hypersensitive reaction which includes the formation of papilla and cell death at the site of fungal penetration. However, long-term control of PM by *Pm* genes has not been effective. These race-specific genes lack broad spectrum activity and durability allowing rapid build-up of PM populations which are virulent on the lines containing these genes (Keller et al., 1999). Thus there is increasing interest in developing germplasm with race non-specific or partial resistance which offers broad-spectrum resistance that could prove durable. These allow the plant to be infected by the pathogen, but significantly retard its development (Lillemo et al., 2008). Such resistance is termed as slow mildewing or adult plant resistance (Lillemo et al., 2008, Keller et al., 1999). The spring wheat line Saar has good partial resistance to

powdery mildew along with partial resistance to leaf and stripe rust. Quantitative genetic studies on a segregating population from a cross between Saar and a susceptible cultivar Avocet showed that three genes in Saar contribute to powdery mildew resistance in the cultivar and have an additive effect (Lillemo et al., 2006).

### 1.4.4. Molecular basis of PM infection

PM development is linked to a restriction of host cell death that can be observed in the 'green island effect' which is indicative of semi-systemic cell death suppression at the infection sites (Ridout, 2001). The formation of haustorium is regulated by sophisticated signalling pathways. Early appresorium development is associated with cyclic AMP-dependant protein kinase A (cPKA) signalling. The involvement of cPKA was studied in the ascomycete *Magnoportha grisea* using gene disruption experiments which resulted in delayed or incomplete appresorium development (Schulze-Lefert and Panstruga, 2003). Gene expression studies suggest that MAPK pathways are also important in appresorium formation. It is suggested that a heterotrimeric G-protein signalling pathway is involved in conjunction with MAPK and cAMP pathway (Hückelhoven, 2005). Some defence responses are triggered by the recognition of some conserved pathogen molecular structures including peptides and carbohydrate units in the fungal cell wall called

as pathogen-associated molecular patterns or PAMPs via the membrane bound LRR receptor like kinases in the plant. Such a carbohydrate elicitor has been identified in *Bgt* (Schulze-Lefert and Panstruga, 2003).

Wheat *MLO*-like genes are homologs of barley MLO gene. The transcription of *TaMLO* is slightly enhanced during *Bgt* infection. The *R*-gene mediated PM recognition triggers multiple cellular responses which include signalling pathways for reactive oxygen species, jasmonic acid, ethylene, nitric oxide, salicylic acid and defence related genes like the PR genes (Niu J., 2009).

## 1.5. Adult plant resistance gene *Lr34*

*Lr34* is associated with resistance to major rusts of wheat like leaf rust caused by *Puccinia triticina* and stripe rust caused by *P. striiformis* f. sp. *tritici* and as well as resistance to powdery mildews caused by *Bgt* (Lagudah et al., 2009). The gene was first reported by Dyck in wheat cultivar Frontana in Canada (Dyck et al., 1966). *Lr34* was localized on short arm of chromosome 7D between the molecular markers *gwm1220* and *SWM10* (Dyck et al., 1994).

### 1.5.1. Molecular mapping and markers

*Lr34* has pleiotropic effects on resistance to adult plant stripe rust (*Yr18*) (Singh, 1992), powdery mildew resistance (*Pm38*) (Spielmeyer et al., 2005) and leaf tip necrosis (*Ltn1*) (Rosewarne et al., 2006). Using high resolution mapping, it has been shown that *Lr34*/*Yr18* cosegregates with stem rust resistance and interacts with unlinked genes in "Thatcher" background to enhance the stem rust resistance (Spielmeyer et al., 2008). *Lr34* was also associated with adult stem rust resistance in around 100 recombinant inbred lines from a cross between "Thatcher" and "RL6058" (Vanegas et al., 2008). RL6058 is a backcross-derived near-isogenic line of cultivar Thatcher with resistance gene *Lr34*. A tightly linked molecular marker, *csLV34* was reported for *Lr34*/*Yr18* that can detect the presence of *Lr34* disease resistance in a wide range of wheat backgrounds (Lagudah et al., 2009). A 0.15cM interval for *Lr34* was identified using high resolution mapping flanked by *XSWSNP3/XcsLVA1* and *XcsLVE17* and a 363 kb physical interval which contained both the markers was isolated and completely sequenced from the Chinese Spring wheat containing *Lr34* (Krattinger et al., 2009).

Based on marker analysis studies, the wheat germplasm containing *Lr34* was classified into three breeding lineages consisting of spring wheat lines from North and South America, germplasm from Far-East, and winter wheat from Europe (Kolmer et al., 2008). Gene specific markers were developed based on these lineages and a third *Lr34* haplotype was identified in winter wheat cultivars Zinal, Allanin, Galaxie, and spelt wheat (*Triticum spelta*) varieties Ostro and Rouquin (Lagudah et al., 2009). Based on microcolinearity studies in *Lr34* region, it was concluded that there is little orthology in different grass species in this interval which is not conserved due to divergence of different grass species because of numerous translocations, deletion events and duplications (Krattinger et al., 2010). It has been suggested that there have been at least two independent changes in the ancestral chromosome region containing *Lr34*/*Yr18* leading to altered gene order and gene content between wheat, rice and *Brachypodium* (Spielmeyer et al., 2008).

### 1.5.2. Gene sequence

The sequence of *Lr34* was a gene rich region containing eight open reading frames. The predicted proteins coded by the ORFs were an ATP-binding cassette (ABC) transporter, a hexose carrier, two cytochromes P450, a cysteine proteinase, two lectin receptor kinases

and a glycosyl transferase. Sequence polymorphisms were identified only in the putative ABC transporter gene on examining the sequence differences of the +/- *Lr34* parental lines of mapping populations suggesting that the ABC transporter gene confers *Lr34* disease resistance (Krattinger et al., 2009). Wheat cultivars, both with and without *Lr34* resistance, express a putatively functional gene at the *Lr34* locus. However these two alleles differ by three polymorphisms, of which two led to amino acid changes in the ABC transporter gene. A 3 base pair (bp) deletion in exon 11 of the resistant allele led to deletion of a phenylalanine at position 546 in the coded protein and a C/T polymorphism in exon 12 changed a tyrosine to histidine at position 634. The proteins coded by these two alleles have 97% homology (Krattinger et al., 2010).

The *Lr34* sequence is 11.8 kbp long containing 24 exons, and is predicted to code for a 1401-amino acid long protein. *Lr34* shares 86% identity in the amino acid sequence with its closest homolog in rice which is OsPDR23 and 56% identity with PEN3 of Arabidopsis suggesting that *Lr34* may confer resistance by exporting metabolites affecting the fungal growth as it has been proposed for PEN3 (Krattinger et al., 2010). The predicted protein belongs to a sub-family of ABC transporters called pleiotropic drug transporters. These

transporters are involved in ATP dependent transport of wide range of structurally unrelated molecules including drugs across the membranes and conferring drug resistance. They share a common basic structure consisting of two hydrophobic domains and two cytosolic nucleotide binding domains (Rogers et al., 2001).

Three unique homologues for *Lr34* have been identified in hexaploid wheat. These homologues have been mapped to chromosome 7A, 4A and 7D. The coding sequence of 7A homologue is interrupted by several repeat elements like a 303bp hAT transposon, a 5.2 kb LTR retrotransposon and a CACTA transposon. The 4A homologue copy putatively encodes a functional protein but the coding sequence is 787 bp longer than *Lr34* sequence on 7D (Krattinger et al., 2010).

### 1.5.3. Characteristics of *Lr34* phenotype

*Lr34* is associated with a lower rate of intercellular hyphal growth and haustorium formation in the early stages of infection with rust with little or no plant necrosis or hypersensitive response (Rubiales and Niks, 1995). The phenotype of *Lr34* resistance is characterized by reduced infection frequency, longer latency period, smaller size of uredinia and a decreasing gradient of uredinia from base to tip (Rubiales and Niks, 1995, Singh and Huerta-Espino, 2003). The

resistance is also stronger at lower temperatures in the seedling stage (Rubiales and Niks, 1995). *Lr34* is also associated with the leaf tip necrosis gene *Ltn1* which causes necrosis progressing from the tip of the leaf to the base over time (Singh, 1992). *Lr34* lines have a higher percentage of early aborted rust sporelings and the size of the colonies is smaller than in the lines without *Lr34* at the seedling stage and these effects were more prominent in adult plants. The reduction in haustorium formation in lines containing *Lr34* is due to reduction in growth of intercellular hyphae in contrast to partially rust resistant lines like Akabozu and BH1146 where resistance is due to high frequency of papilla formation (Rubiales and Niks, 1995).

*Lr34* lines have a low hypersensitivity index with rust isolates both alone and in combination with the gene-for-gene *R*-gene *Lr37*. Hypersensitivity index is a measure of necrotic area per unit of rust colony size. The *Lr34* lines containing *Lr37* also showed very high resistance in the field and glasshouse conditions. Lines with combination of *Lr34* and *Lr13* also had improved resistance against rust (Kloppers and Pretorius, 1997). It was shown that *Lr34* has pleiotropic effects on the components of slow rusting resistance response (Singh and Huerta-Espino, 2003).

### 1.5.4. Associated genes

*Lr34* resistance response is highly energy demanding. It induces multiple metabolic pathways in a co-ordinated fashion like glycolysis, tricarboxylic acid (TCA) cycle, the GABA shunt and CoA biosynthesis during the earlier stages of *Lr34* resistance response. But these responses are not maintained at the later stages of the response (Bolton et al., 2008). This might explain why *Lr34* increases the latent period of the fungus but does not completely inhibit its growth. Microarray analyses have shown that similar genes are up-regulated in both uninfected *Lr34* flag leaves and senescing wheat flag leaves (Gregersen and Holm, 2007, Hulbert et al., 2007). Also HVS40 protein was also shown to be up-regulated in *Lr34* flag leaf tips in Thatcher *Lr34* leaves by northern blot analysis (Krattinger et al., 2009). These findings suggested that *Lr34* resistance might be a result of senescence like processes. *Lr34* lines show a characteristic gene expression pattern of stress response (Hulbert et al., 2007). *Lr34* does not lead to a hypersensitive response but still shares many genes that are typically up-regulated in *R*-gene mediated resistance like the genes coding for PR proteins and phenylpropanoid enzymes (Bolton et al., 2008). *Lr34* resistance doesn’t fit any other disease resistance model and is thus assumed to have a novel resistance mechanism which might involve

abiotic stress response and senescence pathways. These genes might create conditions which enhance the typical defence responses or might just directly play a role in resistance. Many genes are up-regulated in plants with *Lr34* and few of them might be playing the role in resistance (Hulbert et al., 2007). However, in this study only a single time point was studied. Further studies spanning across various time points can give a better understanding of genes which are involved in *Lr34* resistance pathway.

## 1.6. Adult plant resistance gene *Lr46*

*Lr46* is another slow rusting resistance gene that was first described in the wheat cultivar Pavon 76. Pavon 76 showed far less leaf rust severity (5-10%) compared to Jupateco 73S and Avocet S lines (80-100%) indicating the potency of *Lr46*. The gene was localized on chromosome 1B after evaluation of BC2F3 and BC3F3 lines (backcross 2- and backcross 3- derived substitution F3 lines) from crosses between 'LalBahadur' monosomics and Pavon 76 (Singh et al., 1998). The Lalbahadur monosomic series was derived by deletion of one chromosome from each chromosome pair respectively. *Lr46* confers a resistance phenotype in adult plants similar to *Lr34*. It prolongs the latency period, increases percentage of aborted sporelings

and reduces colony size. However the effects of *Lr46* are not as pronounced as *Lr34* in the seedling stage. *Lr46* also confers a similar defence response which is non-hypersensitive like *Lr34* as the high percentage of aborted rust sporelings was not associated with host cell necrosis (Martínez et al., 2001). It has been shown that *Lr46* has an additive effect on leaf rust resistance of *Lr34* (Lillemo et al., 2008).

### 1.6.1. Molecular Markers

AFLP markers have been developed to map *Lr46* on the distal end of 1BL (William et al., 2003). The authors also found that *Lr46* was tightly linked or pleiotropic to a stripe rust resistance gene designated *Yr29*. *Lr46* was mapped distal to *Xwmc44*, approximately 5-15 cM, and proximal to *Xgwm259* (Suenaga et al., 2003). *Lr46/Yr29* is also linked to PM resistance gene designated as *Pm39* (Lillemo et al., 2008). Microsatellite locus *Xbarc80* maps 10-11 cM distal to *Xgwm259* and can be used as an alternative distal marker (http://maswheat.ucdavis.edu/protocols/Lr46/index.htm).

## 1.7. Increasing the durability of resistance

Though it has now become easier to identify and incorporate *R* genes into plants to confer resistance against pathogens, the resistance achieved is usually not durable over the long term. However, there are

several cases where single *R* genes confer highly durable resistance. A classic example is the resistance to cabbage yellows caused by *Fusarium oxysporum* f.sp. *conglutinans*, which has remained effective wherever used since its deployment over 90 years ago (Leach et al., 2001). Partial resistance conferred by several genes with additive effects is usually durable, particularly when it involves developmental or morphological changes in the plants (Stuthman et al., 2007). Thus there is need for identification and incorporation of more durable resistance genes into the plants along with *R* genes to provide an effective disease resistance which has a wider range and more potency. The quality and durability of a plant resistance gene is hypothesized to be a function of the amount of fitness penalty imposed on the pathogen to overcome that resistance gene (Leach et al., 2001, Lo Iacono et al., 2013) though the associated mechanisms are expected to be complex molecular pathways.

The most environment-friendly, low-cost method of controlling leaf rust is to breed and grow durable resistant wheat varieties. Adult plant resistance genes like *Lr34* and *Lr46* are very important for breeding because they have conferred durable resistance over a long period of time in different environments, as well as against multiple pathogens (Imbaby et al., 2014). Both of these genes are widely used in

CIMMYT's rust breeding programs. The durable partial resistance of Frontana and various CIMMYT wheat lines is based on the additive interaction of *Lr34* and two or three additional slow rusting genes (Singh and Rajaram, 1993, Singh et al., 2011). When susceptible cultivars display 100 percent leaf rust severity, cultivars with only *Lr34* display approximately 40 percent severity; cultivars with *Lr34* and one or two additional minor genes display 10 to 15 percent severity; and cultivars with *Lr34* and two or three additional genes display 1 to 5 percent severity. *Lr46* in combination with one or two minor genes can reduce leaf rust severity to 30% from 100%.

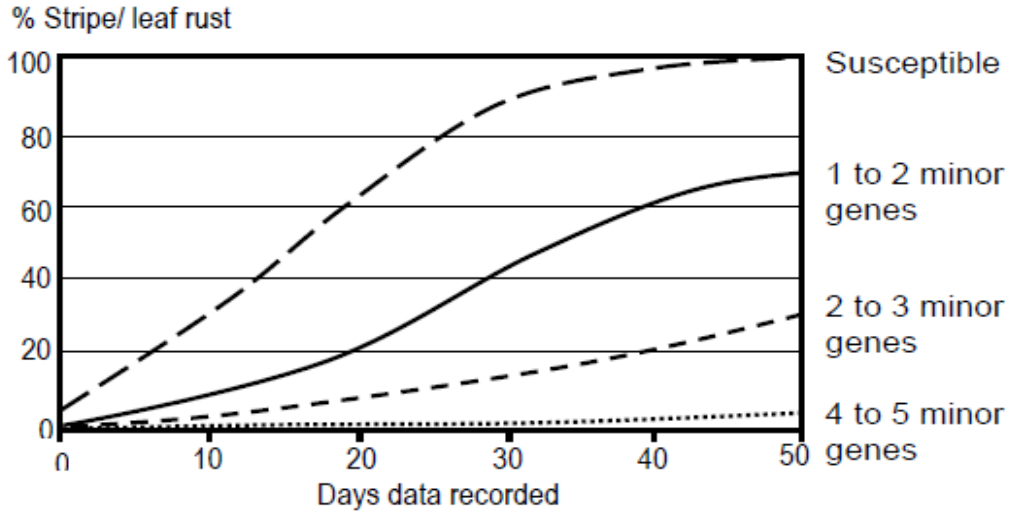



**Figure 1.7.1 Relationship between progress of stripe (yellow) rust in relation to number of minor genes present in a wheat cultivar (2004, R.P.Singh, Proceedings of the 4th International Crop Science Congress)**

Combining several minor rust resistance genes to provide near immunity to rust pathogens is the main strategy followed by CIMMYT

to achieve durable resistance (Fig 1.7.1). Combinations of *Lr34/Yr18* and two to four additional slow rusting genes have resulted in adequate stripe rust resistance levels in most environments (Singh and Rajaram, 1993). Comparative studies in Mexico showed that stripe rust infection caused grain yield losses of 31 to 52 percent in *Lr34/Yr18*-carrying Jupateco 73R and 74 to 94 percent in *Lr34/Yr18*-lacking Jupateco 73S (Ma and Singh, 1996).

## 1.8. Aim of the project

There is mounting evidence that resistance to biotrophic pathogens may increase susceptibility to necrotrophs. In this thesis, I show that *Lr34* and *Lr46* increase susceptibility to virulent isolates of *Z. tritici* and suppress resistance to avirulent isolates (Chapter 3). The effect of *Lr34* in increasing susceptibility to virulent isolates is greater than that of *Lr46*. I also tested the hypothesis that senescence plays an important role in *Lr34* and *Lr46*-mediated defence responses (Chapter 4). *Lr34* responses to STB and mildews were shown to be age-dependant and possibly linked to expression of cell-death or senescence associated genes. Metabolite profiling of Septoria-infected *Lr34* lines indicated a complex mechanism involving a balance between sugar and amino acid concentrations in an age-dependant manner. I also showed that these *Lr*

genes have potential to increase susceptibility of wheat seedlings to the blast and Ramularia leaf spot pathogens (Chapter 5). These trade-offs might have significant consequences for the use of *Lr34* and *Lr46* in rust and mildew control in farms, particularly where biotrophic and non-biotrophic pathogens both occur at the same location (Chapter 6).

# 2. Materials and method

## 2.1. Plant material

For seedling tests, the wheat seedlings were grown for 18days (16-h light/8-h dark, 20°C/16°C, 80% RH) till growth stage 12 on the Zadoks (1974) decimal code in Levington F2 compost media (Scotts Professional, Ipswich, UK). For adult plant tests, the seedlings were grown in rows in trays of 3×3×5cm (P60) trays before being transferred to 1 litre pots for maturity.

Near-isogenic lines (NILs) of *Lr34* and *Lr46* in backgrounds Lal Bahadur (LB), Avocet and Jupateco were used for the experimental studies along with the parents as controls. NILs in the background of the rust susceptible Indian spring wheat variety LB were developed at CIMMYT (Lagudah et al., 2009). Another line with an introgression of *YrPmLr1* gene which is *Lr46* from an unknown source in LB background was used (Lillemo et al., 2007). Jupateco with *Lr34* and Jupateco without *Lr34* were reselections of the heterogenous Mexican spring wheat cultivar Jupateco 73 which originated by pedigree selection up to the F6 generation (Singh, 1992). Avocet NILs with *Lr34* and *Lr46* were developed from Avocet-*YrA* by in Australia by C.

Wellings and CIMMYT, Mexico respectively (Lillemo et al., 2007). Avocet-*YrA* is a line selected from the heterogeneous Australian cultivar Avocet for lacking yellow rust resistance gene *YrA* and has been referred to as Avocet throughout this thesis. The size of the introgressed segments bearing *Lr34* or *Lr46* in each NIL is not known but it is possible that the stock carry genes flanking *Lr34* or *Lr46* and as well as *Lr34* or *Lr46* themselves.

Two sets of mutant pairs (Mutant 19 and Mutant 21) for *Lr34* in the LB background (Spielmeyer et al., 2008) were also used. Mutants with a loss-of-function mutation in *Lr34* used were derived by inducing gamma irradiation for optimal mutagenesis (Mago et al., 2005) in seeds from the single chromosome substitution line Lal Bahadur (Parula 7D) with *Lr34* obtained from CIMMYT. The seeds were irradiated using a $^{60}$Co source at a dosage of 20 krad. Leaf rust susceptible plants identified in M2 generation were selected along with their resistant sibs and were re-tested as M3 and M4 for leaf rust resistance to confirm their M2 genotypes. Mutant 19 and Mutant 21 retained all the single sequence repeat (SSR) markers used to study deletions for mutant selection. Mutant 19 and mutant 21 had single base pair deletions at nucleotide positions 5,035 and 10,620 respectively in *Lr34* gene based

on genomic sequence of Chinese Spring (Krattinger et al., 2009). These deletions resulted in frame shifts after amino acid 499 (M19) and 1269 (M21). They also resulted in premature stop codon after amino acid 516 (M19) and 1269 (M21). The presence of additional mutations in the other genes co-segregating with *Lr34* in a 363 kb interval was tested by sequencing DNA fragments covering 12 to 15 kb of the other five genes and intergenic regions on mutants 19 and 21 (Krattinger et al., 2009). There were no sequence polymorphisms detected in those genes. However, the presence of other mutations in rest of the introgressed 7D chromosomal segment cannot be excluded.

## 2.2. Fungal material

### 2.2.1. *Zymoseptoria tritici* isolates

*Z. tritici* isolates that were used for the work described are as follows:

| Isolate code | Country of origin | Reference |
|---|---|---|
| **IPO323** | Netherlands | (Kema and vanSilfhout, 1997) |
| **IPO88004** | Ethiopia | (Chartrain et al., 2004) |
| **IPO92006** | Portugal | (Arraiano et al., 2001a) |

IPO323 is avirulent to *Stb6* resistance gene (Brading et al., 2002), IPO88004 to *Stb15* (Arraiano and Brown, 2006) and IPO92006 to an uncharacterized resistance present in Bastard II and Chaucer wheat lines (Chartrain et al., 2004).

#### 2.2.1.1. Culture of *Z. tritici* isolates

*Z. tritici* isolates were removed from the –80°C freezer and partially defrosted for five minutes. A small quantity of inoculum was removed from each tube under sterile conditions using a flamed inoculating loop. The sample was densely streaked onto yeast peptone dextrose (YPD) agar plates described as follows:

**Yeast peptone dextrose agar (YPDA)**
1g/L bacto yeast extract
5g/L bactopeptone
5g/L dextrose
5g/L agar
The plates were placed at 15°C under near UV (340/370nm) light in a controlled environment cabinet for 5-7 days. Isolates were sub-cultured by scraping a small amount of *Z. tritici* spores from the petri dishes and transferring onto a new petri dish containing YPDA.

#### 2.2.1.2. Inoculum Preparation

Inoculum was produced from sporulating cultures of *Z. tritici*, grown on YPDA plates for 7 days under near-ultra violet light (340/370nm) for 16h per day at 15°C. Cultures were flooded with sterile distilled water and scraped to release conidia with a flame sterilized glass rod. Spore concentration was assessed using a Fuchs-Rosenthal counting

chamber (Hawksley, Lancing, UK). Average number of spores in the 1mm$^2$ squares (shaded in Figure 2.2.1) was calculated for five random squares.

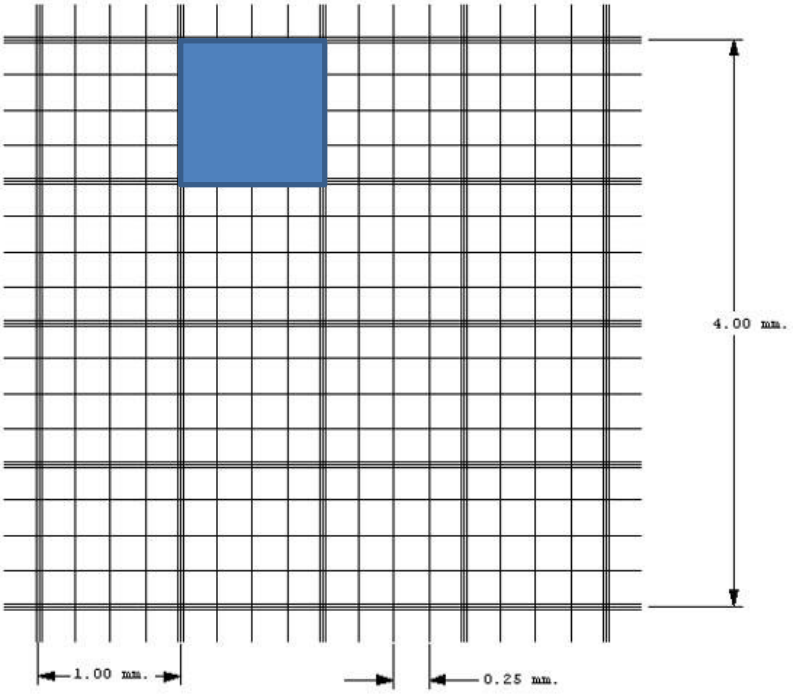



Figure 2.2.1 Fuchs-Rosenthal counting chamber

This was multiplied to give an estimate of spore concentration using the equation:

**(Average number of spores) x 16 x 5000 = Spore concentration**

The concentration of the conidial suspension was adjusted to 1x10$^7$ spores/mL and Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) was added to 0.15% v/v, before inoculation.

**Volume of spore suspension required =[Quantity of inoculum required x $10^7$] / Spore concentration of suspension**

### 2.2.1.3. *Z. tritici* inoculation

Plants were grown in glasshouse conditions (16h light/8h dark, 20°C/16°C, 80% RH) for 17days. The plants were spray inoculated with a fixed amount of inoculum using an airbrush apparatus using a turntable for a uniform coverage (Arraiano et al., 2001a). The plants were covered and kept in dark for 48 hrs.

### 2.2.1.4. Disease evaluation

The symptoms were scored by visually measuring the percentage of leaf area covered with lesions bearing pycnidia which are the small black dot-like fruiting structures. The percentage scores were converted into AUDPC (Area under disease progress curve) scores. AUDPC entails repeated disease assessments. Infected plants were assessed for the percentage leaf area infected three to five times during the course of experiment. The AUDPCs were calculated directly from data and estimated from the described equation (Campbell and Madden, 1990)

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1}\left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

Where “t” is time in days of each observation, “y” is the percentage leaf area covered with pycnidia at each reading and “n” is the number of readings.

### 2.2.2. *Blumeria graminis* f.sp.*tritici* isolates

The isolate JIW48 was used throughout the experiments and obtained from John Innes Centre, UK collection of wheat powdery mildew isolates.

#### 2.2.2.1. Transfer of mildew isolates

The plants were grown for 14 days in the glasshouse conditions (16h light/8h dark, 20°C/16°C, 80% RH). The prophyll leaves were cut and placed on onto agar boxes (Brown and Wolfe, 1990) which have following composition:

**Mildew boxes**
5g/L agar
10g/L benzamidozole
The spores were sprinkled onto the leaves from earlier infected leaves. The leaf boxes were kept at 15°C under 16h low intensity white light/ 8hr dark to induce sporulation.

#### 2.2.2.2. Inoculation of mildew

Plants were grown in glasshouse conditions (16h light/8h dark, 20°C/16°C, 80% RH) for 17days in a single row in trays. The second leaves of the seedlings were fixed horizontally on a plastic platform with the adaxial side up using double sided tape (Keon et al., 2007). Plants were inoculated with spores of *B. graminis* in a settling tower by the method of Boyd et al. (1994). The plants were kept at 15°C under 16hr low intensity white light/ 8hr dark.

#### 2.2.2.3. Disease scoring

The disease was scored by counting the number of mildew colonies per infected leaf. For standardization, leaf area was measured by using the software ImageJ (Collins, 2007) on photographed leaves.

### 2.2.3. *Magnoporthe grisea* isolates

Wheat adapted *Magnaporthe grisea* isolate BR32 was obtained from Rachel Goddard (JIC) and used for the experimental studies. It originated in Brazil and is virulent on both wheat and barley (Faivre-Rampant et al., 2008).

#### 2.2.3.1. Culture of isolates

The cultures were maintained as described in Talbot et al. (2009). The fungal stock samples consist of dried mycelia on pieces of cellulose filter paper. To revive the fungus, a tiny piece of the paper was cut (approximately 1x2 $mm^2$) and placed on a suitable solid medium (CMA supplemented with 100µg/mL Carbenicillin and 60µg/mL Streptomycin) and the plates were incubated at 25°C.

| **Complete Media (CMA)** | 1 litre |
|---|---|
| $NaNO_3$ | 6 g |
| KCl | 0.52 g |
| $MgSO_4$ x $7H_2O$ | 0.52 g |
| $KH_2PO_4$ | 1.52 g |
| Trace elements (1ml of stock; see below) | 0.1% |
| D-Glucose | 10 g |
| Peptone | 2 g |
| Yeast extract | 1 g |
| Casamino acids | 1 g |
| Vitamin solution (see below) | 1 mL |
| Agar | 15 g |

pH was set at 6.5 with NaOH.

| **1000x Trace Elements** | |
|---|---|
| $H_2O$ | 80 mL |
| $ZnSO_4 \cdot 7H_2O$ | 2.2 g |
| $H_3BO_3$ | 1.1 g |
| $MnCl_2 \cdot 4H_2O$ | 0.5 g |

| | |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | 0.5 g |
| $CoCl_2 \cdot 6H_2O$ | 0.17 g |
| $CuSO_4 \cdot 2H_2O$ | 0.16 g |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.15 g |
| $Na_4EDTA$ | 5 g |

The compounds were added in order and boiled. The solution was cooled to 60°C and pH 6.5 was set with KOH. The volume was adjusted to 100 mL after cooling at room temperature.

| **Vitamin solution** | 100mL |
|---|---|
| Biotin | 0.01 g |
| Pyridoxin (vit. $B_6$) | 0.01 g |
| Thiamine (vit. $B_1$) | 0.01 g |
| Riboflavin (vit. $B_2$) | 0.01 g |
| PABA (p-aminobenzoic acid) | 0.01 g |
| Nicotinic acid | 0.01 g |

The solution was stored in a dark glass bottle at 4°C.

#### 2.2.3.2. Inoculum preparation

Culture plates were flooded with approximately 3-4 ml of 0.25% gelatin (or 0.02% Tween 20) and the spores were scraped with a glass spreader. The suspension was filtered through a layer of Miracloth into a 15 ml falcon tube to remove mycelial fragments. Spores were counted under the microscope and the spore concentration was adjusted to $10^5$ spores per ml with 0.25% gelatin.

#### 2.2.3.3. Inoculation of *M. grisea*

The spore suspension was sprayed onto the upper side of the leaf segments using an airbrush. The plant trays were sealed with parafilm and kept under dark for 24h at 24°C.

#### 2.2.3.4. Scoring of symptoms

The leaves were scored after 6 days of inoculation for blast lesions. Number of lesions was counted per infected leaf. Blast lesions or microlesions are generally less than 3 mm in diameter, greyish in colour and ringed by brown, necrotic tissue (Tufan et al., 2009). The lesions usually sit surrounded by chlorotic leaf tissue.

### 2.2.4. *Ramularia collo-cygni* isolate

*R. collo-cygni* Rcc09B4 collected from Bush Estate, Midlothian, Scotland in 2009 by Dr Neil Havis (Scotland's Rural College, Scotland) was used in all disease experiments.

#### 2.2.4.1. Culture of isolates

Fungal cultures were maintained on YPDA plates as described in Makepeace et al. (2007).

#### 2.2.4.2. Inoculum preparation

Rcc09B4 liquid cultures were prepared as described by Peraldi et al. (2014). Inoculum was grown in potato dextrose broth (PDB) liquid culture prepared by adding a 5 mm agar plug excised from a 2-week-old YPDA culture plate. Liquid cultures were supplemented with 10 $\mu$g mL$^{-1}$ streptomycin and incubated at 20°C for 14 days under constant agitation at 175 rpm on an orbital shaker (New Brunswick Scientific Co.) in the dark.

#### 2.2.4.3. Inoculation of *R. collo-cygni*

Plants were inoculated with a slurry of hyphal fragments using an airbrush sprayer and placed under plastic covers to maintain high relative humidity (80–100%) in the dark for 48h, then under fluorescent lighting at 220 µmol m$^{-2}$ s$^{-1}$ for the duration of the experiment.

#### 2.2.4.4. Scoring of symptoms

Disease was scored as the percentage area of the leaf covered with RLS lesions which are reddish brown and rectangular in shape as described by (McGrann et al., 2014a). Leaves were scored three to six times

between 1 and 4 weeks after inoculation and the area under the disease progress curve (AUDPC) was calculated.

## 2.3. DNA isolation

DNA was isolated from infected leaves using DNeasy Plant Mini Kit (Catalogue No. 69104, Qiagen) advanced silica-membrane technology and simple spin procedures to isolate highly pure total cellular DNA from plant tissues and cells or fungi by selective adsorption to silica gel membranes under controlled ionic conditions. Purification using QIAGEN silica gel membrane technology is based on a bind-wash-elute procedure. Nucleic acids are adsorbed to the silica gel membrane in the presence of chaotropic salts, which remove water from hydrated molecules in solution and polysaccharides and proteins are removed. After a wash step, pure nucleic acids are eluted under low- or no-salt conditions in small volumes, ready for immediate use without further concentration.

The sample material was disrupted using mortar and pestle under liquid nitrogen. For large number of samples, leaf material was collected in 96-well collection plates (Catalogue No. AB-0564, ABgene) and sealed with 96 cap sealing mat (Catalogue No. AB0674, ABgene) after

lyophilization. The plate was transferred to Genogrinder 2000 after adding a 3mm tungsten carbide bead (Catalogue No. 69997, Qiagen) to each well and tissue disrupted at 1300 RPM for 3 minutes. DNA was extracted using DNeasy 96 plant kit (Catalogue No. 69181, Qiagen) using the manufacturer's protocol. DNA quantification was done using Nanodrop.

## 2.4. Fungal DNA quantification

*Z. tritici* biomass was estimated by quantifying fungal DNA using the method described by Fraaije et al. (2005) using *cytochrome b* (*cytb*) gene of *Z.tritici*. For testing leaf samples using real-time PCR, 50 ng of infected plant DNA was used. PCRs were carried out in 25µL reaction volumes (capped Thermo-Fast 96 PCR Plates; ABgene). IQ SuperMix (Invitrogen Life Sciences, Carlsbad, CA) was used for quantitative PCRs.

The generalized composition of qPCR mix followed is as below:

| Component | Volume (in µL) | Final concentration |
|---|---|---|
| **Total DNA (10ng/µL)** | 5 | 50ng/reaction |
| **2X IQ SuperMix** | 12.5 | 1X |
| **Forward primer (10µM/µL)** | 1.25 | 0.5 µM |
| **Reverse primer (10µM/µL)** | 0.75 | 0.3 µM |
| **5'CY5/3'BHQ2-labeled probe (10µM/µL)** | 0.25 | 0.1 µM |
| **Nuclease-free Water** | 7.5 | |

Reactions were carried out in CFX96 TouchTM Real-Time PCR detection system (BIO-RAD).

The thermo-cycling parameters for qPCR amplification of *Z. tritici* DNA are as follows:

| Step | Temperature (°C) | No. of cycles | Time | Activity |
|---|---|---|---|---|
| **1** | 50 | 1 | 2 min | Incubation |
| **2** | 95 | 1 | 2 min | Denaturation |
| **3** | 95 | 50 | 15 sec | Denaturation |
| **4** | 60 | 50 | 1 min | Extension |

The increase in fluorescence from probes was recorded at 60°C during every cycle. For each sample, the threshold cycle (cycle at which the increase of fluorescence exceeded the background [Ct]) for the CY5-labeled probe was determined. Cleavage of this probe correlated with the amount of pathogen DNA. Plotting known amounts of DNA against Ct values generated standard curves. The resulting regression equations were used to quantify the amount of pathogen DNA in samples using CFX96 TouchTM Real-Time PCR software.

## 2.5. RNA isolation

Leaf tissue for RNA extraction was collected and frozen in liquid nitrogen. The tissue was stored at -80°C. RNA was extracted from frozen leaf tissue using RNeasy Plant Mini kit (Catalogue No. 74903,

Qiagen) following the manufacturer's protocol. The kit combines the guanidine-isothiocyanate lysis with silica-membrane purification. RNA was quantified using Nanodrop and stored at -20°C.

## 2.6. cDNA synthesis

The first strand cDNA synthesis was carried out using First Strand cDNA synthesis kit (Catalogue no. K1612, Thermo Scientific) from 1 µg of DNA-free total RNA isolated in the following steps:

### 2.6.1. DNase I treatment

DNase I (Catalogue No. EN0521, Thermo Scientific) treatment was carried out prior to cDNA synthesis to remove traces of genomic DNA from RNA samples. Following reaction mix was prepared in an RNase-free tube:

| Component | Amount |
|---|---|
| **RNA** | 1 µg |
| **10X reaction buffer with $MgCl_2$** | 1 µL |
| **DNAse1** | 1 µL(1U) |
| **Water, Nuclease-free** | to 10 µL |

The reaction mix was incubated at 37°C for 30 minutes. One µL EDTA (50mM) was added and incubated at 65°C for 10 minutes followed by addition of one µL RT primer (random hexamers) and incubation at 65°C for 5 minutes.

### 2.6.2. Reverse transcription reaction:

First strand cDNA synthesis was carried out with the above reaction mix by setting the following reaction:

| Component | Amount |
|---|---|
| **5X Reaction buffer** | 4 µL |
| **RiboLock RNase Inhibitor (20 U/µL)** | 1 µL |
| **10mM dNTP mix** | 2 µL |
| **M-MuLV Reverse Transcriptase (20 U/µL)** | 2 µL |
| **Total volume** | 20 µL |

The reaction mix was incubated at 25°C for 5 min followed by 60 min at 37°C. The reaction was terminated by heating at 70°C for 5 minutes. The mix was diluted 1:20 for qRT-PCR and stored at -20°C.

## 2.7. qRT-PCR

qRT-PCR of RNA for gene expression analysis was carried out with EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen). The following reaction mix was used:

| Component | Volume (in µL) | Final concentration |
|---|---|---|
| **DNase treated RNA** | 5 | |
| **2X EXPRESS SYBR Supermix** | 10 | 1X |
| **Forward primer (10µM/µL)** | 0.2 | 200 nM |
| **Reverse primer (10µM/µL)** | 0.2 | 200 nM |
| **Nuclease-free Water** | 4.6 | |
| **Total volume** | 20 | |

The thermocycling conditions were set as follows:

| Step | Temperature | No. of cycles | Time | Activity |
|---|---|---|---|---|
| **1** | 95°C | 1 | 10 min | Denaturation |
| **2** | 95°C | | 15 sec | |
| **3** | 60°C | 39 | 30 sec | Annealing |
| **4** | 72°C | | 30 sec | Extension |
| **5** | 72°C | 1 | 10 min | Final extension |
| **6** | 95°C | | 15 sec | Melting curve |
| | 60°C | 1 | 15 sec | analysis |
| | Ramp | | 20 min | |
| | 95°C | | 15 sec | |

## 2.8. Metabolite analysis

Metabolite analysis of leaf samples was performed using GC-MS. Samples were lyophilized after collection. Dried tissue was ground into a fine powder using mortar and pestle on dry ice. To 20-25 mg of powdered tissue, 1-3 mL 70% ethanol was added. Phenyl α-D-glucoside (Sigma, P6626) was added as internal standard (50mg/L) for extraction. The mix was sonicated for 15 minutes in hot water ~ 80°C and then centrifuged for 10 minutes at room temperature, 2000 rpm (Sorvall RT6000 bench-top centrifuge). The supernatant was poured off into a fresh tube. The solvent was evaporated to dryness using the vortex evaporator for 1 hour at 40°C. 50 µL Methoxyamine hydrochloride – MOX (Pierce Chemical Co. #45950) was added and heated for 90 minutes at 30°C with continuous stirring. Then 100 µL N-Methyl-N trimethylsilyltrifluoroacetamide – MSTFA (Pierce

Chemical Co. #48911) was added and heated at 37°C for 30 minutes. Samples were left to stand at room temperature (25°C) for 2 hours before analysis. The sample analysis was performed on Agilent 6890N GC-MS System. The sample solution was injected into the GC inlet where it was vaporized and swept onto a chromatographic column by the carrier gas (helium). The compounds in the sample were separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase) and came out of the columns at different times (retention times). The mass spectrometer downstream captured, ionized, and detected the ionized molecules separately by detecting these molecules using their mass-to-charge ratio. The molecules were identified using Agilent Chemstation software.

## 2.9. Statistical analysis

The data analyses for experiments were carried out using GenStat 16$^{th}$ Edition (VSN International Ltd.). The experimental designs and statistical models used for different experiments are described in relevant chapters. The general linear model was used when responses were normally distributed with identity link function. The generalized linear model which is a generalization of ordinary linear regression was

used for data sets with variables that had error distribution models other than a normal distribution.

# 3. Effects of *Lr34* and *Lr46* on Septoria tritici blotch

## 3.1. Introduction

Wheat is the one of the oldest and most widely grown crops and has been cultivated since 10,000–8,000 BC (Heun et al., 1997). Increasing and ensuring sustainable wheat production have been major goals to eradicate global hunger (Shiferaw et al., 2013). Septoria tritici blotch is an important wheat disease in Western Europe which is among the largest wheat producing areas in the world. STB is also a major disease in the Americas, Central and West Asia, and parts of North Africa (Ghaffary et al., 2012). STB can cause yield losses exceeding 50%, particularly in low-input agriculture where disease management is frequently suboptimal (Duveiller et al., 2007). The rust diseases of wheat have also been among major production constraints worldwide (Kolmer, 2013). Leaf (or brown) rust caused by *P. triticina*, stripe (or yellow) rust caused by *P. striiformis* and stem (or black) rust caused by *P. graminis* are major threats to wheat production over large areas (Liu et al., 2013, Singh et al., 2011). Many wheat producing areas are affected by both Septoria and rust pathogens.

Pyramiding of minor genes conferring race non-specific resistance is one of the key strategies to breed for durable rust resistance. The combination of minor genes with additive effects has provided long lasting and cost-effective resistance to leaf and yellow rust (Rosewarne et al., 2013). Several CIMMYT wheat lines with rust resistance have APR genes like *Lr34* and *Lr46* in combination with other minor genes with additive effects (Singh et al., 2011, Singh and Rajaram, 1993). These genes are important components of breeding strategies to develop durable rust resistance varieties with reliable yield and quality.

Not much is known about the costs associated with the rust resistance provided by APR genes (Burdon et al., 2014). *Lr34* and *Lr46* are associated with leaf tip necrosis (Rosewarne et al., 2006) which is a form of localized cell death at tip of the flag leaves and might be involved in resistance to biotrophs like rust and mildew which need living plant tissue. Fitness costs associated with trade-offs between different types of pathogen are becoming important in cereal breeding (Brown and Rant, 2013). There is an increasing number of examples where resistance to biotrophic pathogens has led to increased susceptibility to non-biotrophic pathogens. Recessive alleles of *Mlo* which confer durable resistance to powdery mildew (*Blumeria*

*graminis* f. sp. *hordei*) of barley have been shown to increase susceptibility of barley to pathogens like *Magnaporthe oryzae* causing blast (Jarosch et al., 1999), *Cochliobolus sativus* causing spot blotch (Kumar et al., 2001) *Fusarium graminearum* causing head blight (Jansen et al., 2005) and *Ramularia collo-cygni* causing leaf spot (McGrann et al., 2014a) which have a necrotrophic phase. Necrotrophic pathogens interact with their hosts in a distinctly different way to biotrophic ones; and they are often able to survive in a saprophytic phase (Abang et al., 2006). They can complete their infection cycle in dead and/or dying host tissues and a hypersensitive response (HR) can benefit their growth (van Kan, 2006, Van Baarlen et al., 2004).

*Z.tritici* penetrates host wheat leaves only via stomata and has long periods of symptomless phase of leaf infection which can range from 2 to 4 weeks, before eventually triggering disease symptoms (Keon et al., 2007). *Z.tritici* lacks any specialized feeding or penetration structures and remains extracellular in host tissue during the infection cycle (Rudd et al., 2008). Successful plant infection by *Z.tritici* always involves an endophytic growth phase, followed by necrotrophic phase marked by sudden appearance of lesions which bear the spore bearing

structures called pycnidia (Orton et al., 2011). Understanding how non-biotrophic pathogens like Septoria interact with resistance genes which provide durable resistance against pathogens which are obligate biotrophs is thus extremely important to design breeding strategies for introducing resistance genes in areas where there is pressure from more than one disease. There is a risk of a negative impact by increasing resistance of crops to one class of pathogens while exposing them to increased risk of damage by another class which might be difficult and not cost-effective to counter with fungicides and other disease management tools.

This chapter reports a phenotypic analysis of effects of *Lr34* and *Lr46* on STB on both seedlings and adult plants. The tests show that these genes enhance susceptibility to STB in spring wheat in seedling and adult plant tests in controlled environments. This suggests that there is a trade-off between resistance to biotrophs like rust and mildew and susceptibility to necrotrophs like Septoria. The data emphasizes the need to evaluate risks associated with plant breeding strategies solely focussed on combatting one disease but neglecting the effect on other diseases which might be prevalent in an area. Field trials were conducted in UK and Mexico to assess the effect of *Lr34* in different

environments on different varietal backgrounds. However, the results were inconclusive because of heavy rust infection at trial sites in both UK and Mexico.

## 3.2. Materials and methods

### 3.2.1. Septoria disease assessment

Wheat seedlings of LB, Jupateco, and Avocet NILs and mutants were grown for seedling pathology tests as described in sec 2.1. Ten seeds per line per Septoria isolate were sown in P60 pots in peat and sand mix. A minimum of three replicates was set up for each test. Inoculum preparation and inoculations were carried out as described in sec 2.2. Observations of STB symptoms (leaf area covered with pycnidia) were taken as soon as pycnidia were visible and disease percentages were converted to AUDPC as described in Sec 2.2.1.4. AUDPC scores were analysed using generalized linear modelling using Genstat 16.

For adult plant pathology tests, seeds of LB NILs and mutants were germinated and then potted in 1 litre pots (Sec 2.1). The plants were placed in a completely randomized block design created using EDGAR (Experimental Design Generator And Randomiser, James K.M. Brown, John Innes Centre, UK). A minimum of 10 plants were grown per line

per isolate in the polytunnels. *Z. tritici* isolates IPO323 and IPO88004 were used for adult plant tests and inoculum was prepared as described in section 2.2. The inoculations were carried out when the flag leaves were fully expanded using a manual pump-pressure knapsack sprayer at a spore density of $3.33x10^{12}$ spores/ha. The inoculations were carried out in evenings so that moisture was retained on leaves for a longer duration, thus promoting infection. Plants were watered with overhead irrigation system to maintain humidity. Mildew infection was controlled by preventative spray with Talius at 0.25 L/ha. If mildew appeared after the spray with Talius, the plants were treated with Cyflamid at 0.5 L/ha.

### 3.2.2. *Z.tritici* DNA quantification

For *Z.tritici* DNA quantification, three infected leaf samples per replicate per isolate-line combination were collected for DNA isolation (sec 2.3). Fungal DNA quantification was carried out as described in sec 2.4. Primer sequences (Fraaije et al., 2005) used were as follows:

| Primer | Sequence |
|---|---|
| **Stbf1 (forward)** | 5' ACATTAACATGAACAATCGGTACTATAATACTAG 3' |
| **Stbr2 (reverse)** | 5' GGATTTCCTGAACCCGCTG 3' |
| **5'CY5/3'BHQ2 labelled probe** | 5' AATGCAGCTAACACAAACGGTAAAACGA 3' |

**Table 3.2.1 Primers for qPCR reaction to quantify *Z.tritici* DNA in wheat lines infected with different isolates**

### 3.2.3. Arina X Forno field trials

A population of 200 F5 recombinant inbred lines (RIL) derived by single-seed descent from a cross between Arina and Forno, two Swiss winter wheat cultivars which were segregating for *Lr34* was used to evaluate responses of Septoria in the fields (Schnurbusch et al., 2004). Trials were sown at 3 sites (2 at Church Farm, Bawburgh and 1 at Morley) in Norfolk, UK in an alpha-lattice design with two replicates per site in six rows per plot. Church Farm is in an area of glacio-fluvial deposits which extends about 10-12 miles south of Norwich. North end trial was on medium soil, whilst the south end trial was on a much lighter sandy soil. Morley had much more uniform heavier soil. Fungicides, Comet 200 (pyraclostrobin) and Vegas (cyflufenamid), were applied at rates of 1.25L/ha and 0.5L/ha respectively to control rusts and mildew although control of brown rust was not successful. The trials were naturally infected and leaf area covered with pycnidia was scored by visually scoring the diseased plot area as a percentage by me, Laetitia Chartrain and James K.M. Brown. Analysis of data was done by general linear mixed modelling using Genstat 16.

A QTL analysis was performed to identify any disease-responsive locus in the Arina X Forno population using MapQTL® 5 using the

MQM mapping algorithm (Ooijen, 2004). A high density linkage map of Arina X Forno population was used for the analysis provided by group of Prof. Beat Keller, University of Zurich, Switzerland (Appendix 1) (Paillard et al., 2003). Cofactors for mapping algorithm were chosen by the Automatic co-factor tool and a Permutation test to determine significant LOD score value was carried out using default settings.

### 3.2.4. Septoria trial at CIMMYT

NILs and mutant lines for *Lr34* in backgrounds LalBahadur, Jupateco, Thatcher, HUW234, and Yr70 along with the parent lines were evaluated for Septoria responses at CIMMYT's Septoria nursery in Toluca in Mexico. Trials were sown in a randomized block with four replicates in $1m^2$ plots. Toluca had high Septoria disease pressure and the trial was exposed to high natural infection by *Z. tritici*. Septoria disease percent scores were taken weekly for four consecutive weeks and converted to AUDPC scores and analysed by Generalized Linear modelling using Genstat 16. The site is also prone to high levels of yellow rust. No fungicide treatments for rust control were given.

## 3.3. Results

### 3.3.1. Effects of *Lr34* and *Lr46* on Septoria in wheat seedlings

AUDPC values were subjected to a regression analysis using generalized linear modelling using a logit link function, which assumes a binomial distribution of statistical errors as AUDPC values were derived from percentage scores. The AUDPC scores were analyzed as a percentage of maximum possible AUDPC to study the effect of experiment, *Z. tritici* isolate, plant line and their interactions on disease progress. The model used was Exp*Isolate*Line where Exp denotes the Experiment or replicate, Isolate is *Z. tritici* isolate used and Line is the wheat line.

| Factor | d.f. | Mean Deviance | deviance ratio | approx F pr. |
|---|---|---|---|---|
| **Exp** | 2 | 12592.29 | 193.8 | <.001 |
| **Isolate** | 2 | 4540.38 | 69.88 | <.001 |
| **Line** | 17 | 6355.68 | 97.81 | <.001 |
| **Exp.Isolate** | 4 | 3914.84 | 60.25 | <.001 |
| **Exp.Line** | 34 | 767.3 | 11.81 | <.001 |
| **Isolate.Line** | 34 | 3029.53 | 46.62 | <.001 |
| **Exp.Isolate.Line** | 68 | 387.04 | 5.96 | <.001 |
| **Residual** | 1421 | 64.98 | | |

**Table 3.3.1 Accumulated analysis of deviance of AUDPC scores for pathology test on seedlings infected with *Z. tritici* isolates. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

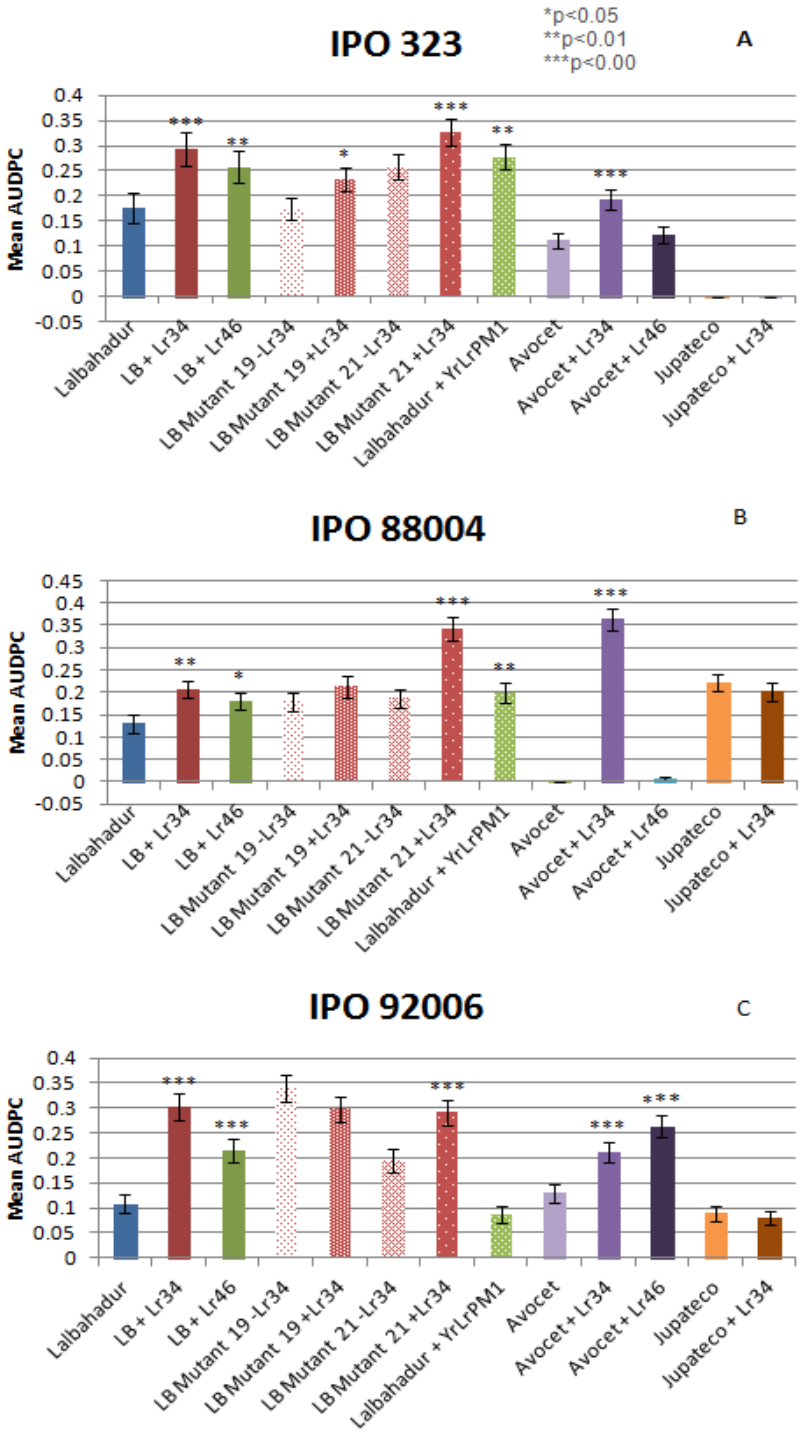



**Figure 3.3.1 Disease levels measured as AUDPC on wheat seedlings infected by *Z. tritici* isolates [A] IPO323, [B] IPO88004, [C] IPO92006. Bars indicate ±1 SE. Data were recorded over 3 experiments with 10 biological replicates per experiment. *p<.05, **p<.01, ***p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34*.**

There was a significant effect of Experiment ($P<0.001$) with the variable nature of Septoria infection highlighted by large differences between experiments in the average amount of disease (Table 3.3.1). There was a significant difference between the behaviour of isolates as well. IPO323 and IPO88004 were not virulent on Jupateco and Avocet respectively (Fig 3.3.1). The isolates were different in their levels of aggressiveness as well in susceptible reactions. The interactions involving Exp were statistically significant but the effects of Exp on the Line and the Line.Isolate interaction were small compared to the Line and Isolate.Line terms themselves, therefore the results are compared across the three experimental replicates (See deviance ratio Table 3.3.1).

In most genotypes and with most isolates, *Lr34* and *Lr46* genes increased susceptibility to Septoria. In Fig 3.3.1, LB NILs with *Lr34* and *Lr46* were significantly more susceptible to all the three Septoria isolates used for the experiment than LB. The Avocet NIL with *Lr34* also had significantly higher AUDPC scores than the parent but the *Lr46* NIL was only more susceptible to IPO92006 (Fig 3.3.1C), not to IPO323 and IPO88004 (Fig 3.3.1A, B). In particular, the presence of *Lr34* in Avocet greatly increased its susceptibility to isolate IPO88004.

In Mutant pair 21, the mutant line without *Lr34* had lower disease levels than the mutant line with *Lr34*. However, the line with *Lr34* in Mutant pair 19 was significantly more susceptible to only IPO323 than the line without *Lr34* (Fig 3.3.1A). LB+*YrLrPm1* which has *Lr46* from an unidentified source also had higher disease than LB for isolates IPO323 and IPO88004 but not IPO92006 (Fig 3.3.1C). There were no significant differences between Jupateco and its *Lr34* NIL.

### 3.3.2. Fungal DNA quantification

Relative fungal DNA values were used for data analysis. Parent lines LB, Avocet and Jupateco were used as references for respective NILs and mutants. Data were analysed by generalized linear modelling using Log-Linear modelling function with Exp*Isolate*Line model.

| Factor | d.f. | Mean Deviance | deviance ratio | Approx F pr. |
|---|---|---|---|---|
| **Exp** | 2 | 0.24515 | 5.51 | 0.008 |
| **Isolate** | 2 | 1760.904 | 39580.64 | <.001 |
| **Line** | 12 | 740.7908 | 16651.09 | <.001 |
| **Exp.Isolate** | 4 | 0.12795 | 2.88 | 0.04 |
| **Exp.Line** | 24 | 0.02555 | 0.57 | 0.9 |
| **Isolate.Line** | 24 | 29.93414 | 672.84 | <.001 |
| **Exp.Isolate.Line** | 48 | 0.01082 | 0.24 | 1 |
| **Residual** | 39 | 0.04449 | | |

**Table 3.3.2 Accumulated analysis of deviance for regression analysis of relative *Z.tritici* DNA levels in wheat seedlings. Generalized linear modelling of relative DNA levels using a log-linear function.**

There were large differences between experiments in the relative amount of *Z. tritici* DNA (Exp term) and interactions involving Exp

were statistically significant ($P<0.01$, Table 3.3.2). However, the effects of Exp on the Isolate and the Isolate.Line interaction were small compared to the Isolate and Isolate.Line terms themselves when comparing deviance ratios for Isolate with Exp.Line, or Isolate.Line with Exp.Isolate.Line. Therefore, the Lines are compared across the replicates. The differences in the relative DNA levels in different NILs and mutants were significant ($P<.001$) as highlighted in Table 3.3.2.

*Lr34* and *Lr46* NILs had higher *Z.tritici* DNA levels compared to LB for all Septoria isolates used (Fig 3.3.2). Mutants 19 and 21 with *Lr34* also had higher *Z.tritici* DNA compared to paired mutants without *Lr34* except for isolate IPO88004 (Fig 3.3.2B) where no significant differences between lines of Mutant pair 19 were seen. LB+*YrLrPm1* also had more fungal DNA than LB except for isolate IPO92006 (Fig 3.3.2C). The Avocet NIL with *Lr34* had significantly more *Z.tritici* DNA than Avocet for all isolates especially for isolate IPO88004 where there was a dramatic difference corresponding to the AUDPC scores (Fig 3.3.2B). Avocet *Lr46* NIL showed increased DNA levels only for isolate IPO92006 compared to the parent. Jupateco lines did not exhibit any significant differences between the parent and *Lr34* NIL in fungal DNA levels.

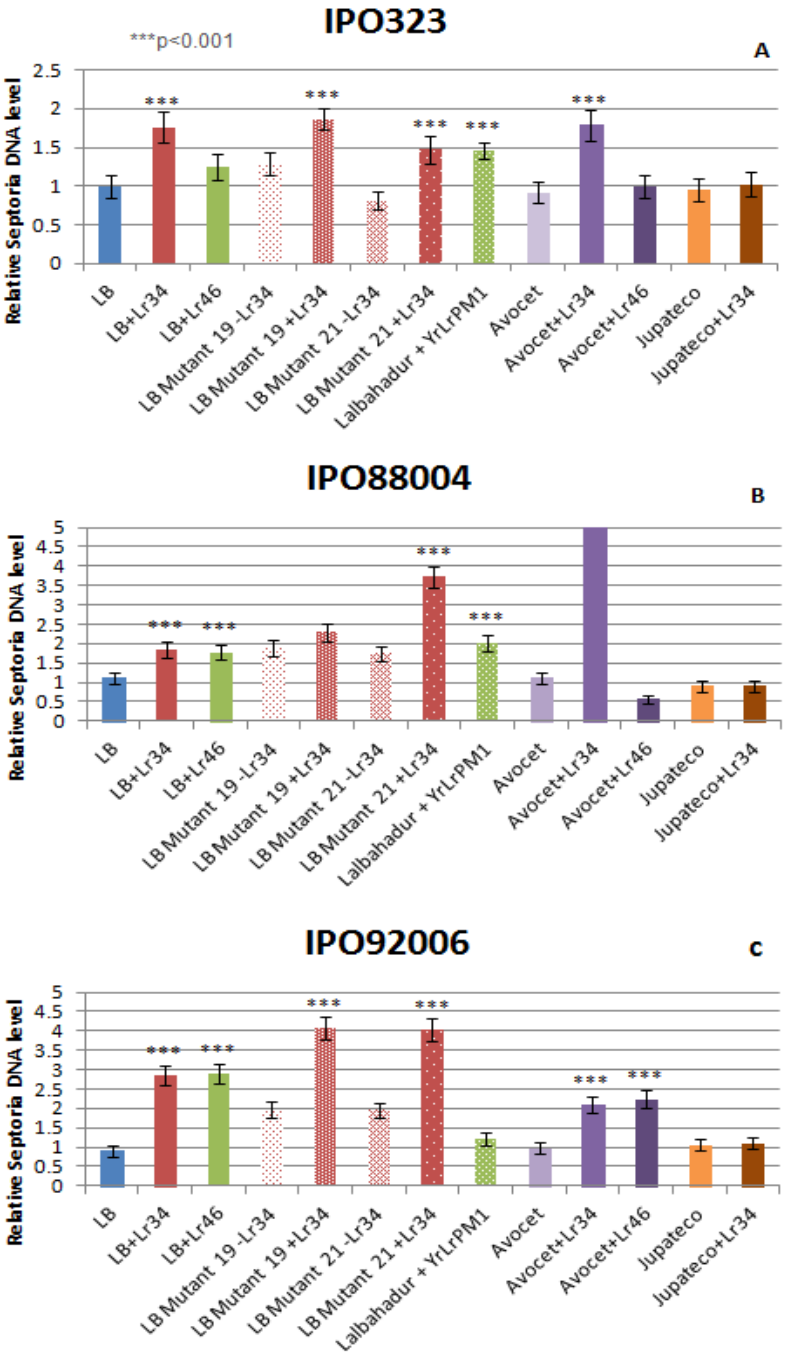



**Figure 3.3.2 Relative *Z.tritici* DNA levels on wheat seedling infected by isolates [A] IPO323, [B] IPO88004, [C] IPO92006. DNA levels in NILs and mutants are relative to DNA levels in their respective parent backgrounds. Bars indicate ±1 SE. DNA was isolated from 3 leaves per experiment for 3 experiments. *p<.05, **p<.01, ***p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34***

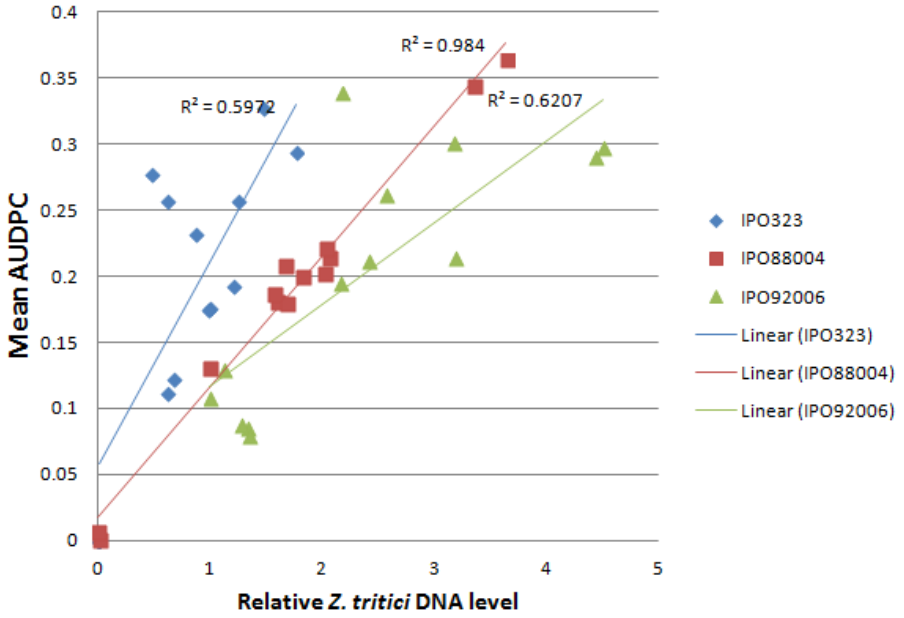



**Figure 3.3.3 Correlation ($r^2$) between relative *Z.tritici* DNA levels and Mean AUDPC scores for wheat seedlings infected with isolates IPO323, IPO88004 and IPO92006**

There was a similar pattern between relative *Z.tritici* DNA levels and mean AUDPC scores for the three isolates on wheat seedlings. Seedlings with higher *Z.tritici* DNA had higher AUDPC scores and vice versa. IPO88004 had strongest correlation ($r^2$=0.98) between *Z.tritici* DNA levels and disease scores (Fig 3.3.3). IPO323 and IPO92006 had weaker correlation values of 0.596 and 0.620 respectively.

### 3.3.3. Effects of *Lr34* and *Lr46* on Septoria in adult plants

Inoculated adult plants were scored for disease percentages over time and regression analysis was carried out using generalized linear modelling based on binomial distribution. The model used was

Exp*Isolate*Line where Exp denotes the experiment or replicate, Isolate is Septoria isolate used and Line is the wheat line.

| Factor | d.f. | Mean deviance | Deviance Ratio | Approx F pr. |
|---|---|---|---|---|
| **Exp** | 1 | 73.07 | 1.21 | 0.3 |
| **Isolate** | 1 | 5622.44 | 92.74 | <.001 |
| **Line** | 7 | 506.84 | 8.36 | <.001 |
| **Exp.Isolate** | 1 | 1.24 | 0.02 | 0.9 |
| **Exp.Line** | 7 | 110.46 | 1.82 | 0.09 |
| **Isolate.Line** | 7 | 71.03 | 1.17 | 0.3 |
| **Exp.Isolate.Line** | 7 | 30.82 | 0.51 | 0.8 |
| **Residual** | 125 | 60.62 | | |

**Table 3.3.3 Accumulated analysis of deviance for regression analysis of AUDPC scores for pathology test on adult plants infected with *Z.tritici* isolates. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

There were no significant differences between the two experiments (Exp term, $P>0.05$) and interactions involving Exp were not statistically significant, therefore the results are presented as an average of two experiments. Though Isolate and Line terms had significant independent effects on the results ($P<0.001$), the interaction between Line and Isolate (Isolate. Line term) was not statistically significant (Table 3.3.3). Adult plants had consistent responses to both isolates used (Fig 3.3.4).

There were significant differences between LB NILs in response to Septoria isolates compared to LB (Fig 3.3.4). Lines with *Lr34* and *Lr46* had higher disease levels of Septoria compared to lines without *Lr*

genes. IPO88004 was more aggressive than IPO323 but both isolates exhibited similar patterns of disease on different NILs and mutants.

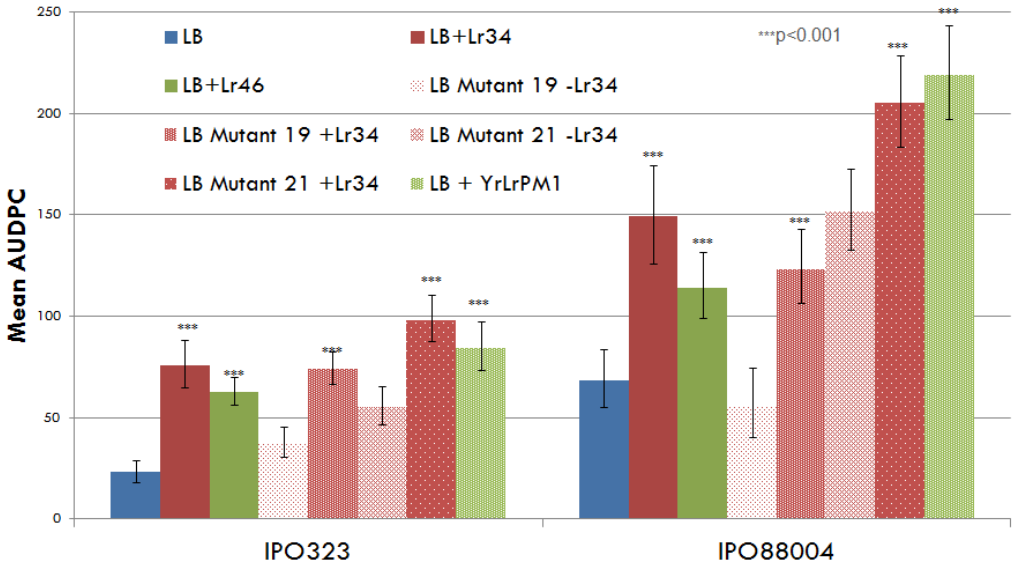



**Figure 3.3.4 Mean AUDPC scores on LB NILs and mutants infected with *Z. tritici* isolates IPO323 and IPO88004. Bars indicate ±1 S.E. Data were collected for 2 independent experiments with 10 biological replicates per experiment. *p<.05, **p<.01, ***p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34*.**

The greater necrosis in flag leaves of *Lr34* and *Lr46* NILs and *YrLrPm1* line than in LB is apparent in Fig 3.3.5. Also the mutant pairs with *Lr34* allele had more symptoms compared to mutants without *Lr34*.

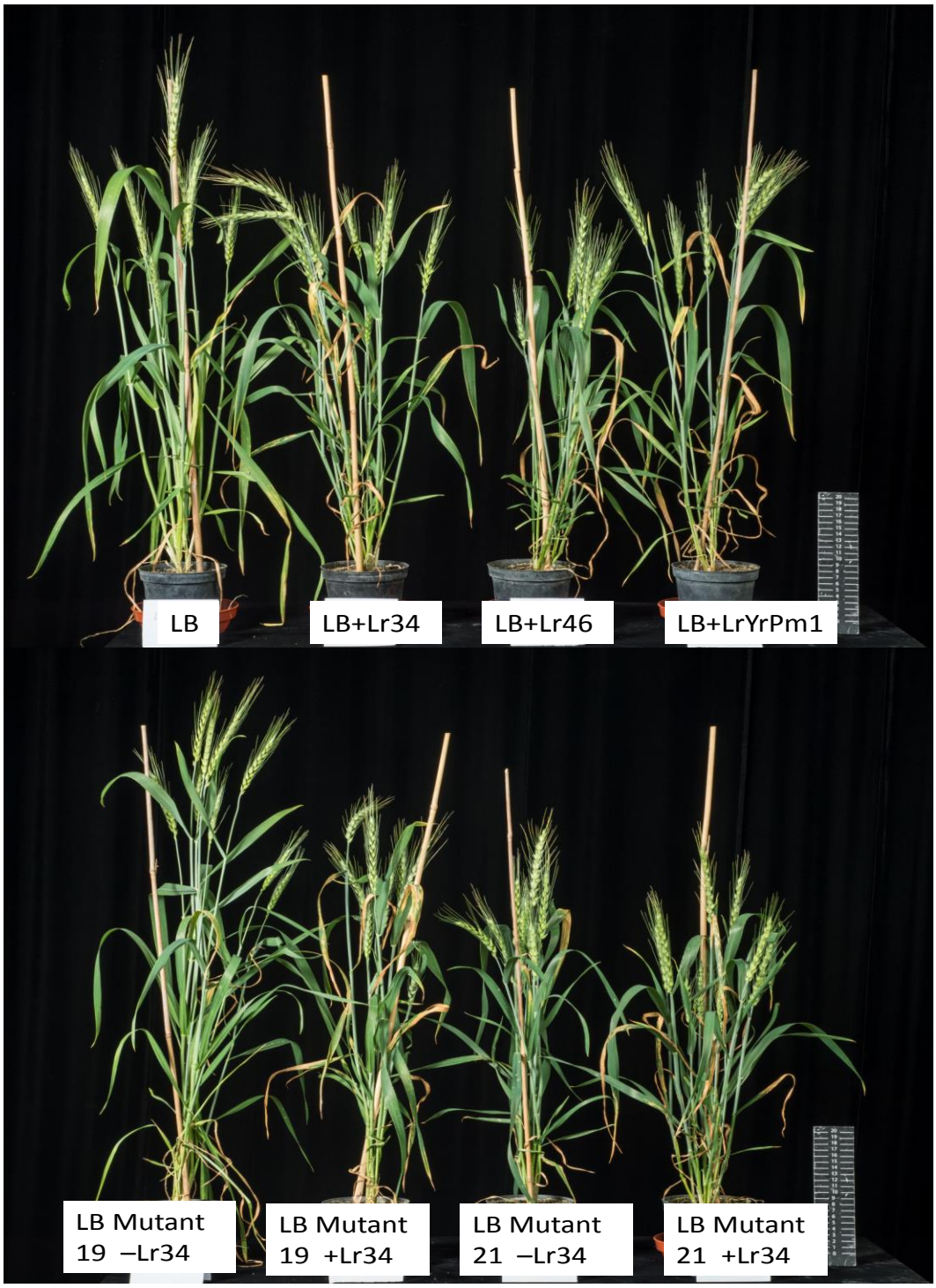



**Figure 3.3.5 Representative infected adult LB NILs and mutants with *Z. septoria* (Isolate IPO323).**

### 3.3.4. Effect of *Lr34* on Septoria in Arina X Forno population

The trials were scored in mid-July 2012 and the range of symptoms varied from 1-90 % infected leaf area. The trial sites were also infected with brown and some yellow rust. Septoria disease percentages for the plots were analyzed using mixed model (REML) analysis. The Random Model was Block.Rep.Rack+Line while the Fixed model was Block/Rep+Scorer+Lr34_allele. Line was included in the random model because the lines included in the study were in effect a random selection from all possible genotypes. Block represents the trial site, Rep represents the replicates within the site and Rack represents the location of the Line in the field. The Scorer term represents the person who scored the Plot and the Lr34 allele term indicates presence or absence of *Lr34* in a line.

| Random term | component | s.e. |
|---|---|---|
| **Block.Rep.Rack** | 0.158 | 0.033 |
| **Line** | 0.44 | 0.048 |

| Fixed term | Wald statistic | n.d.f. | F statistic | d.d.f. | F pr |
|---|---|---|---|---|---|
| **Block** | 31.78 | 2 | 15.89 | 52.6 | <0.001 |
| **Block.Rep** | 16.13 | 3 | 5.38 | 52.5 | 0.003 |
| **Scorer** | 63.71 | 1 | 63.71 | 1391 | <0.001 |
| **Lr34_allele** | 0.52 | 1 | 0.52 | 195.7 | 0.5 |

**Table 3.3.4 REML variance component analysis table for effect of *Lr34* allele on Septoria in a field trial of the Arina x Forno population.**

There was a significant difference between the three trial sites (Block term, $P<0.001$) and a significant effect of the Block.Rep interaction (Table 3.3.4). There was a significant difference between the mean disease scores recorded by different people. More than one person did the scoring because a large number of plots needed to be scored quickly before the rust infection completely masked the Septoria symptoms.There was no significant effect of the *Lr34* allele ($P>0.05$) on mean responses of Lines to Septoria.

QTL analysis of Arina X Forno population revealed no significant QTLs associated with responses to Septoria. The QTL peak with greatest effect was on Chromosome 3B, between markers *cfd000a4* and *gwm0b383*. It had a LOD score of 3.7, less than the minimum significant value of 3.9 calculated by the permutation test, and accounted for 10.2 % of variation in Septoria scores.

### 3.3.5. Field trials to evaluate effects of *Lr34* in different varietal backgrounds in Mexico

The Septoria trials at Toluca were scored in mid-August of 2012. There was high disease pressure in the area. However, the trial sites were heavily infected with yellow rust making it difficult to score some plots for Septoria symptoms. The AUDPC scores for Septoria were analyzed

by generalized linear modelling assuming binomial distribution of the data using the logit link function. The model used was Rep*Line*Lr34 where Rep was the replicate, Line was the genotype and Lr34 indicated presence or absence of *Lr34* allele.

| Factor | d.f. | Mean deviance | Deviance ratio | Approx F pr. |
|---|---|---|---|---|
| **Rep** | 3 | 58.8 | 0.4 | 0.8 |
| **Line** | 5 | 4206.2 | 28.77 | <.001 |
| **Lr34** | 1 | 0.3 | 0 | 1 |
| **Line.Lr34** | 5 | 146.7 | 1 | 0.4 |
| **Residual** | 40 | 146.2 | | |

Table 3.3.5 **Table of accumulated analysis of deviance for regression analysis of AUDPC scores for field study for effect of *Lr34* on Septoria. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

There was no significant effect of the Rep on Septoria trials ($P$>0.05). The Lines were significantly different in their responses to Septoria but there was no significant effect of presence or absence of *Lr34* allele (Table 3.3.5).

There was a slight decrease in susceptibility to Septoria in Jupateco, Tepoca, Thatcher and Yr70 lines with *Lr34* but the effect was not significant (Fig 3.3.6). There was also a non-significant increase in susceptibility to Septoria in HUW234 and Lal Bahadur lines with *Lr34*.

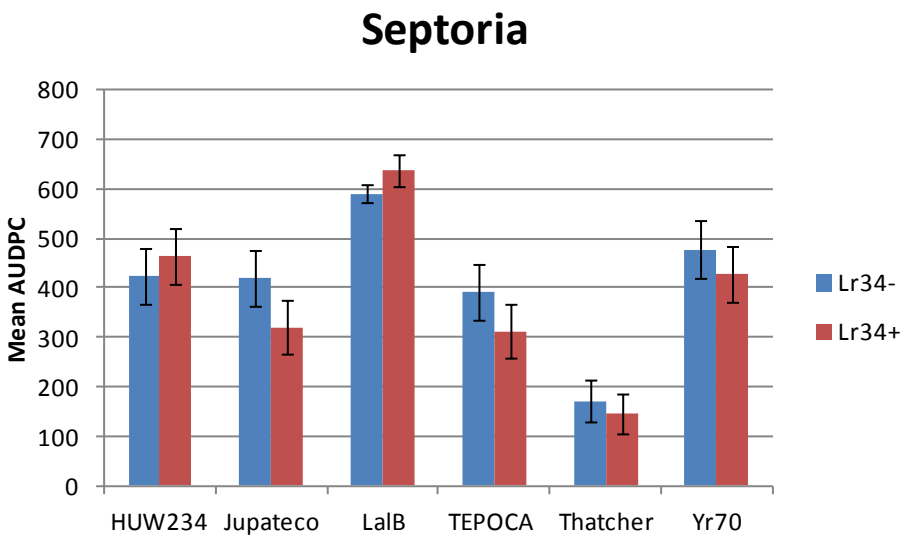



**Figure 3.3.6 Disease levels measured as AUDPC on wheat lines infected by Septoria. Bars indicate ±1 SE. Data was collected over 4 replicates.**

## 3.4. Discussion

The objective of this study was to evaluate the effects of the presence of *Lr34* and *Lr46* genes in wheat lines on STB symptoms. The results indicate that these genes increase susceptibility to virulent Septoria isolates and suppress resistance to non-virulent isolates depending on variety-isolate combinations.

For this study, near-isogenic lines for *Lr34* and *Lr46* in different backgrounds were used. NILs differ mainly in the presence or absence of the target gene and loci surrounding the gene. Depending on the number of backcrosses, pairs of NILs may also differ at chromosome segments not linked to the target locus (Brinkman and Frey, 1977). The

near-identity of NILs means that phenotypical differences (in this study, the disease responses) can be attributed to the gene of interest (Zeven et al., 1983), here *Lr34* or *Lr46*, but the possibility that increased susceptibility to Septoria was caused by other genes present in the LB and Avocet *Lr34* NILs but not the Jupateco NIL cannot be excluded.

The probability that both the LB and Avocet *Lr34* and *Lr46* lines have the same genes enhancing Septoria, not linked to the respective *Lr* gene and absent from the recurrent parents, is low. A stronger possibility is that linkage drag in NILs for *Lr34* and *Lr46* lines might be associated with responses to STB. Kolmer et al. (2008) showed that NILs of *Lr34* (Avocet+*Lr34*, LB+*Lr34* and Jupateco+*Lr34*) contain the *csLV34b* allele of *Lr34* which was first found in Frontana and traced to the Italian variety 'Mentana'. Derivatives of crosses with Frontana like Penjamo 62 are sources of *Lr34* in CIMMYT breeding programs. WW15 and Anza derived from Frontana are donors of *Lr34* in Australian breeding programs. Most probably there was a single source of *Lr34* in plant material used in this study. LB may have obtained it by the CIMMYT route and Avocet by the Australian route.

Nevertheless, two lines of evidence support the hypothesis that the *Lr* genes themselves, rather than linked genes, increase susceptibility to STB. First, both *Lr34* and *Lr46* had similar responses to STB. They have similar phenotypes, with broad-spectrum resistance to biotrophic fungal pathogens and leaf-tip necrosis in adult plants (Lillemo et al., 2012). So it is likely that the effects of these genes are associated with their rust-resistance phenotype. The increase in susceptibility to *Z.tritici* of lines with *Lr46*, which is unlinked to *Lr34*, indicates that two unlinked adult plant resistance genes showed similar effects. It can be predicted that other genes with a similar phenotype, such as *Lr67* (Spielmeyer et al., 2013, Herrera-Foessel et al., 2011) would have a similar effect.

Secondly, in two pairs of LB mutants, the presence of *Lr34* increased STB susceptibility compared to sibling lines lacking *Lr34*. It is very unlikely that those lines would share a mutation in another gene which increased susceptibility to STB. This therefore also suggests that the differences in disease symptoms are more likely to be associated with the *Lr* genes themselves rather than any linked genes on the introgressed chromosome segments.

The CIMMYT bred lines Jupateco+*Lr34* and LB+*Lr34* (Lillemo et al., 2007) exhibited different responses while the Australian bred Avocet+*Lr34* NIL (Lillemo et al., 2007) showed increased susceptibility to *Z. tritici* isolates similar to that of the LB+*Lr34* NIL. The differences between the Jupateco lines on the one hand and the LB and Avocet lines on the other may be related to the genetic background.

*Lr34* and *Lr46* confer partial and durable resistance against the fungal pathogens leaf rust, yellow rust, powdery mildew and stem rust (Lillemo et al., 2008, Singh et al., 2011). Although these genes on their own might not provide enough resistance under high disease pressure areas, they may contribute to achieving acceptable resistance levels in combination with other slow rusting genes (Singh et al., 2011). These genes have been a focal point in breeding for rust resistance around the world using gene pyramiding strategies. Therefore, it is important to appreciate the significance of effects of these genes on Septoria, the occurrence of which coincides with rust in many regions in the world (Boukef et al., 2013, Morgounov et al., 2012).

### 3.4.1. Effect of *Lr34* and *Lr46* on STB in wheat seedlings

Both *Lr34* and *Lr46* lines, with the exception of Jupateco NILs, were consistently more susceptible to Septoria which has a prominent necrotrophic phase (Fig 3.3.1). *Lr46* NILs in LB background had increased susceptibility to all the three isolates but only showed increased symptoms in Avocet with isolate IPO92006. The use of *mlo* mildew resistance alleles in barley breeding has been associated with increased susceptibility to several hemibiotrophic and necrotrophic fungal pathogens (Jarosch et al., 1999, Kumar et al., 2001, McGrann et al., 2014a). It is a possibility that many biotroph resistance genes can increase susceptibility to non-biotrophs, presenting a significant challenge for breeding for broad-spectrum disease resistance (Brown and Rant, 2013).

Relative *Z.tritici* DNA levels followed a similar pattern to AUDPC scores with a strong positive correlation between disease symptoms and fungal DNA levels especially for isolate IPO88004, although IPO323 and IPO92006 showed weaker correlations (Fig 3.3.2, Fig 3.3.3). This reflects the highly variable behaviour of Septoria isolates in terms of fungal establishment and progress of disease. First appearance of symptoms can vary from 14 days to 25 days post

inoculation in a successful compatible reaction (Keon et al., 2007). Different backgrounds also have an effect on behaviour of different isolates. High DNA levels but low AUDPC scores show delay or failure in symptom manifestation which is a common occurrence in pathology tests. McGrann et al. (2014a) showed that *mlo* based powdery mildew resistance is based on *ROR1* and *ROR2* genes. Ramularia leaf spot symptoms were significantly reduced on *mlo-5 ror* double mutants but fungal DNA levels remained as high as in *mlo-5* single mutants which exhibited higher levels of symptoms. These genes regulated the transition of *Ramularia collo-cygni* from endophytic to necrotrophic phase in barley leaves which results in symptoms. The strong correlation between *Z.tritici* fungal DNA levels and STB symptoms in this study suggests that *Lr34* and *Lr46* affect the fungal growth directly which in turn affects the expression of symptoms.

### 3.4.2. Effect of *Lr34* and *Lr46* on STB in adult plants

*Lr34* has been proposed to enhance senescence like mechanisms (Krattinger et al., 2009). LTN associated with *Lr34* and *Lr46* in adult plants can enhance resistance to pathogens with a biotrophic phase and increase susceptibility to pathogens with a necrotrophic phase (Fig

3.3.4) by creating a more favourable feeding environment in the leaf tissue. It is thus important to study this response to other non-biotrophic pathogens of wheat which has been discussed in Chapter 5.

### 3.4.3. Effect of *Lr34* on STB in field conditions

The results of field studies to evaluate effect of *Lr34* on plant responses to Septoria were inconclusive. There is a possibility that this may be due to heavy rust infection at sites in both UK and Mexico. The rust infection led to unreliable plot scores as it was difficult to differentiate between lesions caused by different pathogens on same plots. Rust infection also reduced the leaf area available for *Z. tritici* infection on upper leaves of the plots which might have led to lower scores on otherwise susceptible varieties. Also prior infection of leaves by rust might have suppressed the *Lr34*-mediated susceptibility to Septoria. This can be tested by co-inoculating wheat plants with pathogens for both rust and septoria.

### 3.4.4. Conclusions

The effects of *Lr34* and *Lr46* favouring the necrotrophic pathogen in both the seedling and adult plant stages indicate the potential of these genes to have a negative impact in field conditions, although this needs to be tested rigorously in environments where plants are not severely

affected by other diseases. The trade-offs between resistance to biotrophs and susceptibility to non-biotrophs can have a net detrimental effect in areas where there is pressure from more than one disease. There were significant background interactions of *Z.tritici* isolates with *Lr34* and *Lr46* genes which resulted in either a compatible (susceptible) or non-compatible (resistant) interaction. Jupateco was the only variety with no significant differences between the *Lr34* NIL and the parent irrespective of the isolate used (Fig 3.3.1). Thus there is a possibility to breed for rust resistant lines which mitigate the effect of *Lr34* and *Lr46* on Septoria.

Breeding strategies should consider determining the costs and benefits of introducing varieties with strong resistance for one disease and of controlling other diseases with methods like fungicides. Strategies for optimising resistance to multiple diseases may also need to be developed. Different aspects of a plant's phenotype should be studied alongside resistance to determine true benefits and costs of resistance genes in new cultivars. If resistance to a disease is not commercially viable or the fitness cost or trade-offs with other valuable traits are greater than the benefits, such genes are unlikely to be released in varieties as a result of intense selection within a breeding nursery

(Brown and Rant, 2013). Future research should focus on studying the genotype-by-environment interactions. Septoria is a complex disease which can be dramatically affected by the environmental conditions (Hess and Shaner, 1987). Study of environmental factors like abiotic stress, nutrient and water availability, temperature and soil conditions is essential to fully understand the trade-offs of resistance in field conditions across different environments. The experiments in polytunnels which were comparatively controlled in terms of environmental condition from field trials provided consistent evidence of increased susceptibility to Septoria in *Lr34* and *Lr46* lines (Fig 3.3.4). The effect of rust on Septoria can be tested in similar conditions.

Further studies include study of possible mechanisms of *Lr34* and *Lr46* (Chapter 4) and their effect on other wheat diseases (Chapter 5). Future research should be focussed on implications of these trade-offs on wheat breeding programs and the possibility of mitigating the increased susceptibility to necrotrophs.

# 4. Mechanisms of *Lr34* and *Lr46* mediated disease responses in wheat

## 4.1. Introduction

Pathogen infection results in a number of molecular and physiological changes in plants. Senescence is a form of programmed cell death that is activated in response to physical, developmental, physiological and hormonal stimuli (Buchanan-Wollaston et al., 2003). Microarray studies suggest a significant overlap between pathogen defence and senescence in plants (Bechtold et al., 2013, Obregon et al., 2001, Schenk et al., 2005). Maleck et al. (2000) reported that two senescence-associated genes were represented among the genes of the so-called PR1 regulon which were activated after inoculation with an incompatible isolate of the filamentous pathogen *Peronospora parasitica*. There was a strong induction in expression of transcription factor genes involved in senescence responses in response to pathogen inoculation (Chen et al., 2002, Buchanan-Wollaston et al., 2003).

The colonization by necrotrophic fungus *B. cinerea* is commonly associated with induction of senescence in the host tissue (Swartzberg et al., 2008). It was shown in a recent study that the abscisic acid deficiency in tomato mutants resulted in an anti-senescence defence mechanism for resistance against *B. cinerea* infection while in susceptible wild-type tomato a strong induction of senescence was observed following *B. cinerea* infection (Seifi et al., 2014). Induction of the phytohormones ethylene and jasmonates in biotic interactions also results in accelerated senescence (Nie et al., 2011). An increase in plant- or pathogen-derived cytokinins delays senescence and results in the formation of green islands (Robert-Seilaniantz et al., 2007).

Sugars are important signals in the regulation of plant metabolism and development (Wingler and Roitsch, 2008). Sugars often accumulate during stress and in senescing leaves. Also, both sugar accumulation and stress can induce leaf senescence. There is a rapid induction of sink metabolism during activation of defence responses upon pathogen infection to satisfy the increased demand for carbohydrates as an energy source to sustain the defence-cascade (Heil and Bostock, 2002). Additionally, the pathogen can utilize plant carbohydrate metabolism to derive nutrition. The withdrawal of nutrients by the pathogen will

further increase the demand for assimilates, resulting in the down-regulation of source metabolism (Roitsch and Gonzalez, 2004). Infection by bacterial and fungal pathogens can also influence leaf senescence via modulation of the sugar status, either by directing carbon metabolism or by regulating steady state levels of plant hormones (Wingler and Roitsch, 2008). Many types of biotic interactions involve the source-sink transition and an increased hexose/sucrose ratio. In the tomato–*B. cinerea* interaction, levels of sucrose decrease more strongly than levels of hexoses, leading to an increase in the hexose to sucrose ratio (Berger et al., 2004).

Production of reactive oxygen species (ROS) affects developmental senescence and pathogen response (Bechtold et al., 2005). A link between oxidative stress, pathogen response and senescence was found in ascorbate-deficient Arabidopsis mutant *vitamin c-1* (Conklin and Barth, 2004, Barth et al., 2004). There was an increased expression of pathogenesis-related (PR) proteins and of some Senescence-associated genes (SAGs) which indicated altered pathogen response and accelerated senescence. Transcriptome and proteome analysis of *Z. tritici* revealed that the necrotrophic growth phase of the fungus was accompanied by enhanced host responses involving oxidative stress,

cell-death, increased energy metabolism, defence-signalling and decreased photosynthesis whereas in the biotrophic phase, several defence-related proteins accumulated and several proteins involved in photosynthesis were downregulated (Yang et al., 2013b, Yang et al., 2013a). Genes encoding some ROS scavenging enzymes were highly induced in *Z.tritici* during symptomatic infection stages to probably counter ROS (Keon et al., 2007) as ROS were shown to limit the growth of an endophyte (Tanaka et al., 2004).

This chapter aims to understand the mechanism by which *Lr34* and *Lr46* induce the different responses to biotrophic and non-biotrophic fungal pathogens. The potential of senescence or related processes as regulators of trade-offs between responses to different pathogens was explored. Effect of leaf age at the time of inoculation with a biotroph (mildew) or non-biotroph (Septoria) was studied to deduce the mode of action of the *Lr* genes. ROS-inducers were tested for their ability to induce lesions and senescence on the *Lr34* and *Lr46* NILs to evaluate any difference in stress-response behaviour among the lines. Further studies were carried out to understand *Lr34-Z.tritici* interaction using gene expression analysis and metabolic studies to understand the

specific biological and physiological changes involved in the increased susceptibility of *Lr34* lines to *Z. tritici*.

## 4.2. Materials and methods

### 4.2.1. Infection assays for studying effects of leaf age on Septoria and mildew

A set of 10 seedlings (LB and NILS) were grown for 14, 17, 21 and 25 days in three replicates and 2$^{nd}$ leaves of seedlings of different ages were inoculated simultaneously with *Z. tritici* isolate IPO323 as described in section 2.2.1. Following inoculation, the trays were kept in a fully randomized design. Disease was scored as soon as symptoms started to appear over a period of two weeks and percentage disease scores were converted to AUDPC values which were further analyzed (sec 2.2.1.4). Three infected leaves per line for each age group were collected for DNA isolation for each replicate. *Z. tritici* DNA in collected samples was quantified as described in section 2.4.

For mildew assays, 5-6 seeds of LB and NILs were grown for either 21 or 25 days in three replicates and 2$^{nd}$ leaves of the seedlings were infected with *B. graminis* f.sp. *tritici* isolate JIW48 (sec 2.2.2). 14 and 17 day old leaves were too small to be horizontally affixed for mildew

inoculations. The number of colonies per leaf was counted (sec 2.2.2.3) and further analyzed with Genstat 16.

### 4.2.2. ROS-induced cell death assay

ROS-induced cell death assays were performed on prophyll leaves from 14 day old plants of LB and NILs. Leaves were detached and placed across agar (1% agar w/v supplemented with 100 mg/L benzimidazole strips in clear plastic boxes (Arraiano et al., 2001b). The sensitivity of each line to ROS-inducers was tested by adding 2 µL solution, supplemented with 0.5% v/v Tween20, of the following compounds to the centre of each leaf; 200 mM alloxan (Sigma-Aldrich), 100 mM menadione (Sigma-Aldrich), and 20 µM paraquat (Sigma- Aldrich) (McGrann et al., 2014b). Distilled water with 0.5% v/v Tween20 was used as control. Inoculated leaves were stored under constant light (15-20 µmol $m^{-2}$ $s^{-1}$) for 96 hours at room temperature. After incubation each box was photographed and the lesion size measured using ImageJ. ROS-induced lesions were measured from three independent replicate experiments each consisting of a minimum of five replicate leaves of each line for each ROS inducer.

### 4.2.3. Gene expression studies

Three independent experiments were set up to study expression of genes of interest in Septoria-infected LB and LB-*Lr34* lines. Control samples were inoculated with water with 0.15 % Tween20. Samples were collected at 7, 10, 13, 15 and 17 days post inoculation. Three leaves were pooled together in one sample per line per time point per treatment. RNA extraction and subsequent gene expression analysis was carried out as described in sec 2.6. Two technical replicates per sample were used for each qRT-PCR reaction. Primers used for reference genes and genes of interest are described in Table 4.2.1.

| Gene | Type | Forward primer (5'-3') | Reverse primer (5'-3') | Reference |
|---|---|---|---|---|
| **Ta Elongation factor** | Reference | TGGTGTCATCAAGCCTGGTATGGT | ACTCATGGTGCATCTCAACGGACT | Coram et al. 2008 |
| **Hv GapDH** | Reference | CCTTCCGTGTTCCCACTGTTG | ATGCCCTTGAGGTTTCCCTC | McGrann et al. 2009 |
| **Ta Ubiquitin** | Reference | CCTTCACTTGGTTCTCCGTCT | AACGACCAGGACGACAGACACA | van Riet et al. 2006 |
| **Chlorophyll a/b binding precursor** | GOI | CCTTGGTGAGGCCCGAGTCACTAT | TTGGCAAAGGTCTCGGGGTC | Orton pers. Comm. |
| **TaMAPK3** | GOI | TACATGAGGCACCTGCCGCAGT | GGTTCAACTCCAGGGCTTCGTTG | Rudd et al. 2008 |
| **SAG12 (Cysteine protease)** | GOI | GTTCTCGGACCTCACCAGCGAA | ACGCCCACCAACAACCGCAT | Orton pers. Comm. |
| **Catalase** | GOI | TGCCTGTGTTTTTTATCCGAGA | CTGCTGATTAAGGTGTAGGTGTTGA | Wang et al. 2010 |

**Table 4.2.1 List of primers used for qRT-PCR to measure expression of genes of interest in LB and LB-*Lr34* NILs in response to infection with *Z. tritici***

A modification of the comparative Cq method based on the differences in Cq between target and reference genes was used to normalize gene expression to a calibrator sample which was the sample with the highest Cq value (Pfaffl, 2001). The difference between the samples and a calibrator ΔCq target (Cq sample - Cq calibrator) was calculated and used to determine the relative expression rate (r). Primer efficiencies for the genes of interest were tested for each primer pair using a dilution series from 1:10 to 1:10000 made from a mixture of cDNA samples. Amplification efficiency (E) was calculated from the plot of the Cq values against cDNA input according to the following equation:

$$E = 10^{(-1/slope)}$$

Quantification cycle (Cq) values of three reference genes were checked for stability using the geNORM software (Vandesompele et al., 2002) (http://medgen.ugent.be/~jvdesomp/genorm/). A gene expression normalization factor was calculated for each sample based on the geometric mean of three specified reference genes. The relative expression ratio (Normalized Expression) of a target gene in comparison with the three reference genes was calculated according to the equation:

$r = E_{target}^{-\Delta Cq\ target} / N$

N=Normalization factor calculated by GeNorm for three reference genes.

Mean normalized expression (MNE) was calculated by averaging the r values for technical replicates of one sample. MNE values were subjected to further statistical analysis using Genstat 16 using general linear modelling on log-transformed MNE values which assumed a normal distribution of errors.

### 4.2.4. Metabolite analysis

Metabolite extraction and analysis was carried out as described in sec 2.7. LB and LB-*Lr34* NILs were grown and inoculated with IPO323 and control samples were inoculated with water with 0.15% Tween20 as described in section 2.3. Three independent experiments were set up and three leaves were collected per experiment per line per treatment. Relative abundance of each metabolite detected was calculated as a percentage content of each metabolite out of total metabolite content detected. The relative abundances were subjected to principal component analysis (PCA) using Genstat 16 and JMP® 11. A cluster

analysis of the data was carried out using Minitab 17 statistical software (2010).

## 4.3. Results

### 4.3.1. Effect of leaf age on PM

Leaves of either 21d or 25d old seedlings were inoculated with *B. graminis* f.sp. *tritici* (*Bgt*) isolate JIW48 and the number of mildew colonies was subjected to a regression analysis using generalized linear modelling. A Poisson distribution with a logarithmic link function was used because the data were integer values. The model used was Rep*Age*Line where Rep was the replicate, Age was the age of seedling at time of inoculation (21d or 25d) and Line was LB or its *Lr34* or *Lr46* NIL.

| Factor | d.f. | Mean Deviance | deviance ratio | Approx. F pr. |
|---|---|---|---|---|
| **Rep** | 2 | 4.8785 | 5.03 | 0.03 |
| **Age** | 1 | 256.6569 | 264.49 | <.001 |
| **Line** | 2 | 19.4109 | 20 | <.001 |
| **Rep.Age** | 2 | 2.19 | 2.26 | 0.2 |
| **Rep.Line** | 4 | 0.5851 | 0.6 | 0.7 |
| **Age.Line** | 2 | 5.1189 | 5.28 | 0.09 |
| **Rep.Age.Line** | 3 | 0 | 0 | 1 |
| **Residual** | 11 | 0.9704 | | |

**Table 4.3.1 Accumulated analysis of deviance for effect of age of leaf on *Bgt* infection on LB and NILs. Generalized linear modelling of count of mildew colonies using a Poisson distribution with logarithmic link function.**

Rep, Age and Line were all statistically significant terms ($P<0.05$) but there was no effect of Rep on either Age or Line terms (Table 4.3.1). The interaction between Age and Line was not significant.

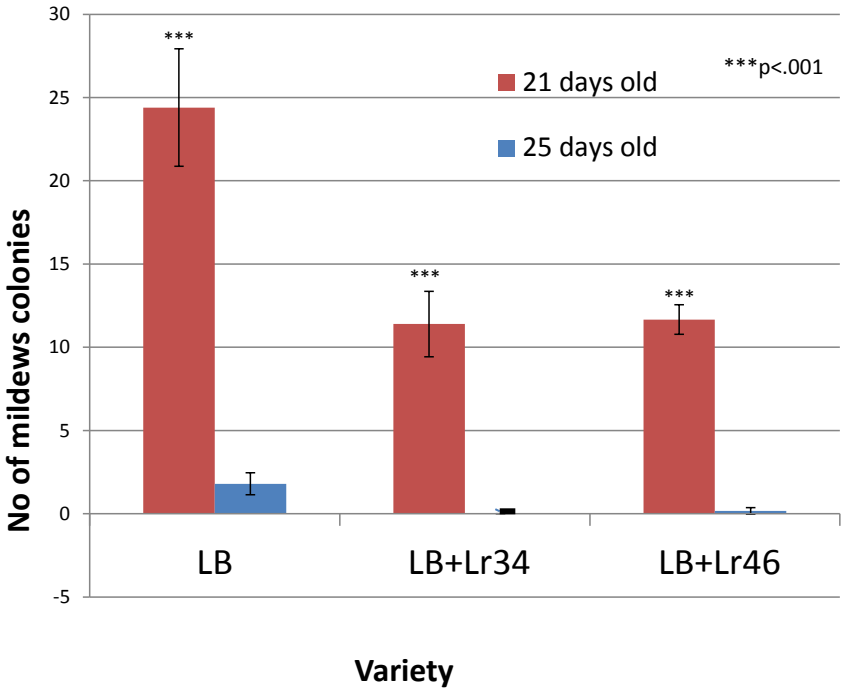



**Figure 4.3.1 Effect of leaf age on susceptibility to *Bgt* on LB and NILs. Data were recorded over 3 replicates with five leaves per replicate per treatment combination, Bars indicate ±1SE, ***p<.001, significant differences between *Lr* NILs and parent LB.**

*Lr34* and *Lr46* NILs were significantly less susceptible to PM infection than LB (Fig 4.3.1). Old seedlings (25d old) were significantly more resistant to PM than younger seedlings (21d old) and old *Lr34* and *Lr46* were almost completely resistant to PM.

### 4.3.2. Effect of leaf age on Septoria infection

Seedlings of LB and *Lr* NILs of different ages (14d, 17d, 21d and 25d) were infected with IPO323 isolate of *Z. tritici* to assess the effect of leaf age on Septoria infection and responses of *Lr34* and *Lr46* NILs. AUDPC scores were analysed using generalized linear modelling assuming binomial distribution of observations with a logit link function. The AUDPC score was analysed as a proportion of the maximum possible AUDPC value of the experiments (1100-1400). The model used was Rep*Age*Line.

| Factor | d.f. | Mean Deviance | deviance ratio | Approx. F pr. |
|---|---|---|---|---|
| **Rep** | 2 | 24.19 | 0.24 | 0.8 |
| **Age** | 3 | 3241.88 | 32.53 | <.001 |
| **Line** | 2 | 130.48 | 1.31 | 0.3 |
| **Rep.Age** | 6 | 270.82 | 2.72 | 0.02 |
| **Rep.line** | 4 | 50.52 | 0.51 | 0.7 |
| **Age.line** | 6 | 5322.57 | 53.41 | <.001 |
| **Rep.Age.line** | 12 | 205.18 | 2.06 | 0.02 |
| **Residual** | 141 | 99.65 | | |

**Table 4.3.2 Accumulated analysis of deviance for effect of age of leaf on Septoria infection on LB and NILs. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

There was no significant effect ($P>0.05$)of Rep and Line but Age was significant ($P<0.001$, Table 4.3.2). The interaction between Rep and Age was significant but had a smaller effect that Age alone (Rep.Age term in Table 4.3.2). The interaction between Age and Line was highly significant, more so than the main effect of Age. Rep affected the

Age.Line interaction but only slightly (Rep.Age.Line term in Table 4.3.2).

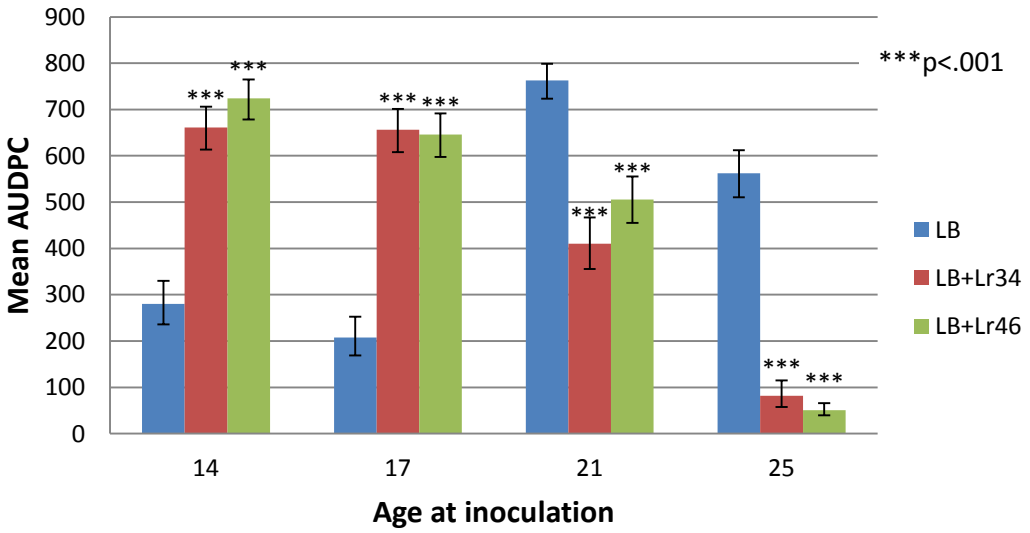



**Figure 4.3.2 Effect of leaf age on susceptibility to *Z.tritici* on LB and NILs measured in terms of mean AUDPC. Bars indicate ±1 SE. Data were recorded over 3 experiments with 10 biological replicates per experiment. ***p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34*.**

*Lr34* and *Lr46* NILs had significantly more disease than LB when inoculated at age of 14d and 17d (Fig 4.3.2). However, LB was more susceptible to Septoria when infected with Septoria at age of 21d or 25d compared to *Lr34* or *Lr46* NILs. This gave rise to the strong Age.Line effect in Table 4.3.2.

Further, *Z. tritici* DNA levels were measured using qPCR at the end of disease scoring. DNA levels were analysed using general linear

modelling assuming a normal distribution of log transformed-DNA level values. The model used was Rep*Age*Line.

Rep, Line and Age were significant terms as were the interactions ($P$<0.001, Table 4.3.3). There were very strong interactions between Rep and Age and Age and Line. Although Rep.Line.Age is significant, the effect was smaller than Age.Line (see variance ratios, Table 4.3.3).

| Factor | d.f. | m.s. | v.r. | F pr. |
|---|---|---|---|---|
| **Rep** | 2 | 1.05152 | 79.19 | <.001 |
| **Age** | 3 | 2.20931 | 166.38 | <.001 |
| **Line** | 2 | 0.41088 | 30.94 | <.001 |
| **Rep.Age** | 6 | 1.19845 | 90.26 | <.001 |
| **Rep.Line** | 4 | 0.16706 | 12.58 | <.001 |
| **Age.Line** | 6 | 0.81339 | 61.26 | <.001 |
| **Rep.Age.Line** | 12 | 0.46648 | 35.13 | <.001 |
| **Residual** | 36 | 0.01328 | | |

**Table 4.3.3 Accumulated analysis of deviance of log-transformed *Z. tritici* DNA levels in LB and *Lr* NILs inoculated at different ages with isolate IPO323. General linear modelling of log-transformed DNA levels using a normal distribution.**

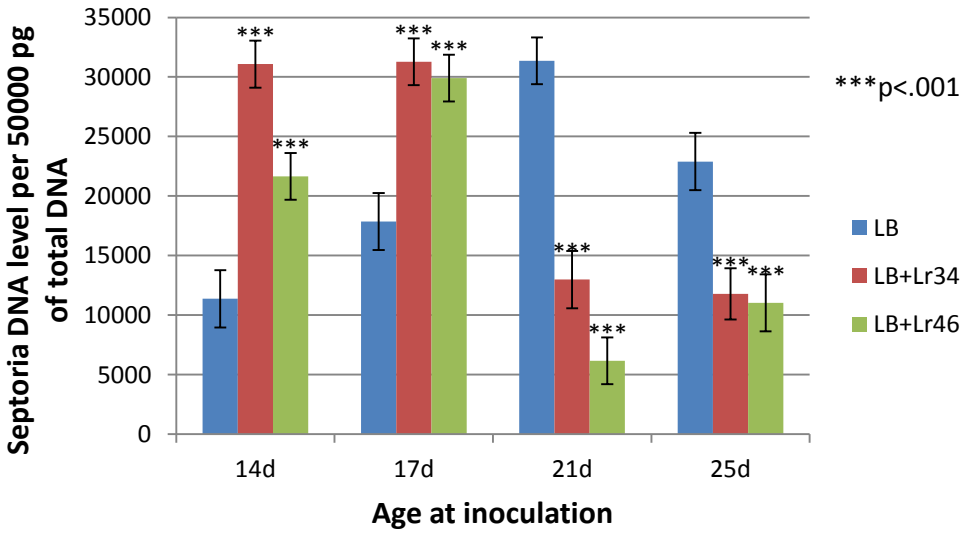



**Figure 4.3.3 *Z. tritici* DNA levels in LB and *Lr* NILs inoculated at different ages with isolate IPO323. Bars indicate ±1 SE. DNA was isolated from 3 leaves per experiment for 3 experiments. ***p<.001, significant differences between *Lr* NILs and parent LB.**

*Lr34* and *Lr46* NILs had significantly higher levels of *Z.tritici* DNA levels compared to LB when inoculated at a younger age (14d and 17d), but lower levels when inoculated at an older age (21d and 25d) (Fig 4.3.3). The DNA levels correspond to the pattern or AUDPC of Septoria infection on LB and *Lr* NIL seedlings infected at different ages (Fig 4.3.2). This led to the large Age.Line effect seen in Table 4.3.3.

### 4.3.3. Effect of ROS inducers on LB and *Lr* NILs

ROS inducers were tested for their effects on LB and NILs for *Lr34* and *Lr46*. Lesion size induced by the chemicals was calculated using ImageJ and subjected to ANOVA assuming a normal distribution of

the observations. The Model used was Rep*Line where Rep was the replicate, and Line was LB or either *Lr34* or *Lr46* NIL. ROS inducers were analysed separately. Methadione did not induce any lesions and was thus excluded from the data analysis.

| Source of variation | d.f. | m.s. | v.r. | F pr. |
|---|---|---|---|---|
| **Rep stratum** | 2 | 4.397 | 3.9 | |
| **Rep.*Units* stratum** | | | | |
| **Line** | 2 | 0.125 | 0.11 | 0.9 |
| **Residual** | 13 | 1.126 | | |

**Table 4.3.4 Analysis of variance for lesion area induced by Paraquat on LB and *Lr34* and *Lr46* NILs**

| Source of variation | d.f. | m.s. | v.r. | F pr. |
|---|---|---|---|---|
| **Rep stratum** | 2 | 1052 | 11.71 | |
| **Rep.*Units* stratum** | | | | |
| **Line** | 2 | 25.6 | 0.28 | 0.7 |
| **Residual** | 13 | 89.88 | | |

**Table 4.3.5 Analysis of variance for lesion area induced by Alloxan on LB and *Lr34* and *Lr46* NILs**

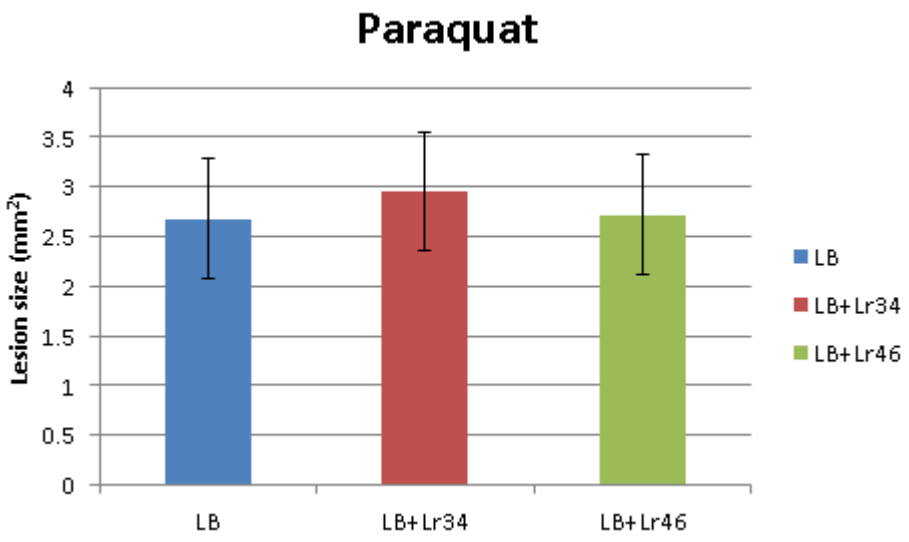



**Figure 4.3.4 Lesion area ($mm^2$) caused by ROS inducer Paraquat on LB and *Lr* NILs (Bars indicate ±1SE). Data were collected for 3 experimental replicates with 5 leaves per replicate.**

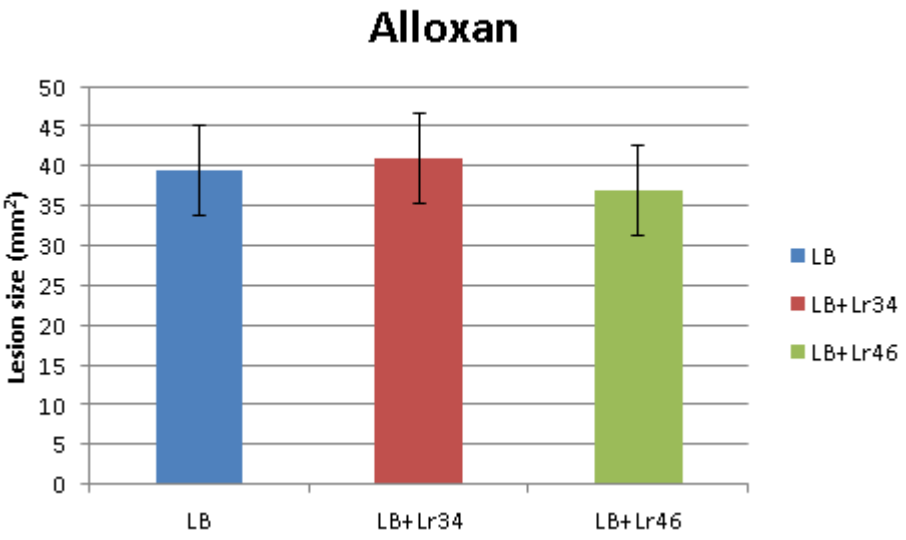



**Figure 4.3.5 Lesion area ($mm^2$) caused by ROS inducer Alloxan on LB and *Lr* NILs (Bars indicate ±1SE). Data were collected for 3 experimental replicates with 5 leaves per replicate.**

There was no significant difference ($P>0.05$) between LB and its NILs in response to either Paraquat or Alloxan (Table 4.3.4, Table 4.3.5). The lesions induced by Alloxan were larger than lesions induced by Paraquat (Fig 4.3.4, Fig 4.3.5).

### 4.3.4. Gene Expression analysis

The gene expression data were analysed by converting the Cq values to mean normalized expression values (MNE) as described in section 4.2.2. Log-transformed MNE values were subjected to a general linear model analysis assuming a normal distribution. The model used was Exp*Time_Point*Pathogen*Line*Gene. Exp term represented the biological replicate, Time_point represented the days post inoculation when samples were analysed, Pathogen represented the treatment

(Mock or IPO323-inoculated), Line represented LB or LB-*Lr34* NIL and Gene represented Gene of interest.

The effects of Exp and Time_Point were not significant ($P>0.05$) but Pathogen ($P<0.001$), Line ($P<0.01$) and Gene ($P<0.001$) had significant effects (Table 4.3.6). There was a significant interaction between Pathogen.Gene but the effect was small compared to individual terms (Variance ratios, Table 4.3.6). The interaction between Gene and Line although significant had a small effect. Exp.Pathogen.Line term also had a small effect. Pathogen.Gene.Line was also a significant interaction but the effect of Pathogen.Gene on Line was small.

| Factor | d.f. | m.s. | v.r. | F pr. |
|---|---|---|---|---|
| **Exp** | 2 | 1.7931 | 2.36 | 0.1 |
| **Time_point** | 4 | 0.3198 | 0.42 | 0.8 |
| **Pathogen** | 1 | 14.8837 | 19.57 | <.001 |
| **Gene** | 3 | 48.7229 | 64.05 | <.001 |
| **Line** | 1 | 5.9239 | 7.79 | 0.006 |
| **Exp.Time_point** | 8 | 0.4561 | 0.6 | 0.8 |
| **Exp.Pathogen** | 2 | 6.2194 | 8.18 | <.001 |
| **Time_point.Pathogen** | 4 | 2.9764 | 3.91 | 0.005 |
| **Exp.Gene** | 6 | 1.9135 | 2.52 | 0.02 |
| **Time_point.Gene** | 12 | 1.7539 | 2.31 | 0.01 |
| **Pathogen.Gene** | 3 | 3.9873 | 5.24 | 0.002 |
| **Exp.Line** | 2 | 1.4487 | 1.9 | 0.2 |
| **Time_point.Line** | 4 | 2.9835 | 3.92 | 0.005 |
| **Pathogen.Line** | 1 | 0.7171 | 0.94 | 0.3 |
| **Gene.Line** | 3 | 2.6042 | 3.42 | 0.02 |
| **Exp.Time_point.Gene** | 24 | 1.4861 | 1.95 | 0.009 |
| **Exp.Pathogen.Gene** | 6 | 4.9718 | 6.54 | <.001 |
| **Exp.Pathogen.Line** | 2 | 2.7031 | 3.55 | 0.03 |
| **Time_point.Pathogen.Line** | 4 | 4.1381 | 5.44 | <.001 |
| **Pathogen.Gene.Line** | 3 | 4.2426 | 5.58 | 0.001 |
| **Residual** | 144 | 0.7607 | | |

**Table 4.3.6 Accumulated analysis of variance for log-transformed mean normalized expression of wheat genes in response to *Z. tritici* infection in LB and LB-*Lr34* NILs. Higher-order terms not shown in this table were not significant (*P*>0.05) and were therefore removed from the analysis.**

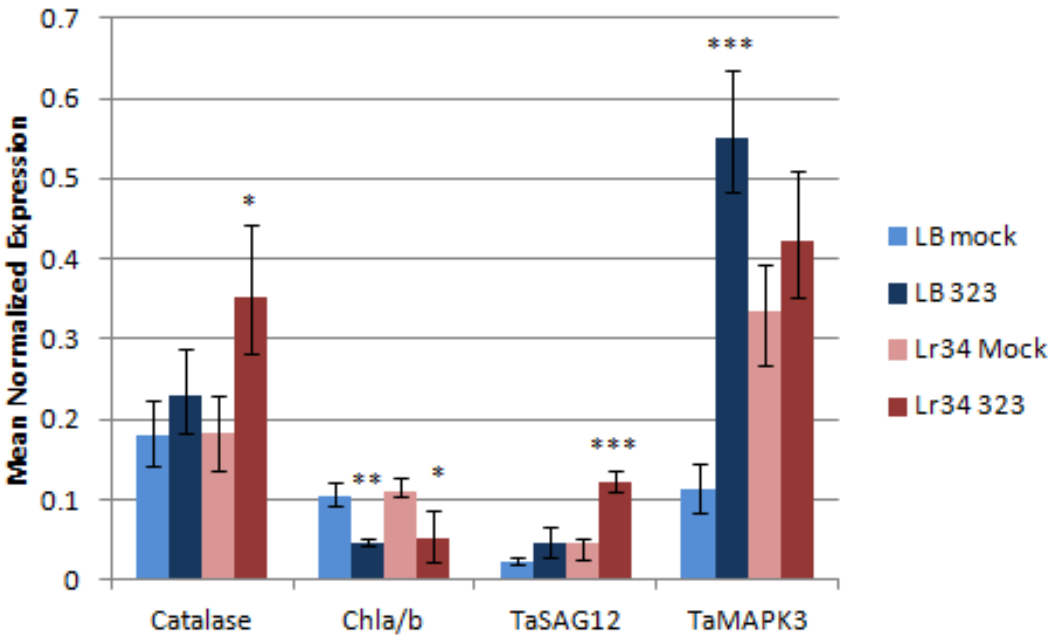



**Figure 4.3.6 Average of mean normalized expression values of *Catalase*, *Chla/b*, *TaSAG12* and *TaMAPK3* across different time points in Mock and IPO323 infected LB and LB-*Lr34* NIL. Bars indicate ±1SE, *p<.05, **p<.01,***p<.001, significant differences between Mock and IPO323 infected line.**

There was an overall significant induction of *Catalase* and *TaSAG12* expression in the IPO323-infected *Lr34* NIL compared to mock-*Lr34* (Fig 4.3.6). There was also a significant decrease in *Chla/b* transcript levels in infected samples for both lines. There were higher transcript levels of *TaMAPK3* in infected LB leaves compared to non-infected samples. There was no significant difference between mock-*Lr34* and IPO323-infected *Lr34* leaves. There was a large significant difference in expression of *TaMAPK3* between mock LB and mock *Lr34*.

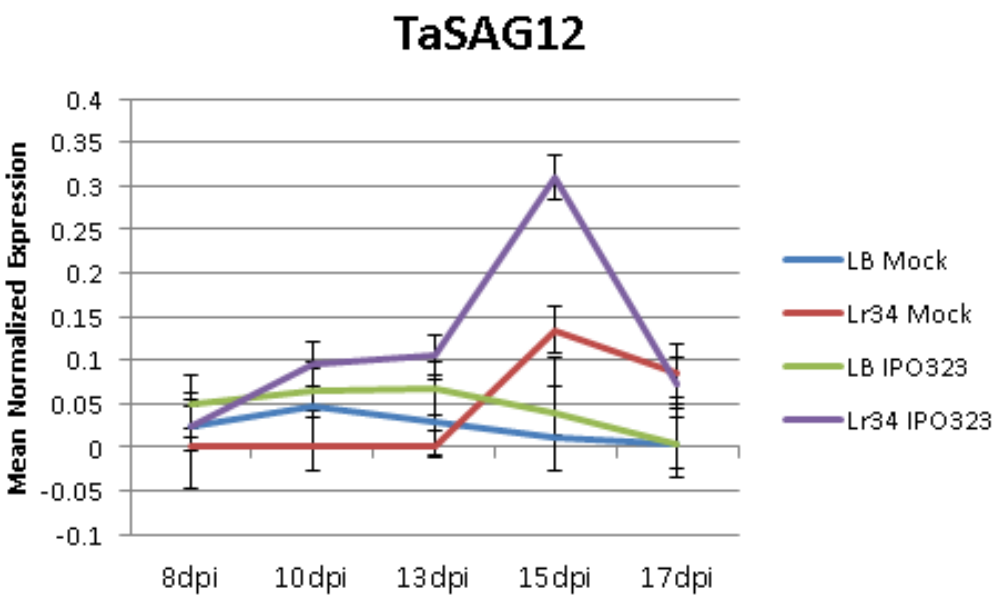



**Figure 4.3.7 Mean normalized expression of *SAG12* gene in Mock and IPO323 infected LB and LB+*Lr34* wheat seedlings (dpi- days post inoculation). Data for 3 independent biological replicates with 2 technical replicates within each experiment, bars indicate ±1 S.E.**

IPO323-infected samples had generally higher expression of *SAG12* compared to mock-infected samples but the differences were not statistically significant except for 15 dpi samples for the *Lr34* mock and infected pair (Fig 4.3.7). *Lr34*-IPO323 15dpi and 17dpi had higher expression of *SAG12* compared to respective LB-IPO323 samples but only differences between 15dpi samples were significant indicating an earlier onset of senescence like processes in infected *Lr34* leaves compared to LB.

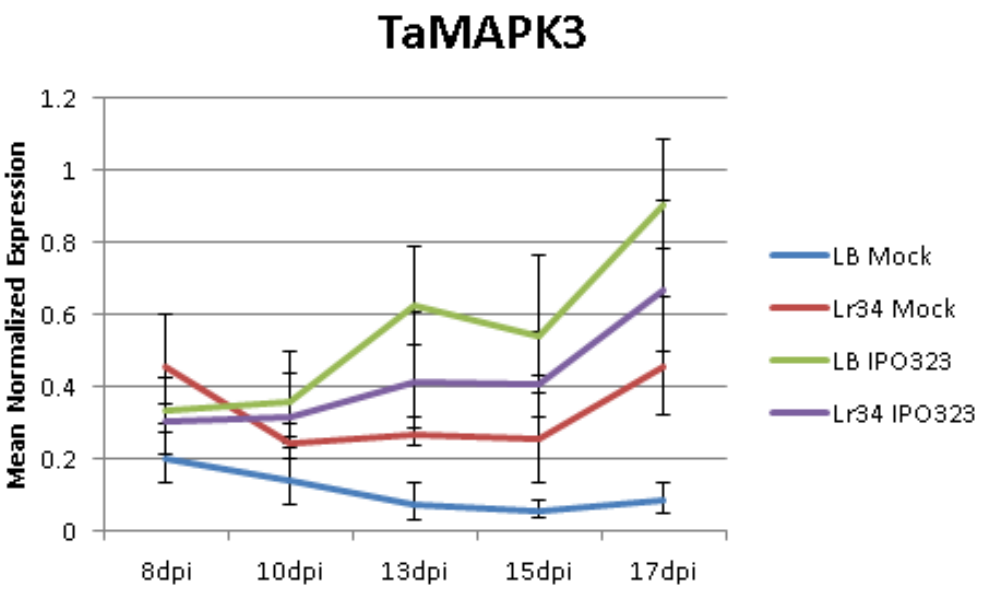



**Figure 4.3.8 Mean normalized expression of *TaMAPK3* gene in Mock and IPO323 infected LB and LB+*Lr34* wheat seedlings (dpi- days post inoculation). Data for 3 independent biological replicates with 2 technical replicates within each experiment, bars indicate ±1 S.E**

*TaMAPK3* expression was higher in IPO323-infected samples compared to Mock-infected samples and this difference was statistically significant for LB-IPO323 samples for 13, 15 and 17dpi (Fig 4.3.8). Mock-infected *Lr34* leaves had higher *TaMAPK3* levels than mock-infected LB leaves at 13, 15 and 17dpi.

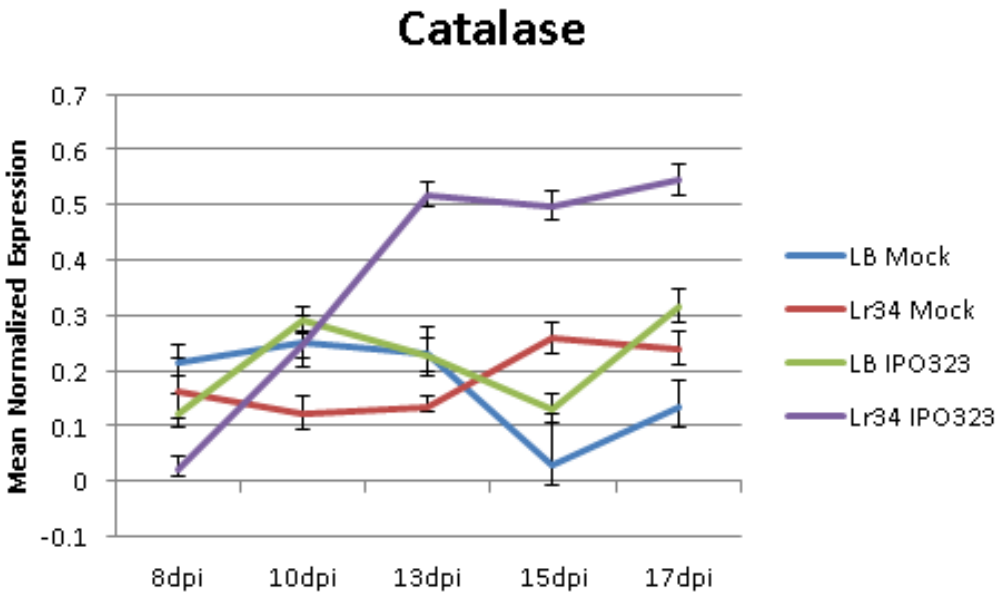



**Figure 4.3.9 Mean normalized expression of *Catalase* gene in Mock and IPO323 infected LB and LB+*Lr34* wheat seedlings (dpi- days post inoculation). Data for 3 independent biological replicates with 2 technical replicates within each experiment, bars indicate ±1 S.E.**

Lower expression of *catalase* was seen in IPO323-infected LB samples than in mock-infected samples collected after 8 and 13dpi but the difference was not statistically significant (Fig 4.3.9). At 17dpi there was a significant increase in *catalase* expression in IPO323-infected LB compared to mock-infected LB. IPO323-infected *Lr34* leaves had significantly higher *catalase* expression in 13, 15 and 17dpi samples but lower expression at 8dpi compared to mock-infected *Lr34*.

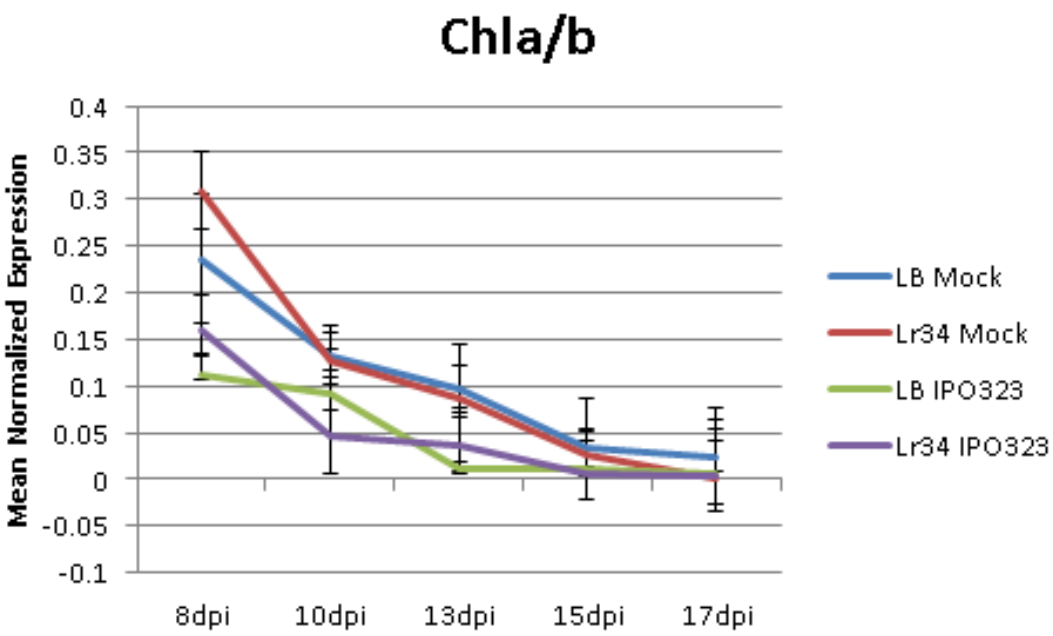



**Figure 4.3.10 Mean normalized expression of *Chl a/b* gene in Mock and IPO323 infected LB and LB+*Lr34* wheat seedlings (dpi- days post inoculation). Data for 3 independent biological replicates with 2 technical replicates within each experiment, bars indicate ±1 S.E, *p<.05, **p<.01, ***P<.001)**

*Chl a/b* transcript levels decreased over time in four treatment classes but IPO323-infected samples had a steeper decline compared to mock-infected counterparts (Fig 4.3.10). The differences between IPO323-infected and mock-infected samples were significant for LB at 8, 10, and 13 dpi and for *Lr34* at 8 and 10dpi only. There were no significant differences between LB and *Lr34* samples.

### 4.3.5. Analysis of metabolite relative abundance in LB and *Lr34* NILs in response to *Z. tritici* infection

Samples collected at 7, 10 and 14 dpi were subjected to GC-MS analysis as described in section 2.x. More than 500 peaks were detected out of which only 44 were significantly abundant (amount in samples was above the minimum threshold required for identification) after analysis of raw data by GC-MSD ChemStation Software (Agilent).

#### 4.3.5.1. Principal component analysis

The relative abundance values for metabolites obtained from ChemStation analysis were subjected to a principal component analysis using Genstat and the top 3 PC scores were selected for further analysis based on a scree plot which graphically depicts the variance (Eigenvalues) explained by principal components against the rank order of the size of the respective Eigenvalues, from largest to smallest (Fig 4.3.11).

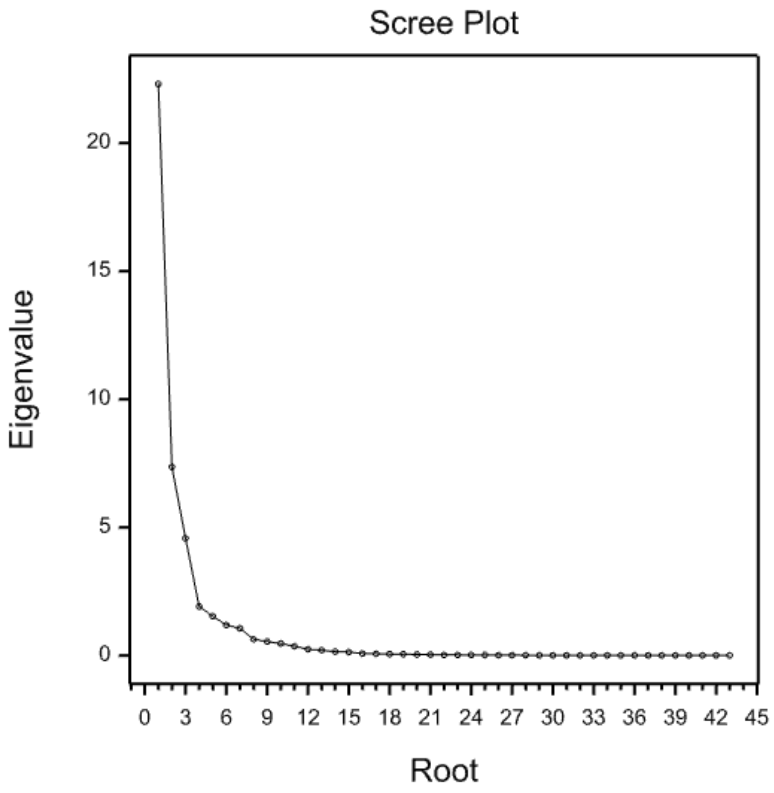



**Figure 4.3.11 Scree plot of Eigenvalues of principal components in descending order of magnitude of the correlation matrix generated from Principal Component analysis for relative abundance of metabolites in mock and IPO323-infected LB and *Lr34* samples**

The first three PC components explained 51.85%, 17.09% and 10.63% variation in the data respectively. A scatterplot matrix of the 3 PC score values was generated using JMP® 11.2.0 (Fig 4.3.12). PC1 divided the samples into three different age groups where 7dpi was most positive and14dpi was most negative. PC2 also explained some variation by age and 10dpi (positive) was separated from 7dpi and 14dpi (more negative) on PC2. PC3 explained the variation due to

Septoria infection and genotype. PC3 separated LB-*Lr34* (most positive) and with LB (most negative) for 7dpi and 10dpi samples. It also separated mock-infected (negative) and IPO323-infected samples (positive) for 14dpi samples irrespective of genotype.

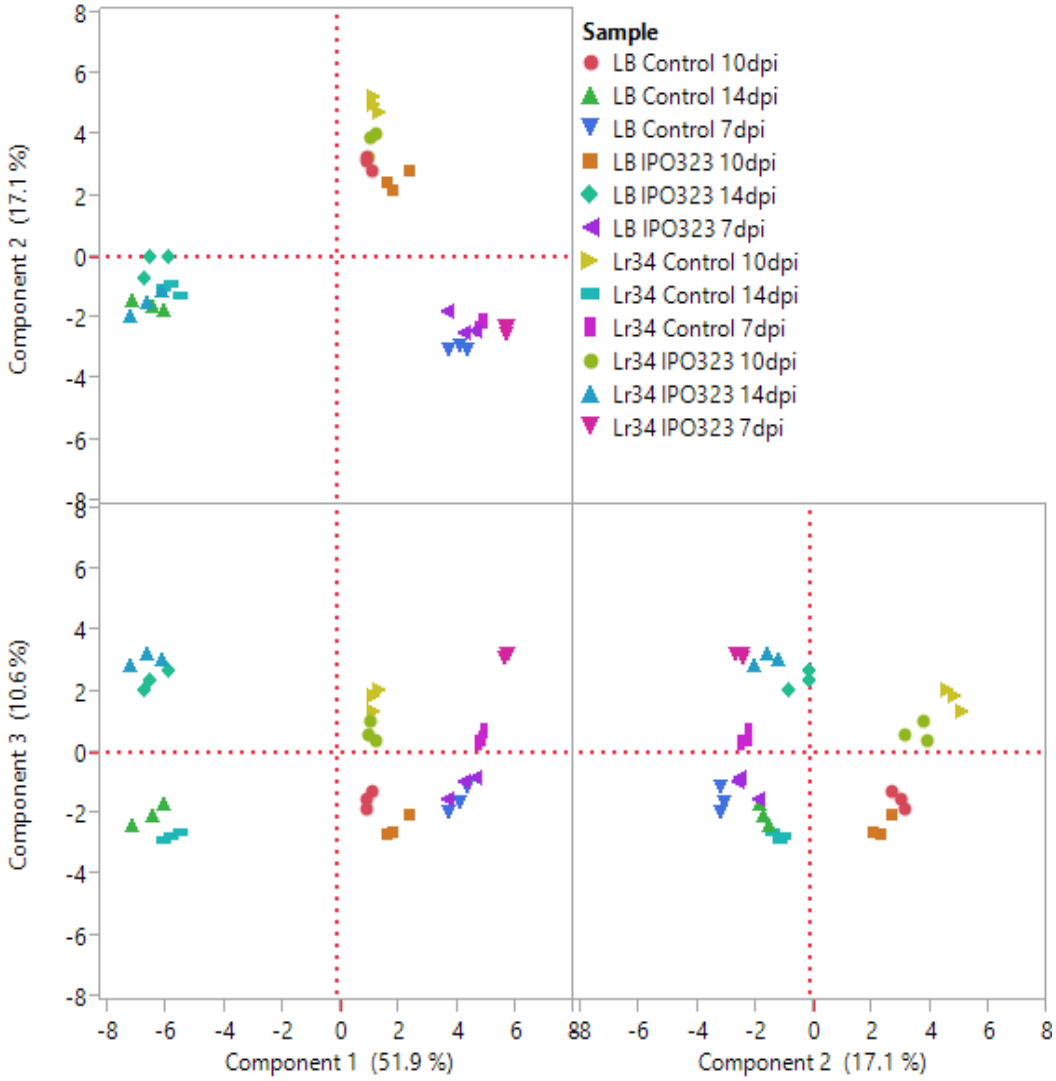


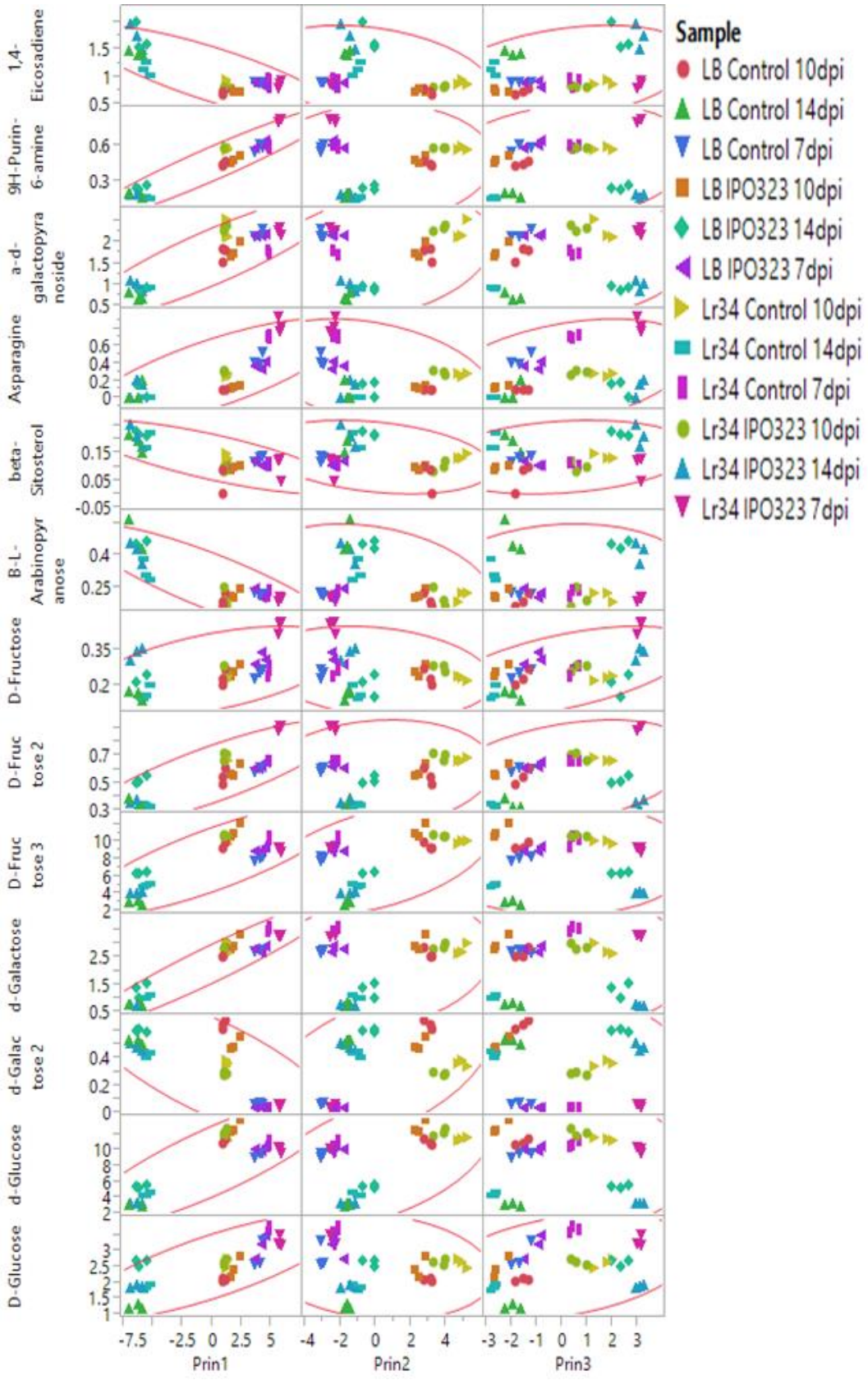

**Figure 4.3.12 Score plot of PC1, PC2 and PC3 identified after PC analysis of relative abundance data of metabolites identified after GC-MS analysis of uninfected and IPO323-infected LB and LB-*Lr34* NILs.**

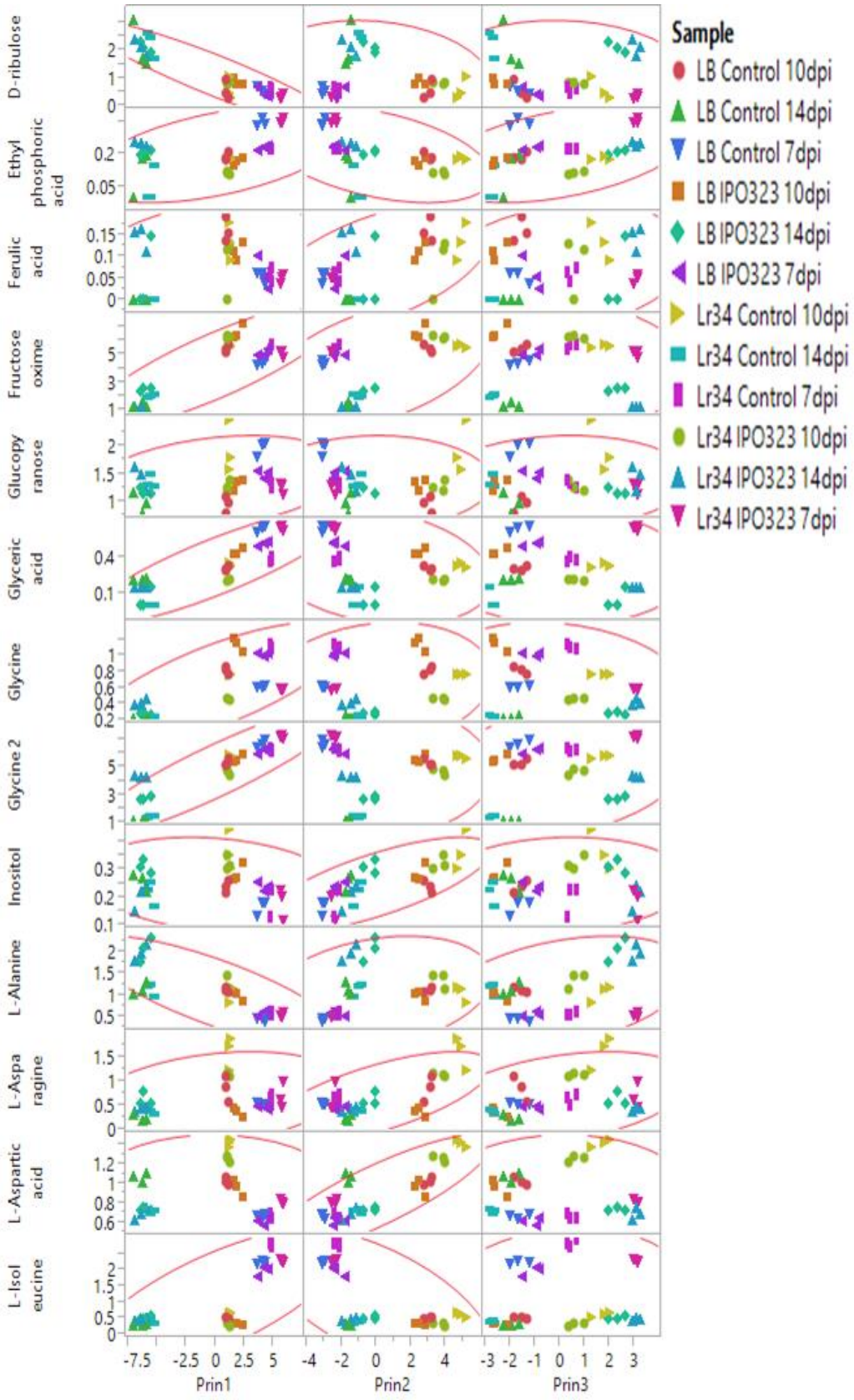
#### 4.3.5.2. Correlation analysis by scatterplot matrix

A scatter plot matrix of the correlation of the relative abundance of the detected metabolites with PC scores was generated to identify patterns of metabolite abundance using JMP® 11.2.0. Density ellipses were drawn around the samples in the plots to demonstrate correlation. Narrower and more diagonally placed ellipses indicated tighter and stronger correlation. Horizontal and more circular ellipses indicated weak correlation of the metabolites with the PC scores.

PC1 had strong negative correlation of L-alanine, pyrrolidone, D-ribulose, 1,4-eicosadiene, β-1-arabinopyrose, myo-inositol, sucrose and melibiose but a positive correlation for L-norleucine, glycine, glyceric acid, D-fructose, 9H-purin-6-amine (adenine), asparagine, fructose oxime, D-glucose and α-D-galactopyranoside (Fig 4.3.13). The negatively correlated compounds were comparatively abundant in 14dpi samples and the positively correlated compounds were abundant in 7dpi and 10dpi samples.

PC2 had a positive correlation with L-aspartic acid, putrescine, mannose and inositol and PC3 had positive correlation with serine and L-threonine and negative correlation with trimethyl-phosphate.


1,4-Eicosadiene
9H-Purin-6-amine
a-d-galactopyranoside
Asparagine
beta-Sitosterol
B-L-Arabinopyranose
D-Fructose
D-Fructose 2
D-Fructose 3
d-Galactose
d-Galactose 2
d-Glucose
D-Glucose
Prin1
Prin2
Prin3
Sample
LB Control 10dpi
LB Control 14dpi
LB Control 7dpi
LB IPO323 10dpi
LB IPO323 14dpi
LB IPO323 7dpi
Lr34 Control 10dpi
Lr34 Control 14dpi
Lr34 Control 7dpi
Lr34 IPO323 10dpi
Lr34 IPO323 14dpi
Lr34 IPO323 7dpi


D-ribulose
Ethyl phosphoric acid
Ferulic acid
Fructose oxime
Glucopyranose
Glyceric acid
Glycine
Glycine 2
Inositol
L-Alanine
L-Aspa ragine
L-Aspartic acid
L-Isoleucine
Prin1
Prin2
Prin3
Sample
LB Control 10dpi
LB Control 14dpi
LB Control 7dpi
LB IPO323 10dpi
LB IPO323 14dpi
LB IPO323 7dpi
Lr34 Control 10dpi
Lr34 Control 14dpi
Lr34 Control 7dpi
Lr34 IPO323 10dpi
Lr34 IPO323 14dpi
Lr34 IPO323 7dpi

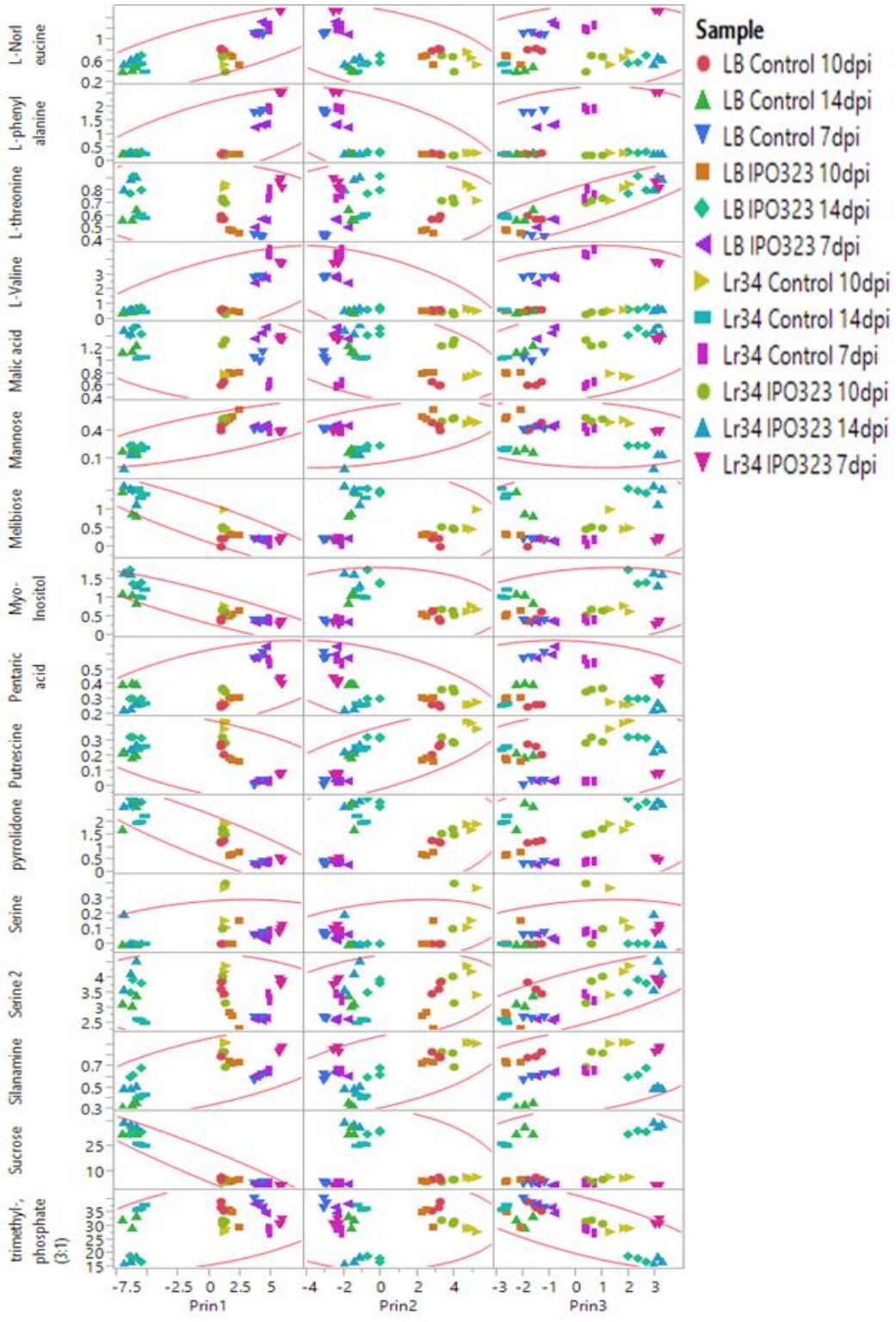



**Figure 4.3.13 Scatterplot analysis of PC scores for top three components and relative abundance values of metabolites detected. Ellipses drawn in red indicate correlation between PC scores of the samples and relative abundance of metabolites; narrow, oblique ellipses indicate a tight correlation.**

#### 4.3.5.3. Biplot analysis

Biplots were plotted for PC scores and loading scores for different metabolites detected in different samples. The biplots illustrate what compounds contribute most strongly to the loadings on each PC and how those contrasts between compounds relate to differences between the samples.

The loading of the concentration of each metabolite on each principal component is given in Table 4.3.7, which describes the contribution of metabolite concentrations to the PC scores and thus the variation explained by the PC. The metabolites with positive PC scores and positive loading scores were positively correlated to the PC while the positive PC score and negative loading scores indicated a negative correlation. Higher loading scores in PC1 indicated a high concentration of metabolites in 7dpi and 10dpi samples and a lower concentration in 14dpi samples.

| Compound[1] | PC1 | PC2 | PC3 |
|---|---|---|---|
| **1,4-eicosadiene** | -0.17483 | -0.11801 | -0.11801 |
| **9H-purin-6-amine** | 0.20176 | 0.02330 | 0.10012 |
| **Asparagine** | 0.16148 | -0.13371 | 0.18916 |
| **D-Fructose** | 0.11118 | -0.08075 | 0.26797 |
| **D-Fructose_1** | 0.17752 | 0.05023 | 0.18122 |
| **D-Fructose_2** | 0.17312 | 0.17713 | 0.00949 |
| **D-galactose** | 0.20210 | 0.07399 | 0.01547 |
| **D-galactose_1** | -0.15781 | 0.17729 | -0.05653 |
| **D-glucose** | 0.16587 | -0.06332 | 0.16370 |
| **D-glucose_1** | 0.17975 | 0.17851 | -0.03027 |
| **D-ribulose** | -0.19496 | -0.06007 | -0.02239 |
| **Ethyl_phosphoric_acid** | 0.10475 | -0.16446 | 0.20487 |
| **Ferulic_acid** | 0.02955 | 0.19811 | 0.10771 |
| **Fructose_oxime** | 0.17863 | 0.17464 | -0.03027 |
| **Glucopyranose** | 0.05909 | 0.00368 | 0.03860 |
| **Glyceric_acid** | 0.18243 | -0.09227 | -0.02061 |
| **Glycine** | 0.15645 | 0.05900 | -0.08187 |
| **Glycine_1** | 0.19138 | 0.00307 | 0.12406 |
| **Inositol** | -0.04116 | 0.26934 | 0.04220 |
| **L-alanine** | -0.16829 | 0.10141 | 0.17211 |
| **L-asparagine** | 0.05777 | 0.25177 | 0.17814 |
| **L-aspartic_acid** | -0.00319 | 0.31141 | 0.01839 |
| **L-isoleucine** | 0.15295 | -0.22690 | 0.07062 |
| **L-norleucine** | 0.16693 | -0.17760 | 0.11596 |
| **L-phenylalanine** | 0.14951 | -0.23335 | 0.10365 |
| **L-threonine** | -0.04188 | 0.02005 | 0.41957 |
| **L-valine** | 0.14814 | -0.22971 | 0.08833 |
| **Malic_acid** | -0.07460 | -0.15255 | 0.19464 |
| **Mannose** | 0.16671 | 0.18978 | -0.08570 |
| **Melibiose** | -0.19647 | -0.02254 | 0.07944 |
| **Myo_Inositol** | -0.19489 | -0.01226 | 0.12569 |
| **Pentaric_acid** | 0.12656 | -0.22381 | -0.07464 |
| **Putrescine** | -0.12562 | 0.27066 | 0.10522 |
| **Pyrrolidone** | -0.19437 | 0.07311 | 0.12353 |
| **Serine** | 0.05710 | 0.13590 | 0.13503 |
| **Serine_1** | -0.03197 | 0.11707 | 0.36853 |
| **Silanamine** | 0.13861 | 0.21787 | 0.17947 |
| **Sucrose** | -0.20365 | -0.07432 | 0.06463 |
| **Trimethylphosphate** | 0.10137 | 0.00377 | -0.35707 |
| **α-D-galactopyranoside** | 0.18742 | 0.10149 | 0.05345 |
| **β-sitosterol** | -0.16582 | -0.10808 | 0.09482 |
| **β-L-Arabinopyranose** | -0.18283 | -0.11122 | 0.04354 |

**Table 4.3.7 Loading scores of metabolites identified on each principal component (PC) in mock and IPO323-infected LB and *Lr34* NIL at 7dpi, 10dpi and 14dpi.**

[1] Chemstation software predicts the identity of detected compounds in GC-MS system by matching the MS profile of the compounds to a reference library. Different compounds with highly similar MS profiles were given same designation.

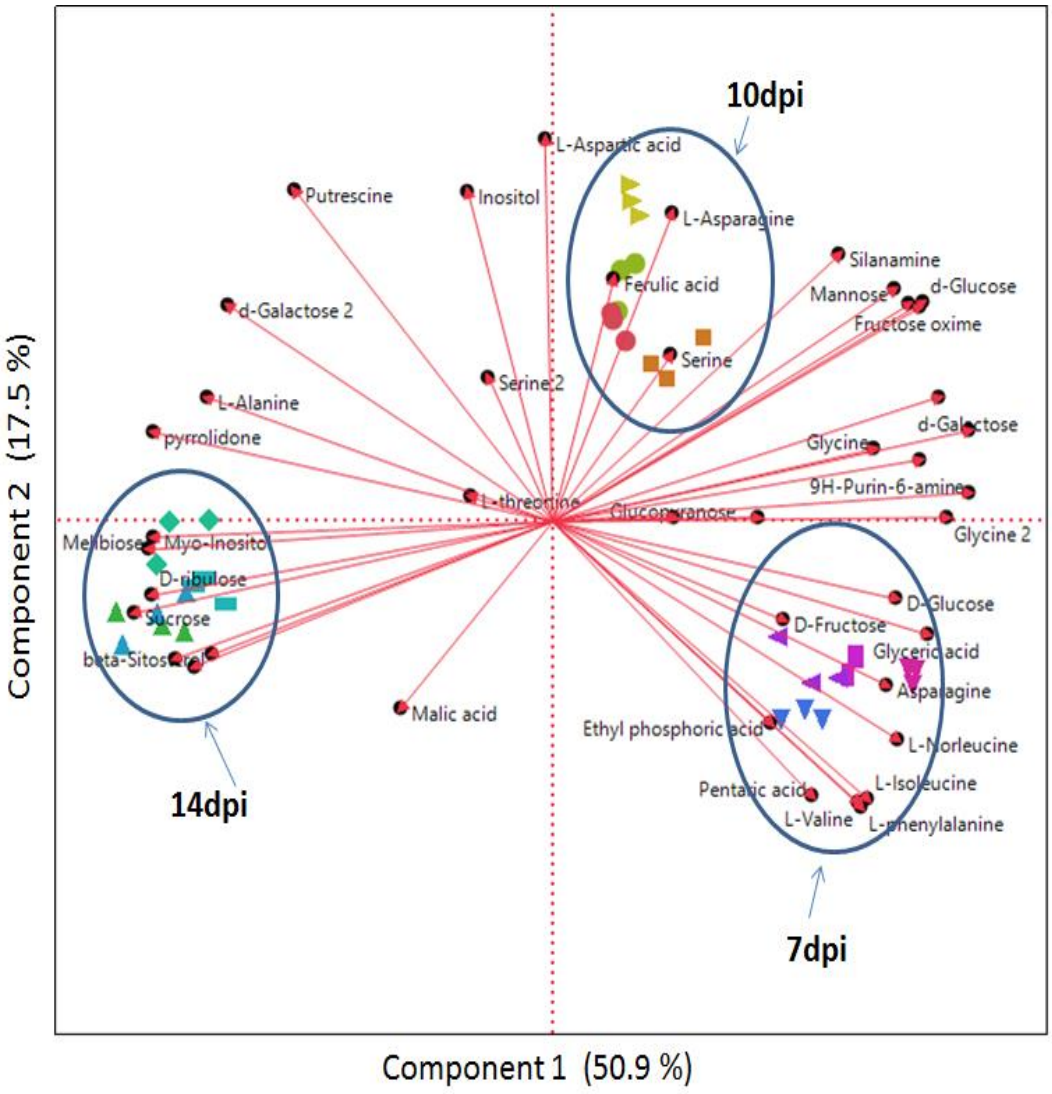



**Figure 4.3.14 Biplot analysis of score plots of PC1 and PC2 with loading scores for metabolites**

Compounds like D-glucose, D-fructose, glyceric acid, L-norleucine, pentaric acid, L-valine and L-phenylalanine had a significant role in 7dpi samples (Fig 4.3.14). 10d samples had higher concentrations of L-asparagine, ferulic acid and serine. 14d samples were correlated with D-ribulose, sucrose, β-sitosterol, melbiose and myo-inositol.

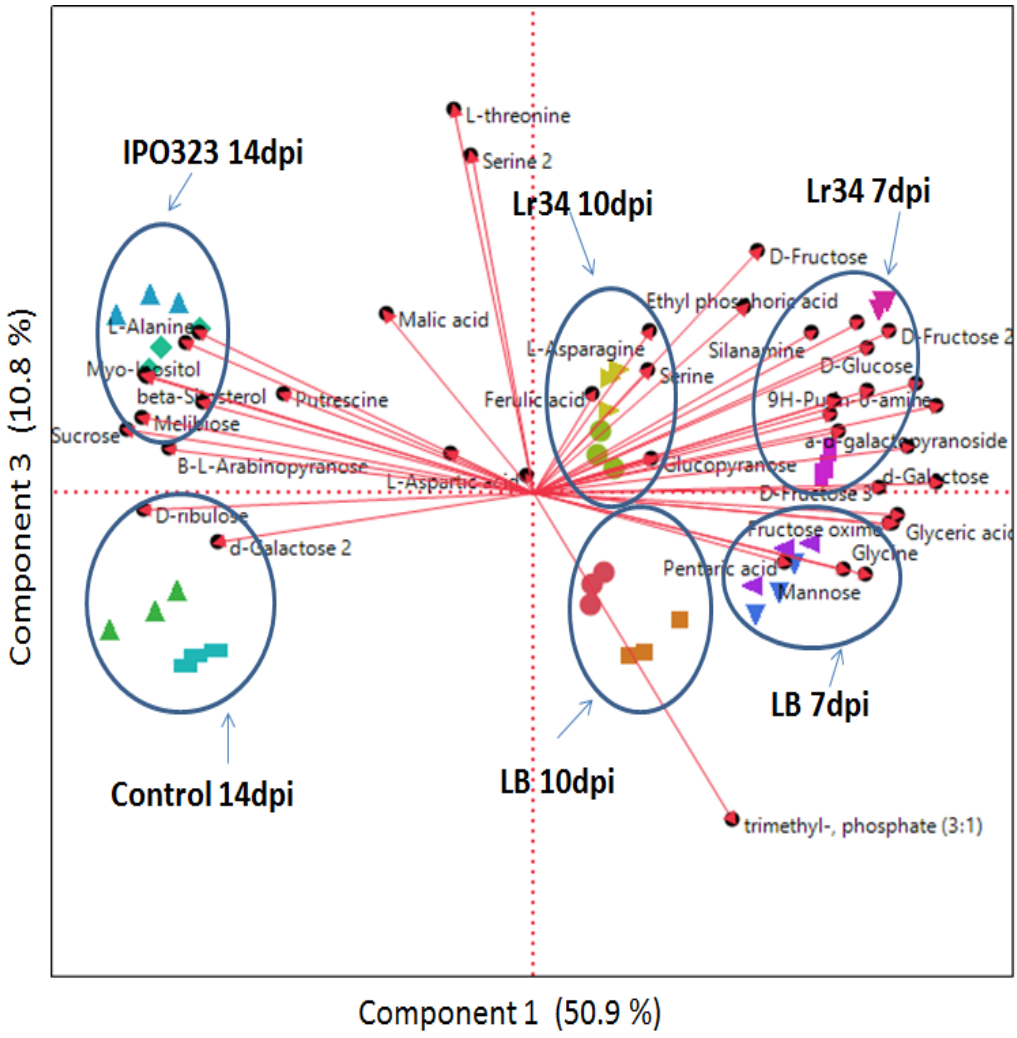



**Figure 4.3.15 Biplot analysis of score plots of PC1 and PC3 with loading scores of metabolites**

In the biplot for PC1 and PC3 (Fig 4.3.15), *Lr34* samples at 7dpi and 10dpi had higher loadings for D-fructose, ethyl phosphoric acid, L-asparagine, 9H-purin-6-amine and L-isoleucine while the LB samples had higher loadings of mannose, pentaric acid and trimethyl-phosphate. IPO323-infected samples for LB and LB-*Lr34* for 14d had higher loadings for L-threonine, serine, L-alanine, pyrrolidone, putrescine,

malic acid and melibiose while control samples for 14d had higher loadings of D-galactose.

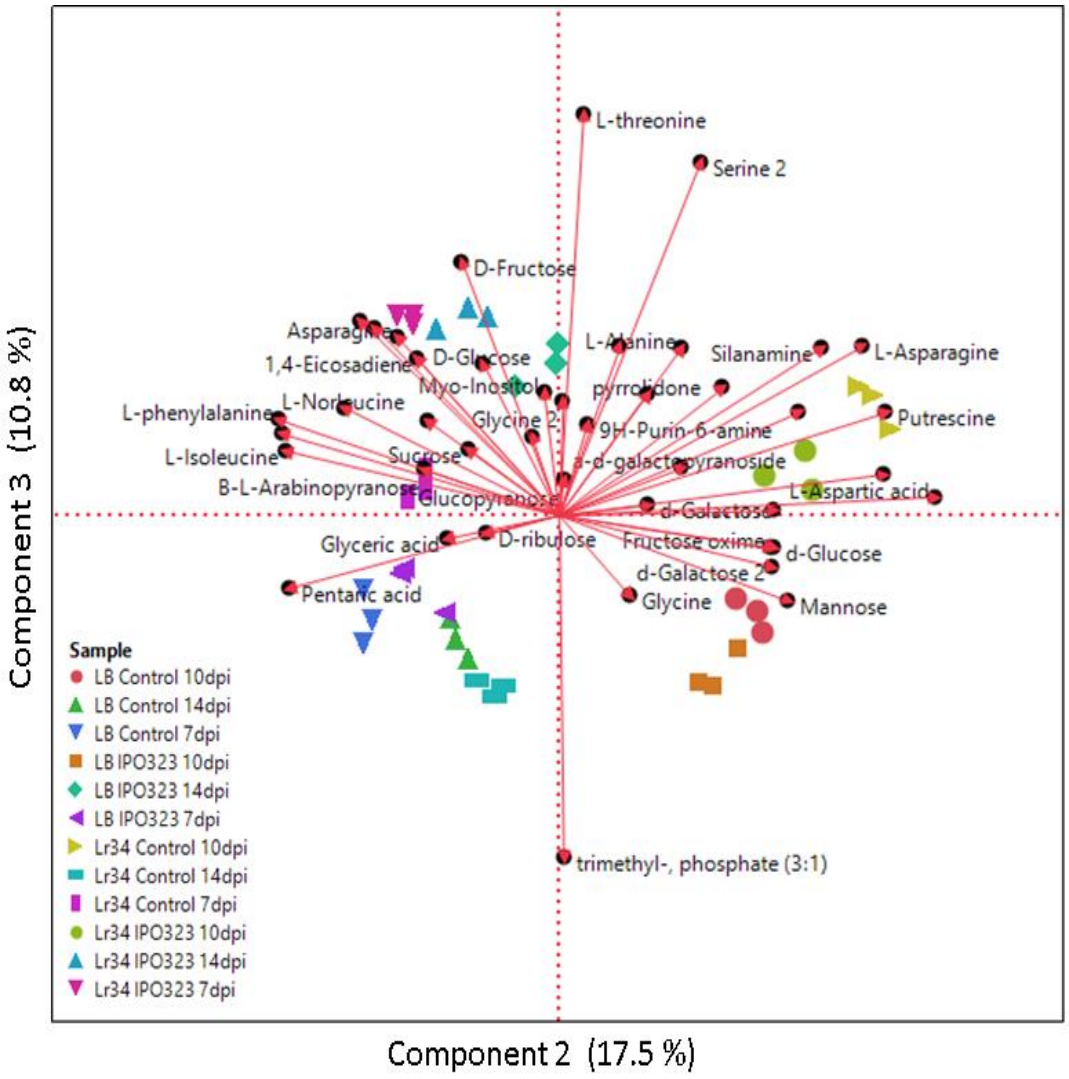



**Figure 4.3.16 Biplot analysis of score plots of PC1 and PC3 with loading scores of metabolites**

In the PC2/PC3 biplot (Fig 4.3.16), *Lr34* 10d samples had higher loadings of L-threonine, serine, L-asparagine, putrescine, 9H-purin-6-amine and inositol while LB 10d samples had higher loadings for

mannose, glycine, D-glucose and D-galactose. 14dpi IPO323-infected samples and LB-*Lr34* 7dpi samples had higher loadings for D-fructose, asparagine, D-glucose, and L-isoleucine. 14d control samples and LB 7d samples had higher loadings of D-ribulose, glyceric acid and pentaric acid.

#### 4.3.5.4. Analysis of variance

The PC scores were further analysed using multivariate analysis of variance (MANOVA) to calculate the effects of different experimental factors and their interactions on PC scores. The model used was Rep*Age*Pathogen*Genotype where Rep was the biological replicate, Age was age of leaf/stage of infection, Pathogen was treatment (Mock or IPO323 infection) and Genotype was line used (LB or LB-*Lr34* NIL).

Age, Pathogen and Genotype terms had significant effects with Age being the biggest contributor (Table 4.3.8). Age.Pathogen, Age.Genotype and Pathogen.Genotype terms were highly significant as well.

| Term | d.f. | Wilk's lambda | n.d.f. | d.d.f. | F prob. |
|---|---|---|---|---|---|
| **Rep** | 2 | 0.4838 | 6 | 4 | 0.9 |
| **Age** | 2 | 0.001 | 6 | 4 | 0.001 |
| **Pathogen** | 1 | 0.0045 | 3 | 2 | 0.007 |
| **Genotype** | 1 | 0.0034 | 3 | 2 | 0.005 |
| **Rep.Age** | 4 | 0.2296 | 12 | 6 | 0.9 |
| **Rep.Pathogen** | 2 | 0.3588 | 6 | 4 | 0.8 |
| **Age.Pathogen** | 2 | 0.0006 | 6 | 4 | 0.003 |
| **Rep.Genotype** | 2 | 0.7113 | 6 | 4 | 0.9 |
| **Age.Genotype** | 2 | 0.0008 | 6 | 4 | 0.004 |
| **Pathogen.Genotype** | 1 | 0.0326 | 3 | 2 | 0.04 |
| **Rep.Age.Pathogen** | 4 | 0.2233 | 12 | 6 | 0.9 |
| **Rep.Age.Genotype** | 4 | 0.0976 | 12 | 6 | 0.7 |
| **Rep.Pathogen.Genotype** | 2 | 0.587 | 6 | 4 | 0.9 |
| **Age.Pathogen.Genotype** | 2 | 0.04 | 6 | 4 | 0.2 |

**Table 4.3.8 Multivariate analysis of variance for PC1, PC2 and PC3 for relative abundance of metabolites in LB and *Lr34* samples in response to IPO323 infection**

### 4.3.5.5. Cluster analysis

A cluster analysis of the relative abundance of metabolites was performed based on Complete Linkage using Euclidean distance using Minitab 17.

The analysis revealed two major clusters; 7dpi and 10dpi samples were more closely related to each other than 14dpi samples (Fig 4.3.17). Within the first cluster, LB 7dpi samples were more similar while rest of the samples clustered together except LB 10dpi control which was placed next to LB 7dpi samples. *Lr34* 7dpi control was similar to *Lr34* IPO323-infected 7dpi sample. IPO323-infected 10dpi samples were also placed close with *Lr34* 10dpi control next to them. In the second

cluster, 14dpi controls were closer to each other than 14dpi IPO323-infected samples.

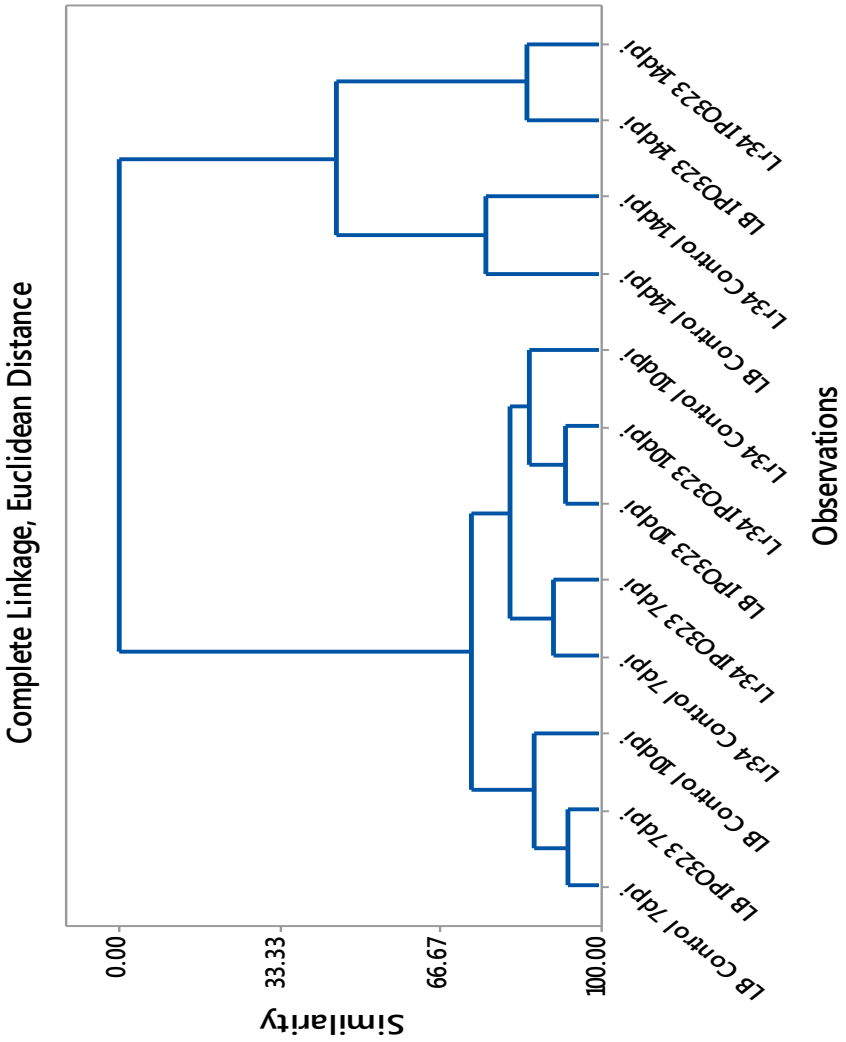



**Figure 4.3.17 Cluster analysis of relative abundance of metabolites in wheat plants with and without *Lr34*, infected with *Z. tritici* isolate IPO323 or mock-inoculated. Samples collected at 7dpi, 10dpi and 14dpi (dpi-days post inoculation).**

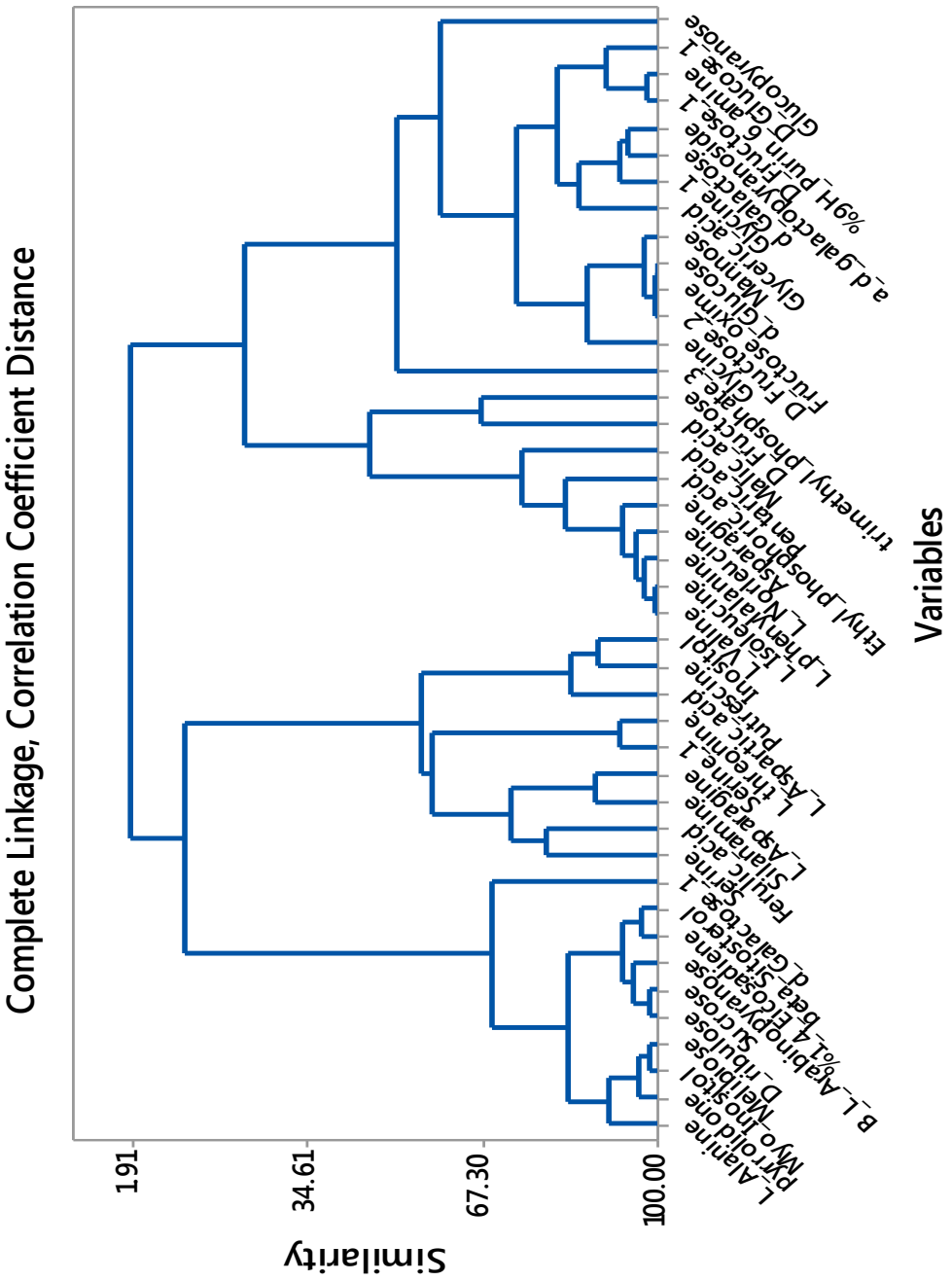



**Figure 4.3.18 Cluster analysis of variables for similarity between the abundances of different metabolites in wheat plants with and without *Lr34*, infected with *Z. tritici* isolate IPO323 or mock-inoculated.**

A further cluster analysis of variables was performed using correlation coefficient distance to find similarities between the patterns of abundance of different metabolites. Two major clusters of metabolites were identified with sugars like glucose, fructose and mannose closer together while amino acids like serine, L-threonine, L-asparagine were placed together (Fig 4.3.18). Amino acids with hydrophobic chains like L-valine, L-isoleucine, phenylalanine and L-norleucine were very similar and clustered very closely together. Polar uncharged amino acids like serine and L-threonine were very close to each other. They were also close to asparagine and its derivative L-aspartic acid.

## 4.4. Discussion

This chapter is aimed at improving understanding the underlying mechanisms of *Lr34* and *Lr46* in their roles in disease responses. The hypothesis tested was that these genes manipulate senescence or cell death pathways that increase resistance to biotrophs, which in turn increases susceptibility to pathogens with a necrotrophic phase.

### 4.4.1. Effect of seedling age on disease responses of *Lr34* and *Lr46*

To understand the role of senescence, seedlings of different ages were inoculated with either *B. graminis* or *Z. tritici*. Older seedlings had

fewer mildew colonies and there was an enhanced suppression of biotrophic growth in *Lr34* and *Lr46* NILs, the effect of age on these NILs being highly significant (Fig 4.3.1, Table 4.3.1). The *sr1-4D* mutation in Arabidopsis suppressed ethylene-induced senescence and in turn suppressed powdery mildew resistance (Nie et al., 2012). Similarly, *B.graminis* f.sp. *tritici* which is a biotrophic pathogen of wheat can be inhibited by host senescence.

There was a very strong effect of leaf age on STB susceptibility (Table 4.3.2). There was an increase in STB disease levels in older LB seedlings compared to younger LB seedlings indicating that natural senescence can promote STB (Fig 4.3.2). However older *Lr34* and *Lr46* seedlings had lower STB levels than younger *Lr* NILs indicating that old age of the leaf hampered the growth of fungus *Z. tritici* in the leaves. *Lr34* and *Lr46* promoted necrotrophy in younger seedlings but did not favour fungal growth in old leaves. *Z. tritici* DNA levels in the seedlings confirmed reduced fungal growth in *Lr34* and *Lr46* seedlings infected at 21d and 25d compared to seedlings infected at 14d and 17d (Fig 4.3.3). It can be concluded that the environment in older *Lr34* and *Lr46* leaves was not optimal for *Z.tritici* growth and the fungus could

not establish itself during its endophytic phase leading to reduced symptoms while it flourished in young *Lr34* and *Lr46* leaves.

It can be hypothesized that *Lr34* and *Lr46* enhance senescence-like mechanisms which promote resistance to biotrophs and that this effect is magnified in older seedlings (Rosewarne et al., 2006, Krattinger et al., 2009). Increased senescence is expected to inhibit the growth of a biotroph which needs greener and healthier tissue to survive and thus impart resistance to pathogens like mildew and rusts. *Z. tritici* is a necrotroph with a very long endophytic or non-symptomatic phase which can last 14-25 days (Orton et al., 2011). Though there is no proof of the fungus feeding from host tissues (Keon et al., 2007), this phase appears to be important for fungus establishment in my experiments. If *Lr34* and *Lr46* do enhance senescence, it can shorten the time period available to fungus *Z.tritici* in old seedlings to establish itself as an endophyte before leaves start dying, reducing fungal growth and producing fewer symptoms. However, in younger seedlings, the fungus can still have the minimum time period required to establish and after that can take advantage of the senescence induced by *Lr34* and *Lr46* to quickly switch to the necrotrophic phase.

### 4.4.2. Effect of ROS inducers on *Lr34* and *Lr46*

Different ROS inducers were also tested to induce cell death and exploit the differences between LB and *Lr* NILs to understand the potential mechanisms. However, no differences were seen among the lines in response to different ROS inducers (Fig 4.3.4, Fig 4.3.5).

### 4.4.3. Gene expression analysis of *Z.tritici* infected *Lr34* NILs

Expression of different senescence and cell-death related genes was measured in uninfected and IPO323-infected LB and LB-*Lr34* lines. The expression of the genes varied among different combinations of pathogen infection and line (Table 4.3.6). There was slight difference between the gene expression between the two lines but it largely depended on pathogen infection (Pathogen.Gene.Line term, Table 4.3.6). The time course of expression of different genes did not differ significantly between Pathogen and Line combinations because there was no significant interaction with Time point.

*Chla/b* is a gene coding for chlorophyll a/b binding protein and was used as a marker of photosynthetic activity (Xu et al. 2012). There was a sharp decline in photosynthesis in both LB and *Lr34* NILs infected with *Z. tritici* compared to uninfected plants, which was not dependent on genotype (Fig 4.3.7. Fig 4.3.10). A barley leaf senescence marker

HvS40 was found to be highly expressed in flag leaf tips of *Lr34* lines (Krattinger et al. 2009). Transcript levels of *TaSAG12*, a cysteine protease gene, were upregulated during leaf senescence (Lohman et al. 1994; Sun-Noh et al. 1999). There was a higher expression of *TaSAG12* in IPO323-infected LB-*Lr34* NILs compared to infected LB in our experiments (Fig 4.3.6, Fig 4.3.7) suggesting that either *Lr34* might enhance senescence or there is an increase in senescence in *Lr34* NILs due to higher susceptibility to *Z. tritici* which causes necrosis later in its life cycle. In plants, cysteine protease activities have been reported to be important factors in the attainment of ROS-driven programmed cell-death or PCD (He and Kermode, 2003, Wingler and Roitsch, 2008). A higher expression of *TaSAG12* in *Lr34* lines indicated potential deployment of PCD pathways in *Lr34*-mediated biotroph resistance and susceptibility to necrotrophs.

The wheat MAPK protein, TaMPK3, has been found to induce a cell death pathway that is implicated in production of STB symptoms (Rudd et al., 2008). *TaMPK3* gene expression is induced during a compatible *Z.tritici*-wheat interaction indicating manipulation of the host responses to initiate cell death by the fungus (Rudd et al., 2008). The Arabidopsis orthologue AtMPK3/WIPK is involved in responses

to PAMPs and avirulence-resistance (AVR-R) incompatible interactions (Stulemeijer et al. 2007). There was a higher expression of *TaMAPK3* in uninfected *Lr34* lines than in LB lines (Fig 4.3.6, Fig 4.3.8). This suggests that *Lr34* lines may have a higher level of PAMP-triggered immunity compared to parent background. There were similar levels of induction in both infected LB and *Lr34* lines indicating both lines were susceptible but the degree of susceptibility might have been related to native *TaMAPK3* levels. Hammond-Kosack and Rudd (2008) have proposed that compatible interactions between wheat and *Z. tritici* result from the fungus utilizing the host disease resistance signalling pathways deployed against biotrophs. Higher *TaMAPK3* levels in *Lr34* can lead to increased host resistance pathways against the biotrophic or endophytic phase of *Z. tritici*. The fungus can use these enhanced responses for a quicker switch to necrotrophy compared to parent background without these enhanced disease responses.

Hydrogen peroxide, $H_2O_2$, is an important regulator of STB and accumulates in a resistant cultivar early in infection (Shetty et al., 2007, Shetty et al., 2003). $H_2O_2$ inhibited the growth of *Z.tritici* both *in vitro* and *in planta* at early and late stages of infection (Shetty et al.,

2008). Genes encoding some ROS-scavenging enzymes are highly induced in *Z.tritici* during symptomatic infection stages (Keon et al., 2007). $H_2O_2$ is the most stable of the ROS and is degraded by catalase and peroxidase, enzymes that act synergistically to protect cells (Anderson, 2002). There was a strong induction of *catalase* gene in IPO323-infected *Lr34* NILs (Fig 4.3.6, Fig 4.3.9) which indicates more favourable conditions for *Z. tritici* growth in *Lr34* NIL compared to LB. Increased *catalase* expression in *Lr34* lines will lead to faster degradation of $H_2O_2$ which inhibits *Z. tritici* growth into non-toxic molecules compared to parent background making the *Lr34* lines more susceptible to STB.

### 4.4.4. Metabolite analysis of *Z. tritici* infected *Lr34* NILs

*Lr34* has been predicted to code an ABC transporter protein which might involve transport of plant metabolites (Krattinger et al. 2009). A metabolite screening of mock and septoria-infected LB and *Lr34* NILs was performed to identify metabolites with possible roles in *Lr34*-mediated disease responses.

Age of the leaves or natural senescence played the biggest role in differences in metabolite content among different samples (Fig 4.3.12, Table 4.3.8). The largest principal component, PC1, corresponded to

differences among three age groups and explained around 52% variation. The scatterplot and biplot analyses (Fig 4.3.13, Fig 4.3.14, Fig 4.3.15) revealed accumulation of amino acids like glycine, asparagine, serine, L-valine etc. and hexoses like D-fructose and D-glucose in samples for 7dpi and 10dpi while there was a higher abundance of sugars like sucrose, myoinositol, arabinopyrose and melibiose in 14dpi samples. A higher level of sugars in 14dpi samples indicated induction of senescence.

PC3 identified some metabolite differences in LB and *Lr34* in 7dpi and 10dpi samples (Fig 4.3.15). There were fewer differences between the lines at 14dpi. These results suggest that *Lr34*-mediated disease responses are more pronounced during initial stages of infection.

There was a distinct contrast between metabolite profiles of the infected and uninfected samples at 14dpi but not at 7dpi and 10dpi samples (Fig 4.3.15) suggesting that there is minimal metabolite activity in response to Septoria infection during initial stages. Metabolites like sucrose, melibiose, myo-inositol, serine and threonine etc. accumulated in infected samples indicating possible roles in responses to *Z. tritici* and making them potential PR (pathogen-responsive) candidates. The observation that sugars accumulate in

infected leaves could merely be a consequence of changes in metabolism related to STB-related necrosis which usually presents after a couple of weeks of infection.

Differences in metabolite concentrations in LB and *Lr34* NILs were identified which might be related to senescence or cell death. There was a higher loading score for L-asparagine, serine and hexose sugars in *Lr34* lines at 7dpi and 10dpi, meaning there were higher concentrations of these compounds at 7dpi and 10dpi relative to other compounds than at 14dpi (Fig 4.3.15, Fig 4.3.16). Serine/threonine protein kinases (STK) are important proteins responsible for defence signal transduction and signalling domains are conserved across plant species (Xu and Deng, 2010). Serine proteases with specificity to aspartate have been reported to be associated with the control of cell death in *Avena sativa* (Coffeen and Wolpert, 2004). It is therefore possible that increased levels of serine in *Lr34* NILs are indicative of involvement of *Lr34* in cell-death related defence pathways.

Infection of wild-type tomato with *B. cinerea* leads to a strong transcriptional up-regulation of a senescence-associated gene, asparagine synthetase, followed by a severe depletion of asparagine and promotion of pathogen-induced host senescence (Seifi et al.,

2014). The higher levels of asparagine accumulation in *Lr34* lines during initial stages of infection might act as a reservoir to support enhanced activity of asparagine synthetase during later stages of infection to induce senescence and support pathogen-induced necrosis.

It was found that expression of senescence-associated genes, such as *SAG12* is increased during growth on low nitrogen medium containing higher amounts of glucose in Arabidopsis (Pourtau et al., 2006). The higher glucose content in *Lr34* lines might be accelerating senescence of the leaves or it can be a consequence of accelerated senescence.

A cluster analysis of relative abundance of the metabolites grouped various samples based on their similarities in their accumulation levels (Fig 4.3.17). The 7dpi LB samples were closer to each other than to 7dpi LB-*Lr34* samples irrespective of their inoculation status. LB control 10dpi samples were placed closer to 7dpi LB samples. The *Lr34* 7dpi infected samples were closely related to 10dpi infected samples for both LB and *Lr34* 10dpi. This suggests an earlier pathogen related response was triggered in *Lr34* NIL compared to LB at 7dpi. LB 7dpi infected samples were similar to LB 7dpi and 10dpi control samples. This suggests that pathogen responsive processes were not yet triggered in LB. The 14dpi samples were clearly distinguished between

uninfected and infected samples indicating very strong pathogen responses which were no longer genotype-dependent.

Cluster analysis of the metabolites also revealed that compounds with similar chemical properties clustered together (Fig 4.3.18). Sugars and amino acids clustered separately. This indicated that different classes of compounds had specific profiles based on genotype, age and infection by *Z. tritici* and thus different functions can be attributed to different classes of metabolites. Amino acid content has been shown to decline in senescing leaves (Masclaux et al., 2000, Jongebloed et al., 2004)), while sugar content increases in several plant species during senescence (Wingler and Roitsch, 2008).

In general, this metabolite screening suggests involvement of senescence pathways by manipulation of sugar and amino acids in *Lr34*-mediated, time-dependent responses to Septoria. Levels of metabolites like hexoses, serine and asparagine which are involved in senescence or cell death pathways differed in *Lr34* lines compared to LB. Since LB-*Lr34* lines were more susceptible to *Z. tritici* (chapter 3) than parental LB, it can be inferred that the enhanced susceptibility may have been due in part to these metabolic differences which might enhance senescence or cell death in *Lr34* lines. Higher expression of

*SAG12*, *catalase* and *TaMAPK3* in *Lr34* NIL also support this hypothesis. The data from leaf age experiments suggested an early onset of senescence in *Lr34* and *Lr46* which promoted the necrotrophic phase of *Z. tritici* but hindered pathogen growth during its biotrophic phase. The presence of senescence/cell-death inducing molecules during early time points (7dpi and 10dpi) further support this hypothesis that there is an early induction of senescence in *Lr34* lines and the responses of *Lr34* to pathogens might be age-related.

Metabolic profiling associated with principal component analysis can be used as a powerful tool for decoding plant defence responses. Several metabolites that are related to *Lr34*-mediated response and STB-response were identified. Further studies need to be carried out to identify different metabolites not identified by the extraction and screening methods used in this study.

# 5. Effects of *Lr34* and *Lr46* on various fungal diseases of wheat

## 5.1. Introduction

In the past few decades, necrotrophic diseases of wheat like blast, tan spot and spot blotch have become economically significant by causing substantial yield losses and reducing grain quality. Necrotrophic fungal pathogens benefit from causing damage to the plant tissues, in contrast to biotrophs that benefit from maintaining the host cells in a living state. They typically deploy diverse phytotoxins and cell wall–degrading enzymes (CWDEs) to induce cell necrosis and nutrient leakage to facilitate pathogen nutrient uptake (Mengiste, 2012).

Wheat blast disease caused by *Magnoporthe grisea* was first identified in 1985 in Brazil (Urashima et al., 1993) and is becoming a major concern for wheat production in humid subtropical regions throughout the world. The disease can attack all above ground plant parts, limit grain development and kill the spike completely resulting in severe yield losses up to 100% (Cardoso et al., 2008). Partially infected spikes turn bleached and affected ears do not produce grain. Warm and humid

conditions (>25°C) during flowering stage of wheat appear to favour the wheat blast pathogen but the absence of detailed epidemiological studies makes it difficult to determine potential risk zones (Urashima et al., 2004, Kohli et al., 2011). Currently wheat blast is restricted to the tropical regions of South America but climatic changes associated with global warming may cause its spread to other parts of the world (Kohli et al., 2011). Besides wheat, the fungus can also grow on a number of cereal crops like triticale, barley, black oats, foxtail millet and many grassy weeds (Marangoni et al., 2013). Integrated management including some level of host resistance, avoidance of early seeding and chemical control, have been applied to successfully control disease (Kohli et al., 2011, Marangoni et al., 2013).

*Ramularia collo-cygni* is an increasingly important pathogen of barley in Northern Europe (Oxley and Havis, 2004) and has also been reported to infect oats and wheat (Sachs, 2006). It causes Ramularia leaf spot (RLS) disease which is characterized by rectangular reddish-brown necrotic lesions surrounded with a chlorotic halo (Salamati and Reitan, 2006). *R. collo-cygni* can survive as a saprophyte and also grow systemically and asymptomatically in the plant (Salamati and Reitan, 2006, Walters et al., 2008). Based on taxonomic studies, *R. collo-cygni*

is argued to be a species of *Mycosphaerella* (Verkley et al., 2004, Braun et al., 2005), a group closely related to *Z.tritici* (Stukenbrock et al., 2012). *R. collo-cygni* produces a number of coloured metabolites called rubellins which can induce light- and concentration-dependent necrosis when applied to barley leaves (Heiser et al., 2004).

Spot blotch is caused by the hemibiotrophic fungus *Cochliobolus sativus* (Maraite et al., 1998). *C. sativus* causes blight on the seedlings and spot blotch on adult plants. Early lesions are characterized by small, dark brown lesions which are 1-2mm long with no chlorotic margins. These lesions extend to form oval elongated light to dark brown blotches ranging several centimetres in size which coalesce and result in the death of leaf tissue (Bockus et al., 2010). An integrated approach including good genetic resistance, good agronomical practices and reasonable chemical control has been described to be most effective to reduce losses by spot blotch (Dubin and Duveiller, 2011).

Fusarium head blight (FHB), or scab, is one of the most important diseases in wheat and is caused by several *Fusarium* species (Parry et al., 1995). Symptoms of FHB include brown, water-soaked spots on glumes and pink or orange discoloration of mature ears (Sutton, 1982).

Kernels colonized by the fungus are characteristically smaller than normal and shriveled in appearance (Abramson et al., 1993). There is a growing concern for food safety as several *Fusarium* species causing FHB produce mycotoxins that are harmful to human and animal health (De Nijs et al., 1996, Bottalico and Perrone, 2002). The mycotoxins of primary concern are the trichothecenes like deoxynivalenol (DON), produced by *Fusarium graminearum* and *F. culmorum* (Logrieco et al., 2007, Haidukowski et al., 2005, Bottalico and Perrone, 2002).Thus, research aimed at minimizing mycotoxin levels in cereals is becoming a high priority.

Tan spot is caused by the ascomycete fungus *Pyrenophora tritici-repentis* and affects both cultivated durum and common wheat (Faris et al., 2013). The fungus causes large, tan-coloured lesions often surrounded by chlorotic haloes which coalesce to form large necrotic areas. This results in decreased photosynthetic capacity, causing yield loss (Bergstrom and Schilder, 1998). Tan spot can be controlled by using appropriate crop rotations or tillage practices (Bockus and Claassen, 1992, Wegulo et al., 2009) in combination with the use of fungicides and biological control methods including genetically resistant varieties (Singh et al., 2006).

Chapter 3 identified the potential of *Lr34* and *Lr46* genes to increase susceptibility of wheat cultivars to *Z. tritici* isolates. The current chapter aims to test if *Lr34* and *Lr46* have similar effects on different non-biotrophic pathogens of wheat in laboratory and field conditions. Wheat blast and RLS studies were carried out on seedlings and spot blotch, FHB and tan spot were studied in field trials carried out in Mexico.

## 5.2. Materials and methods

### 5.2.1. Seedling tests for wheat blast and RLS

Wheat seedlings of LB NILs and mutants were grown for seedling pathology tests as described in sec 2.1. Ten seeds per line per were sown in P60s. A minimum of three replicates was set up for each test in a completely randomized design. Inoculum preparation and inoculations were carried out as described in sec 2.2.3. The number of blast microlesions on leaves were counted 5-6 days after inoculation. The percentage of leaf covered with disease spots was noted for RLS after the appearance of symptoms usually 14-17 days after inoculation. The data was analysed by generalized linear modelling using Genstat 16.

### 5.2.2. Field trials at CIMMYT to evaluate effect of *Lr34* on different wheat diseases

NILs and mutant lines for *Lr34* in backgrounds Lal Bahadur, Jupateco, Thatcher, HUW234 and Yr70 along with their parents were evaluated for responses to FHB, spot blotch and tan spot at CIMMYT in Mexico. FHB and Tan Spot trials were located at El Batan station and scored in mid-August 2012.

FHB trials were sown in 4 replicates of two 1m rows in a completely randomized design. For FHB trials, ten spikes per row were tagged at anthesis and the whole row was inoculated. The inoculum consisted of a mixture of *F.graminearum* isolates collected during the preceding year in naturally-infected fields. Precision $CO_2$ backpack sprayers were used to apply liquid inoculum (50,000 conidia/mL) at a rate of 39 mL of inoculum per meter. The inoculation was repeated 2 days later. Humidity was maintained by mist spraying every two hours. The FHB trials were scored once after appearance of symptoms on heads and an FHB index was calculated (sec 5.3.3).

Spot Blotch and Tan Spot trials were sown in a randomized block with 4 replicates in $1m^2$ plots. Spot Blotch trials were repeated over two years in Agua Fria and scored in early February of 2012 and 2013.

Spot Blotch and Tan spot trials were infected with natural inoculum. Disease observations (percentage of diseased plot area) were taken once a week for 4-5 consecutive weeks after first appearance of symptoms. No fungicide treatments for rust control were given to trials for three diseases studied.

## 5.3. Results

### 5.3.1. Effect of *Lr34* and *Lr46* on wheat blast in seedlings

Microlesions on infected leaves were counted and subjected to regression analysis using generalized linear modelling using the logarithm link function because the data had integer values so the statistical errors were expected to fit a Poisson distribution. The model used was Rep*Line where Rep represents the replicate and Line represents different lines used in the experiment.

| Factor | d.f. | Mean Deviance | Deviance Ratio | approx F pr. |
|---|---|---|---|---|
| **Rep** | 2 | 7.2179 | 31.02 | <.001 |
| **Line** | 7 | 73.078 | 314.06 | <.001 |
| **Rep.Line** | 14 | 2.2463 | 9.65 | <.001 |
| **Residual** | 48 | 0.2327 | | |

**Table 5.3.1 Accumulated analysis of deviance for regression analysis of number of microlesions for pathology test on seedlings infected with *Magnoporthe grisea* isolate BR32. Generalized linear modelling of number of microlesions using a Poisson distribution with logarithm link function.**

Different replicates were significantly different from each other ($P$<0.001, Table 5.3.1). The differences between the lines used for the study were highly significant but the interaction between Rep and Line only had a small effect so Lines are compared across replicates (see deviance ratio, Table 5.3.1).

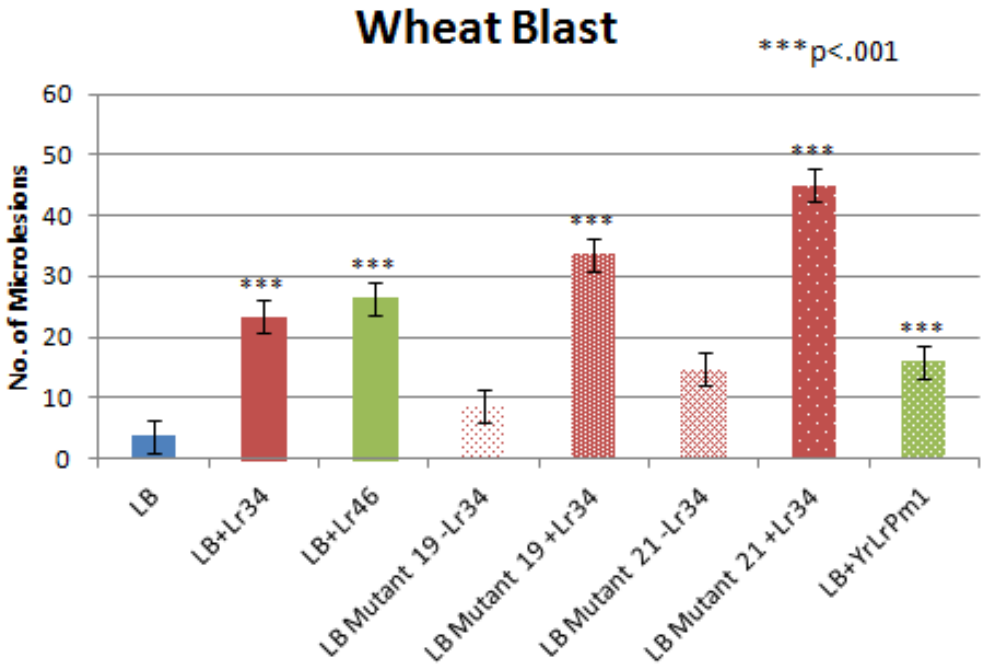



**Figure 5.3.1 Disease measured as mean number of microlesions on wheat seedling infected by *Magnoporthe grisea*. Bars indicate ±1 SE. Data were recorded over 3 experiments with 10 biological replicates per experiment. ***p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34*.**

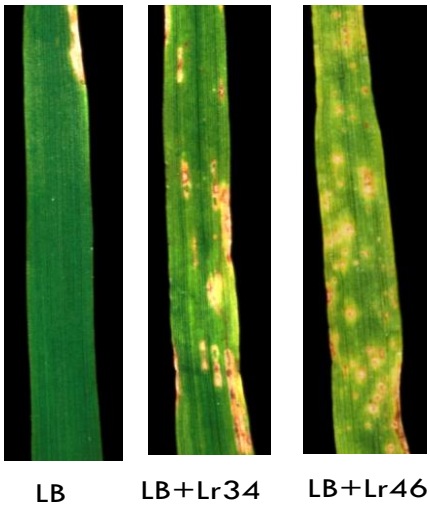



**Figure 5.3.2 Microlesions caused by *M. grisea* isolate BR32 on seedlings of wheat lines LB and its NILs for *Lr34* and *Lr46.***

LB NILs with *Lr34* and *Lr46* were much more susceptible to blast disease than the parent line LB (Fig 5.3.1 and 5.3.2). Similarly Mutant 19 and 21 lines with *Lr34* were significantly more susceptible to blast than respective paired mutant lines without *Lr34*. LB+*YrPm1* also had more blast lesions than parent LB.

### 5.3.2. Effects of *Lr34* and *Lr46* on Ramularia leaf spot in wheat seedlings

Percent disease area covered by RLS was subjected to regression analysis using generalized linear modelling using the logit link function because the percent disease values are expected to follow a Binomial distribution of statistical errors. The model used was Exp*Line where

Exp represents the Rep and Line represents different lines used for experiment.

| Factor | d.f. | Mean Deviance | deviance ratio | approx F pr. |
|---|---|---|---|---|
| **Exp** | 2 | 2.993 | 1.24 | 0.3 |
| **Line** | 2 | 51.004 | 21.13 | <.001 |
| **Exp.Line** | 4 | 5.923 | 2.45 | 0.05 |
| **Residual** | 84 | 2.413 | | |

**Table 5.3.2 Accumulated analysis of deviance for regression analysis of percentage disease scores for pathology test on seedlings infected with *Ramularia collo-cygni* isolate Rcc09B4. Generalized linear modelling of percentage disease using a Poisson distribution with logarithmic link function.**

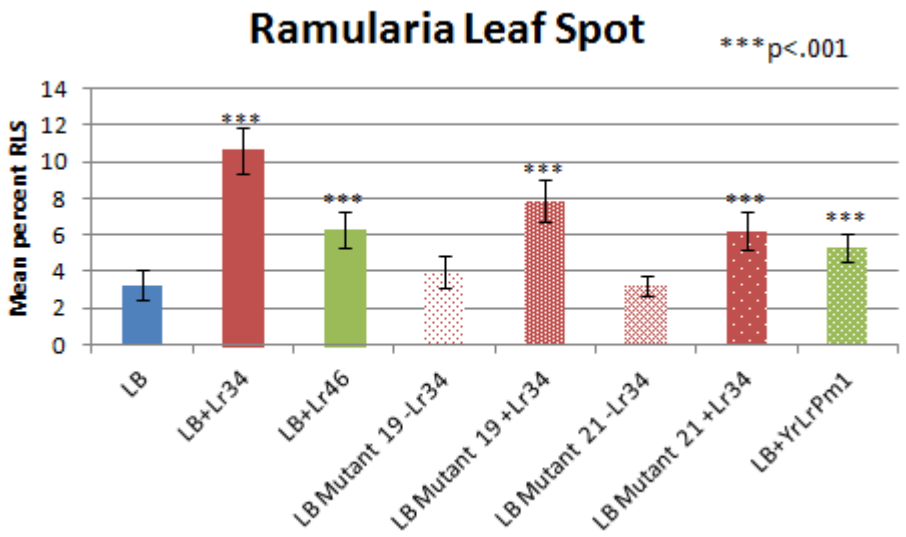



**Figure 5.3.3 Disease measured as mean percentage on wheat seedlings infected by *Ramularia collo-cygni*. Bars indicate ±1 SE. Data were recorded over 3 experiments with 10 biological replicates per experiment. *p<.001, significant differences between NILs and respective parent lines and between mutants with and without *Lr34***

There were no significant differences ($P$>0.05) between the different replicates. The Lines had significant differences in their responses to RLS (Table 5.3.2). The interaction between the Line and Exp was

slightly significant but was small in comparison to the Line term so mean RLS scores for Lines across experiments are presented.

The presence of *Lr34* and *Lr46* significantly increased the susceptibility of wheat lines to RLS (Fig 5.3.3). LB NILs and mutants with *Lr34* or *Lr46* were more susceptible to RLS compared to the parent and mutants without *Lr34* or *Lr46*. LB+*YrPm1* also had more RLS symptoms than the parent LB.

### 5.3.3. Field trials to assess effect of *Lr34* on various fungal diseases of wheat in different varietal backgrounds at CIMMYT

Tan spot and FHB trials located at El Batan were heavily infected with brown rust. For Tan Spot and Spot Blotch trials, percentage scores of the target diseases over time were converted into AUDPC scores. For FHB trials, an FHB index was calculated by averaging the number of Fusarium infected spikelets per spike. AUDPC scores and FHB index were analyzed by regression analysis using generalised linear modelling using a logit link function, which assumes a binomial distribution of statistical errors because the data values were a proportion of a maximum possible disease score. The model used was Rep+Line.Lr34 where Rep represents the plot replicates, Line

represents the variety used and Lr34 represents presence or absence of *Lr34* allele. Rust severity was not scored.

### 5.3.3.1. Tan Spot

| Factor | d.f. | Mean deviance | Deviance ratio | Approx F pr. |
|---|---|---|---|---|
| **Rep** | 3 | 11.795 | 3.32 | 0.1 |
| **Line** | 5 | 699.728 | 197.16 | <.001 |
| **Lr34** | 1 | 23.439 | 6.6 | 0.01 |
| **Line.Lr34** | 5 | 58.796 | 16.57 | <.001 |
| **Residual** | 40 | 3.549 | | |

**Table 5.3.3 Accumulated analysis of deviance for regression analysis of AUDPC scores for field study for effect of *Lr34* on Tan Spot. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

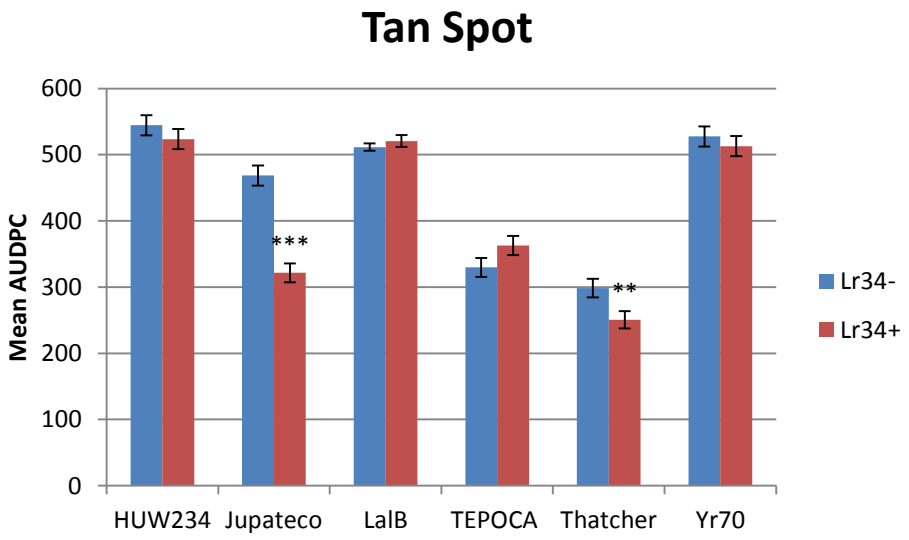



**Figure 5.3.4 Disease levels measured as AUDPC on wheat lines infected by *P. tritici-repentis*. Bars indicate ±1 SE. Data were collected for four replicates.**

There was no significant effect of the Rep ($P$>0.05) on the Tan Spot AUDPC scores. There was a significant difference between the lines and presence or absence of *Lr34* allele had a small significant effect

($P$<0.05) on AUDPC scores (Table 5.3.3). There was a significant interaction between the Line and *Lr34* allele but the effect of *Lr34* allele was smaller than that of Line when comparing the deviance ratios.

There was a difference in disease levels and responses in different backgrounds with *Lr34*. There were significantly fewer Tan Spot symptoms in Jupateco and Thatcher lines with *Lr34* while there were slightly more in Tepoca with *Lr34* compared to the NIL without *Lr34* (Fig 5.3.4). There were no significant differences between HUW234, Lal Bahadur and Yr70 lines with or without *Lr34*.

### 5.3.3.2. Fusarium head blight

| Factor | d.f. | Mean deviance | Deviance ratio | Approx F pr. |
|---|---|---|---|---|
| **Rep** | 3 | 0.08134 | 0.93 | 0.4 |
| **Line** | 5 | 0.38187 | 4.36 | 0.003 |
| **Lr34** | 1 | 0.00292 | 0.03 | 0.9 |
| **Line.Lr34** | 5 | 0.04454 | 0.51 | 0.8 |
| **Residual** | 40 | 0.08753 | | |

**Table 5.3.4 Accumulated analysis of deviance for regression analysis of AUDPC scores for field study for effect of *Lr34* on Fusarium Head Blight. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using a binomial distribution with logit link function.**

There was no significant effect of the Rep on FHB symptoms in the disease trials. The Lines were significantly different in their responses

to FHB but there was no significant effect of the presence or absence of *Lr34* allele (Table 5.3.4).

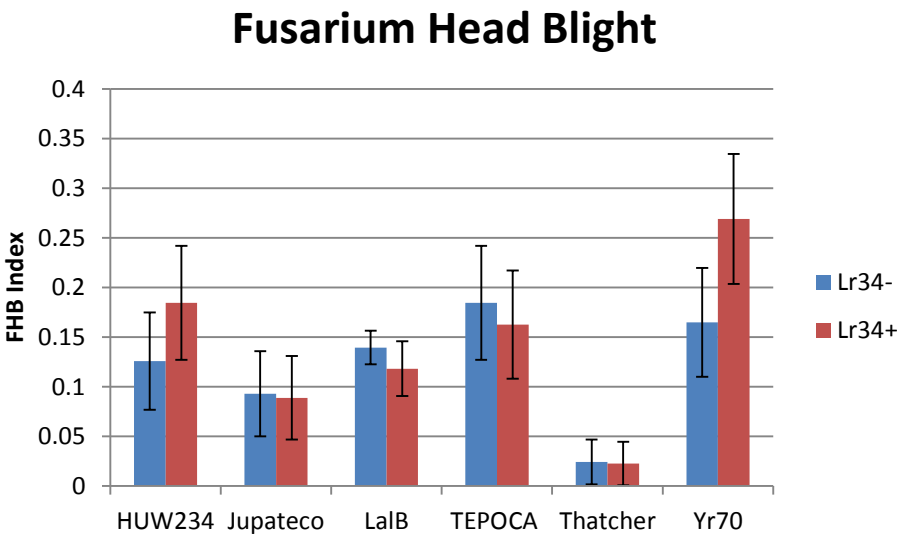



**Figure 5.3.5 Disease levels measured as FHB index on wheat lines infected by *F. graminareum*. Bars indicate ±1 SE. Data were collected over four replicates.**

HUW234 and Yr70 lines with *Lr34* had a higher FHB index compared to non *Lr34* lines but the differences were insignificant (Fig 5.3.5). There was slightly but not significantly less FHB disease on Lal Bahadur and Tepoca with *Lr34* compared to parents without *Lr34*.

### 5.3.3.3. Spot Blotch

| Factor | d.f. | Mean deviance | Deviance ratio | Approx F pr. |
|---|---|---|---|---|
| **Rep** | 1 | 129.39 | 2.65 | 0.1 |
| **Line** | 5 | 1692.93 | 34.7 | <.001 |
| **Lr34** | 1 | 1476.32 | 30.26 | <.001 |
| **Line.Lr34** | 5 | 74.27 | 1.52 | 0.2 |
| **Residual** | 152 | 48.79 | | |

**Table 5.3.5 Accumulated analysis of deviance for regression analysis of AUDPC scores for field study for effect of *Lr34* on Spot Blotch. Generalized linear modelling of AUDPC as a proportion of the maximum possible AUDPC using binomial distribution with logit link function.**

There was no significant difference between the replicates ($P>0.05$) for Spot Blotch trials, therefore results are presented across the replicates. Different lines were significantly different from each other in respective responses to Spot Blotch (Table 5.3.5). Presence or absence of *Lr34* also made a significant effect on disease scores ($P<0.001$). However, there were no significant interactions between the Line and *Lr34* terms in the model.

In general, the presence of *Lr34* decreased the susceptibility of lines to Spot Blotch. There was significantly less Spot Blotch symptoms in HUW234, Jupateco, Lal Bahadur and Yr70 lines with *Lr34* compared to lines without *Lr34* (Fig 5.3.6). In Tepoca, there was an insignificant decrease in Spot Blotch levels in *Lr34* line. There was no difference between Thatcher lines.

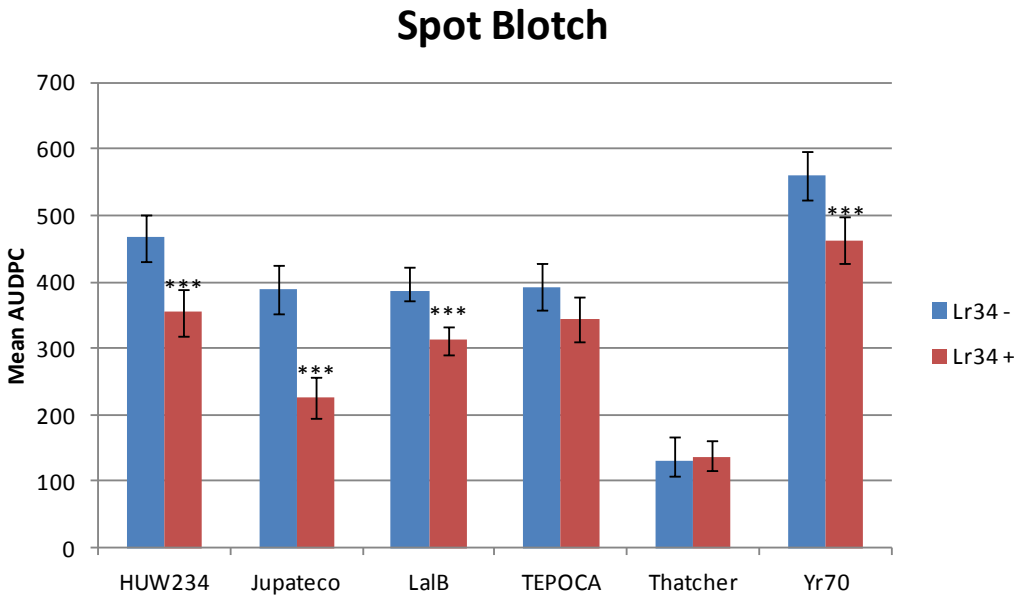



**Table 5.3.6 Disease levels measured as AUDPC on wheat lines infected by *C. sativus*. Bars indicate ±1 SE. Data were collected over two years (2012-2013) with four replicates each year.**

## 5.4. Discussion

The aim of this study was to evaluate effects of *Lr34* and *Lr46* on plant responses to a range of non-biotrophic pathogens of wheat. In chapter 3, it was established that *Lr34* and *Lr46* have a potential to increase plant susceptibility to Septoria in both seedling and adult plant stages with controlled inoculations though the response was not seen in field conditions probably due to contamination of trial sites with heavy rust infection.

### 5.4.1. Effects of *Lr34* and *Lr46* on wheat blast and RLS in seedlings

LB seedlings were not very susceptible to *M. grisea* with little to no symptoms on the leaves but *Lr34* and *Lr46* NILs had a far greater number of blast lesions (Fig 5.3.2). The differences between lines were highly significant and though there was an interaction between different replicates and lines (Table 5.3.1), the effect of the interaction was small suggesting a consistent pattern of responses of different LB lines to *M. grisea*. The presence of *Lr34* and *Lr46* alleles greatly increased the susceptibility of wheat seedlings to blast infection both in NILs and mutants (Fig 5.3.1).

*R. collo-cygni* is not a usual pathogen of wheat but can infect under suitable conditions (Walters et al., 2008). The different LB lines were significantly different from each other in their responses to *R. collo-cygni* and there was a slight difference between their responses in different reps (Table 5.3.2). However, the pattern was consistent with *Lr34* and *Lr46* lines having more symptoms (Fig 5.3.3). Though the average level of disease was not severe, the difference between LB and *Lr34* and *Lr46* NILs was quite significant. Mutants with the *Lr34* allele were more susceptible to RLS than mutants without the *Lr34* allele.

*R. collo-cygni* like *Z. tritici*, has a long non-symptomatic phase lasting 14-17 days before switching to the necrotrophic phase. It is also taxonomically close to *Z. tritici*. *M.grisea* has a very short biotrophic phase lasting a couple of days and a very aggressive necrotrophic phase. Based on the observations of Chapter 3 and the current chapter, it can be summarized that *Lr34* and *Lr46* promote susceptibility to pathogens with a necrotrophic phase. This is consistent with findings suggesting that several resistance genes against biotrophs favour necrotrophy and vice versa (Brown and Rant, 2013). The *mlo* mildew resistance gene has been shown to increase susceptibility to *M. oryzae*, the rice blast pathogen (Jarosch et al., 1999). The presence of *mlo* alleles increased severity of RLS in both seedling tests and field trials (McGrann et al., 2014a). The *Rht-B1b* and *Rht-D1b* semi-dwarfing genes which encode GA-insensitive DELLA proteins confer higher susceptibility to the biotrophic fungus *B. graminis* but reduce susceptibility to a necrotrophic pathogen *Oculimacula* spp. which causes eyespot and to *F. graminearum* (Saville et al., 2012). A loss of function allele of a DELLA encoding gene *Slender 1*, *sln1c*, increases resistance to mildew but enhanced susceptibility to eyespot in barley.

### 5.4.2. Effect of *Lr34* on different fungal pathogens of wheat in field

Scoring for resistance in disease caused by non-biotrophic pathogens in the field can be somewhat difficult because it is sometimes hard to distinguish between pathogen-induced necrosis and physiological necrosis caused due to abiotic stress and natural phenomena like senescence. Also the presence of different pathogens on same tissue can make it difficult to score each disease separately as the symptoms can look similar. No consistent effect of *Lr34* was seen in FHB and tan spot trials (Fig 5.3.4, Fig 5.3.5). Both these trials were heavily infected with brown rust. It is possible that, as in the STB field trials (Chapter 3), there were significant genotype-by-rust interactions which would have affected the plant responses to tan spot and FHB pathogens. A significant inverse relationship was shown between the severity of leaf rust and Septoria nodorum leaf blotch on soft red winter wheat (Spadafora and Cole 1987). On the one hand, the agricultural significance of these interactions should be analyzed in environments where several diseases, including rusts and necrotrophs, are present simultaneously. On the other hand, experimental analysis to understand the separate and joint effects of these pathogens on the plant must be done in controlled conditions. Such experiments would require joint

inoculation of wheat plants with more than one fungal pathogen species; this is technically difficult but has been reported by Madariaga and Scharen (1984) and Orton (2012).

Spot blotch trials were scored in early February when the trial sites were free of rusts and other pathogens. There was no significant Line.Lr34 interaction (Table 5.3.5) indicating a consistent effect of *Lr34* among different genetic backgrounds. *Lr34* consistently decreased spot blotch symptoms with exception of the Thatcher lines (Fig 5.3.6). *C. sativus* starts infection with the formation of appressorium-like structures on the leaf surface and subsequently penetrates the cell wall to form an infection-hyphae network within epidermal host cells (Kumar et al., 2002). This is the biotrophic phase which is confined to a single epidermal cell. The necrotrophic phase begins when the pathogen invades the mesophyll layer accompanied by host cell death (Kumar et al., 2002). The process of cell wall penetration is similar to the infection process of biotrophs and defence reactions to *C. sativus* involve both papilla formation and hypersensitive cell death (Schäfer et al., 2005), which is a shared feature with biotrophic disease resistance. It is understandable that *Lr34* and *Lr46* which provide resistance against multiple biotrophs will

increase resistance to spot blotch, which has a lengthy biotrophic phase. The CIMMYT bread wheat line Saar which carries *Lr34* and *Lr46* was also shown to exhibit low levels of spot blotch disease in field trials carried out in Asia and South America (Lillemo et al., 2012). Although the molecular functions of *Lr34* and *Lr46* are still unknown, these genes are likely enhancing the basal disease resistance against biotrophic diseases in a pathogen non-specific manner which might be LTN-associated.

### 5.4.3. Implications for plant breeding

The positive association of *Lr34* and *Lr46* with resistance to spot blotch but a negative association with wheat blast and Ramularia present major implications. These genes are widely deployed in international wheat breeding programs to improve partial and durable resistance to rusts and powdery mildew (Singh et al., 2011). Rust is a major disease problem in South Asia where spot blotch is endemic (Lillemo et al., 2012), therefore the use of these genes can be beneficial to control rust in these areas with added resistance to spot blotch as well. However, in Northern Europe, Ramularia is becoming an important disease (Walters et al., 2008). In Southern America, wheat blast is a major concern for adequate wheat production (Kohli et al.,

2011). Detailed studies are required to fully understand the impact of *Lr* genes in field on these pathogens in field if they are to be introduced in these risk zones to control rusts and other biotrophs. Plant breeding programmes need to be designed to select for resistance to locally important necrotrophic diseases as well as rusts (discussed further in Chapter 6).

# 6. General Discussion

Plant diseases are major biotic constraints that reduce crop yields worldwide especially in developing countries due to lack of appropriate solutions and resources devoted to their study. It is estimated that 10-15% of the already low yields in developing countries is lost due to disease attack, and losses can be higher if post-harvest diseases are considered (Khoury and Makkouk, 2010). However, reported crop losses due to plant diseases are sometimes exaggerated and do not consider all possible constraints on production. A recent study of production constraints (including diseases) for major crops including wheat, rice and sorghum among others in 13 African and Asian countries showed that yield losses caused by diseases ranged from 3 to 14%, whereas losses due to all biotic stresses ranged from 16 to 37% and yield losses due to all crop production constraints ranged from 36 to 65% (Waddington et al., 2010). Effective and sustainable crop management requires an integrated disease management approach combining biological, cultural, physical and chemical control strategies in a holistic manner (Khokhar and Gupta, 2014).

Plant diseases are complex and every interaction between the host and pathogen is potentially subjected to natural selection which acts on genetic diversity (Brown and Rant, 2013). Disease and plant responses to pathogens are closely related to aspects of biology of all organisms participating in a biotic interaction. Therefore it is necessary for studies on any aspect of disease resistance to include plant interactions with all positive (symbionts, irrigation, fertilization) and negative (pathogens, pests, drought, water-logging) biotic and abiotic factors encountered by the plant.

A simple model for yield can be described as

$$Y = Ymax - Lp1 - Lp2 - Ls + Gsym + Gcp$$

Where Y is final yield, $Y_{max}$ is the theoretical maximum yield without any external input, L is the loss by pathogens (p1), pests (p2) and stress (s) and G is gain in yield due to symbionts and beneficial biotic partners (sym) and better cropping practices (cp). All these components form the environment component. However, these interactions are far more complex because presence of one component can severely affect the other components. Disease resistance breeding is now extremely challenging because of the need to produce resistant varieties with a wide range of agronomic properties. Plant breeders in particular must

maintain and improve resistance to continually evolving pathogen populations without incurring significant costs in terms of other factors (Brown and Rant, 2013).

The aim of this study was to investigate trade-offs related to *Lr34* and *Lr46* mediated resistance to biotrophic pathogens in terms of increased susceptibility to non-biotrophic fungal pathogens. These genes seem to involve a novel mechanism that is not completely understood. Through this study hypotheses have been tested for underlying mechanism of the trade-off between responses to biotrophs and non-biotrophs by these genes.

Chapter 3 researched interactions between *Lr34* and *Lr46* with STB. Presence of these genes significantly increases the susceptibility of wheat plants to *Z. tritici* in both the seedling and adult plant stages though there was a significant variety by isolate effect. There was an increase in susceptibility of seedlings of LB lines with *Lr34* and *Lr46* to *Magnoporthe grisea* and *Ramularia collo-cygni* in Chapter 5. The STB tests on adult plants were carried out on only one background LB with two isolates but different backgrounds need to be tested at adult stage as well. An attempt was made to evaluate different backgrounds together but the inoculations were made difficult because of differences

in plant height and more importantly their heading dates. As a result it was difficult to get a uniform infection. For blast and RLS experiments as well, multiple backgrounds and isolates need to be tested. Both controlled adult plant tests and field trials to evaluate multiple necrotrophic pathogens are required in the absence of rust.

These are important cereal pathogens with significant yield losses every year. *Lr34* is an important gene to control different wheat rusts as it is effective against all known pathotypes of *Puccinia* especially when combined with genes like other rust resistance gene like *Lr13* and *Lr37* (Park, 2008). These trade-offs definitely raise concerns about the use of *Lr34* and *Lr46* for breeding programs for rust resistance.

Durability of resistance genes can possibly be extended considerably if they are deployed in combination of two or more genes so the pathogen might be required to evolve against multiple avirulence factors simultaneously (Wallwork, 2009). Further studies should be conducted on combinations of similar adult plant resistance genes for their effects on necrotrophic pathogens. Also these effects need to be tested rigorously in fields. The field trials in UK and Mexico for Septoria and other necrotrophs did not provide any useful information probably because of high levels of rust infection. Plants are faced with

challenges to fend off multiple pathogens in fields along with complicated interactions with the environment. Numerous factors can affect the disease responses in field trials like plant growth, temperature, rain, irrigation, other pathogens, soil quality etc. and it is hard to control all these factors. Carefully designed experiments to simulate multiple infections in field can be conducted in a controlled environment to dissect out the complex environment-pathogen-plant interactions. Dual inoculations of wheat adult plants with rust and Septoria at different stages of plant growth can give meaningful insights to how both these pathogens interact in field and also how resistance gene responses may be affected. The specific hypothesis of how rust infection can illicit plant responses to suppress different pathogens like *Z. tritici* needs to be tested. Also multiple site field trials are necessary to obtain a robust and clear understanding of environment-resistance gene interactions.

Chapter 4 dealt with mechanisms of *Lr34* and *Lr46* and role of senescence-like processes in *Lr34* and *Lr46* mediated disease responses to biotrophs and non biotrophs. There is a possible role of plant age in directing the disease responses by these genes. ROS scavengers like catalase and senescence-associated genes like *TaSAG12* are

differentially expressed in *Lr34*-mediated disease responses indicating involvement of a ROS-mediated programmed cell death pathway. A metabolite screen illustrated the potential involvement of sugar and amino acids in *Lr34*-STB responses through cell death or senescence processes which may further involve multiple signaling pathways.

Different age groups of seedlings were tested for their responses to STB and mildew. The results indicate that the *Lr* genes may suppress Septoria by shortening the biotrophic phase so the fungus is not able to reach a point where necrotrophy and asexual reproduction are triggered. But if the pathogen is established on the leaf well before senescence starts as seen in younger seedlings, *Lr34* or *Lr46* may enhance susceptibility to *Z.tritici* if they are inducing senescence or cell-death mechanisms. Similar studies in adult plants where plants are infected at various developmental stages can help identify crucial time points for *Lr34* and *Lr46* activity which would be useful from breeding perspective for developing mitigation strategies to control STB in *Lr34* and *Lr46* lines. Fungicide control can be employed more effectively if the time points when wheat lines are more vulnerable to *Z. tritici* are identified.

The results presented in chapter 4 showed that there was no clear pattern of gene expression that differentiated *Lr34* NIL from the parent LB over time involving the genes tested. The genes tested have been linked to a role in defence and plant senescence. It has been found in other pathosystems like rusts (Bozkurt et al., 2007) and powdery mildew (Boyd et al., 1994) that defence-related genes are up-regulated in both incompatible and compatible interactions. The up-regulation of these genes may be a general defence response to inoculation with the pathogen, independent of virulence or level of susceptibility. Because of the large variability seen between data sets it is important to correlate the growth and development of the pathogen to the response of the plant. For STB-wheat interactions in this study, fungal DNA levels were measured only at the end of the experiment. A time course analysis of fungal growth within leaf tissue is necessary using *Z.tritici* DNA levels. This can also be achieved by microscopically visualizing fungal growth within the leaf tissue but there are no reliable staining methods for *Z.tritici in planta*. By establishing correlations between the pathogen development and gene expression data, accurate inferences can be made about role of different developmental stages in responses of *Lr34*. The metabolic status of powdery mildew as it infects its host

and completes its life cycle was studied by comparing the transcriptome profile of *B. graminis*. f.sp. *hordei* with the developmental stages of the fungus in the infected tissue (Both et al., 2005).

Variability in gene expression data may partly be due to entry of opportunist bacteria or fungi during inoculations by *Z.tritici.* It has been observed that wounded barley plants grown in non-sterile conditions accumulated transcripts of *chitinase, peroxidase* and pathogenesis related *PR-R* genes with no discernible pattern but plants grown under sterile conditions accumulated no *chitinase* or *peroxidase,* although they accumulated *PR-R* at 0 hours after wounding, which may have been due to physiological stress (Boyd et al. 1994). Carrying out pathology experiments on sterile wheat plants can help identify specific pathogen induced responses, though a sterile environment is unnatural for plants and there may be fitness costs associated.

There has been tremendous progress in genomic initiatives to profile genomes and gene expression of plant-pathogen interactions. A better understanding of metabolite profiling is needed to understand plant defence responses against various environmental and biotic stresses. Chapter 4 presents probably the first study of the metabolic profile of

*Lr34* lines in response to pathogens. The proposed mode of mechanism is transport of toxins or metabolites to induce resistance (Krattinger et al., 2009) and metabolomics approach was used to identify such resistance molecules in *Lr34* lines. Wheat breeders are looking for fast and precise tools for screening resistance against different pathogens along with understanding the functions of different resistance genes to facilitate breeding programs. In this study, GC-MS metabolic profiling of wheat leaves at different time points has helped identify metabolites involved in endophytic and necrotrophic phases of wheat-STB interactions and also specific *Lr34* related metabolites. Many of these compounds are known to be significant in defence and senescence related metabolism pathways. The screening was limited to detection of polar compounds because of lack of knowledge of specific metabolites involved in STB-wheat interactions and also there have been no prior studies which might have narrowed down the compounds with a possible role in *Lr34*-mediated disease responses. Plant defence and metabolite synthesis are active and dynamic processes but metabolites were profiled only at three time points following pathogen inoculation in this study. Further studies involving detailed temporal analysis of metabolites, different wheat genotypes and *Z.tritici* isolates

are needed to better understand metabolite function in plant defence in responses to resistance genes.

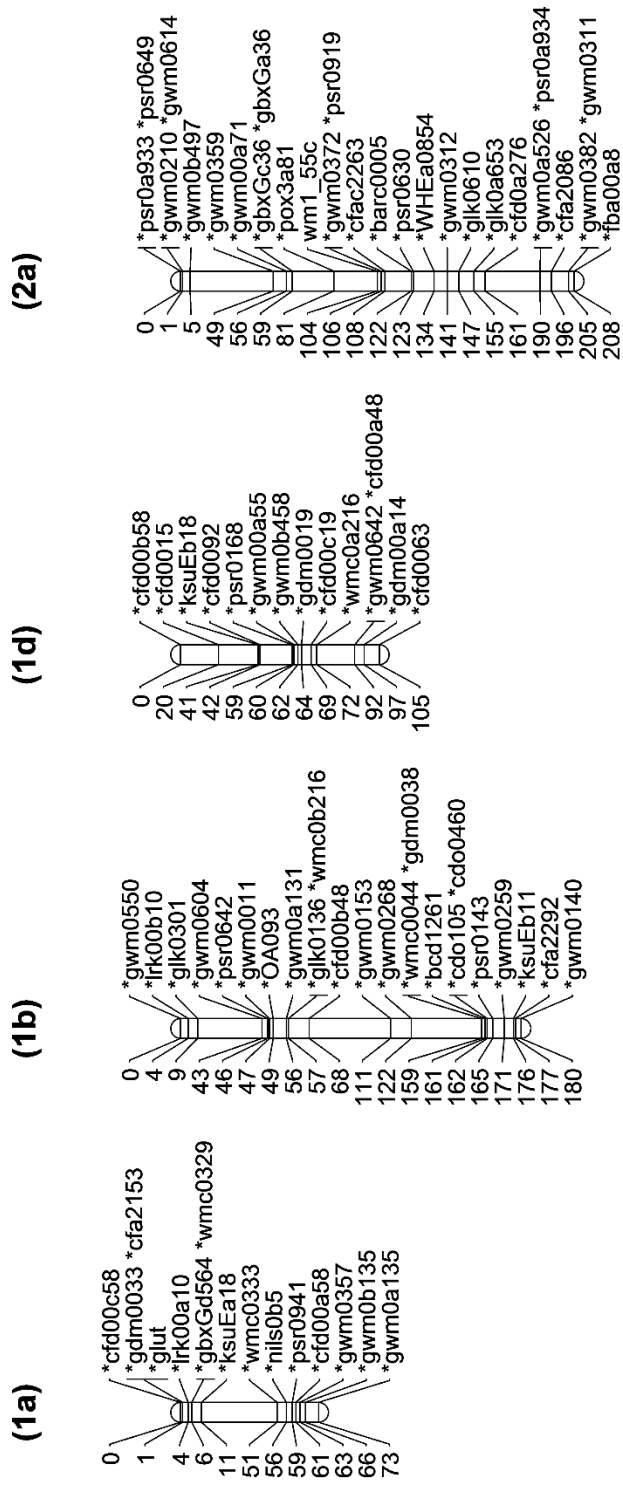
GC-MS technology was a useful approach to follow as there was no prior knowledge of the metabolite identities. However, the identity of the compounds reported here are only predictions and they need to be confirmed with further studies involving spiking with pure compounds or use of other instruments to identify the compound structure (Bollina et al., 2010, Hamzehzarghani et al., 2005). More extraction methods and analytical approaches are needed to detect different classes of compounds to better explain the plant-pathogen interactions. Metabolic profiling can help better understand the functions of metabolites, assist in selecting suitable genes for disease-resistance breeding program by coupling this knowledge with pathology, genomics and proteomic studies.

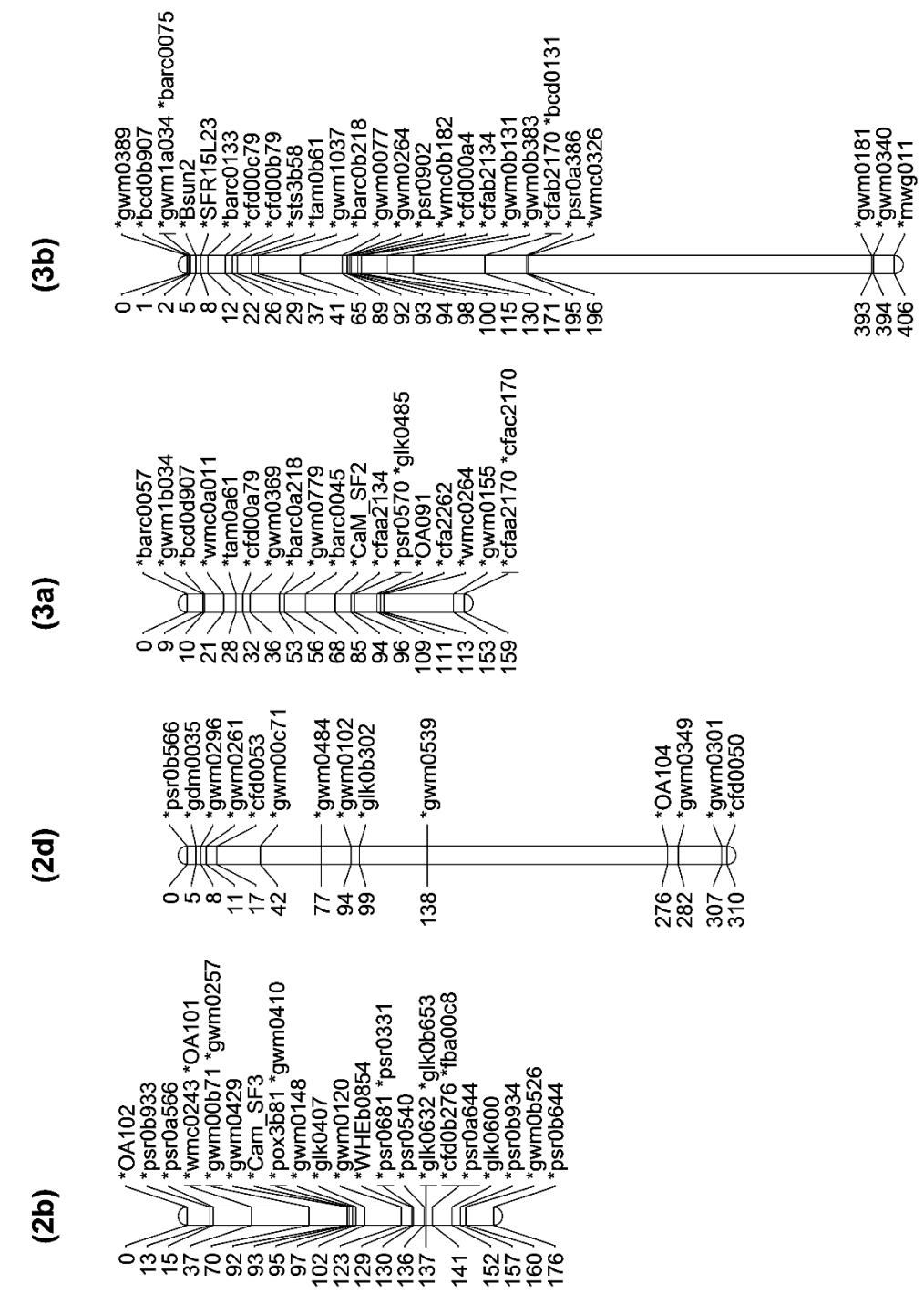
It is necessary to devise strategies to mitigate undesirable trade-offs associated with introduction of disease resistance genes. Some genes that provide a significant increase in resistance have negative pleiotropic effects and therefore have a net detrimental effect on yield as discussed in this thesis. Many barley breeders continue to use *mlo* alleles for powdery mildew resistance despite yield penalties and

increased susceptibility to a number of pathogens (Brown and Rant, 2013). Kjær et al. (1990) found that the associations between the three *mlo* alleles and agronomic traits were pleiotropic and it was possible to select for high yielding barley lines with mlo-based resistance to powdery mildew. Bjørnstad and Aastveit (1990) found that the genetic background had a significant effect on negative effects of *mlo* genes on yield in barley and high yielding resistant backgrounds could be selected. Jupateco NILs with *Lr34* did not exhibit increased susceptibility to different isolates of *Z. tritici* in this study. This implies that it is possible to select for genetic background of plants adapted to the presence of *Lr34* and *Lr46* genes to enhance resistance to rusts and powdery mildew without increasing susceptibility to non-biotrophs. Detailed studies of genetics of resistance genes and associated traits are necessary to form mitigation strategies.

## APPENDIX 1.

Linkage map of wheat derived from Arina X Forno Population




(2b)
0 *OA102
13 *psr0b933
15 *psr0a566
37 *wmc0243 *OA101
70 *gwm00b71 *gwm0257
92 *gwm0429
93 *Cam_SF3
95 *pox3b81 *gwm0410
97 *gwm0148
102 *glk0407
123 *gwm0120
129 *WHEb0854
130 *psr0681 *psr0331
136 *psr0540
137 *glk0632 *glk0b653
141 *cfd0b276 *fba00c8
152 *psr0a644
157 *glk0600
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(3a)
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(4a)
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(4b)
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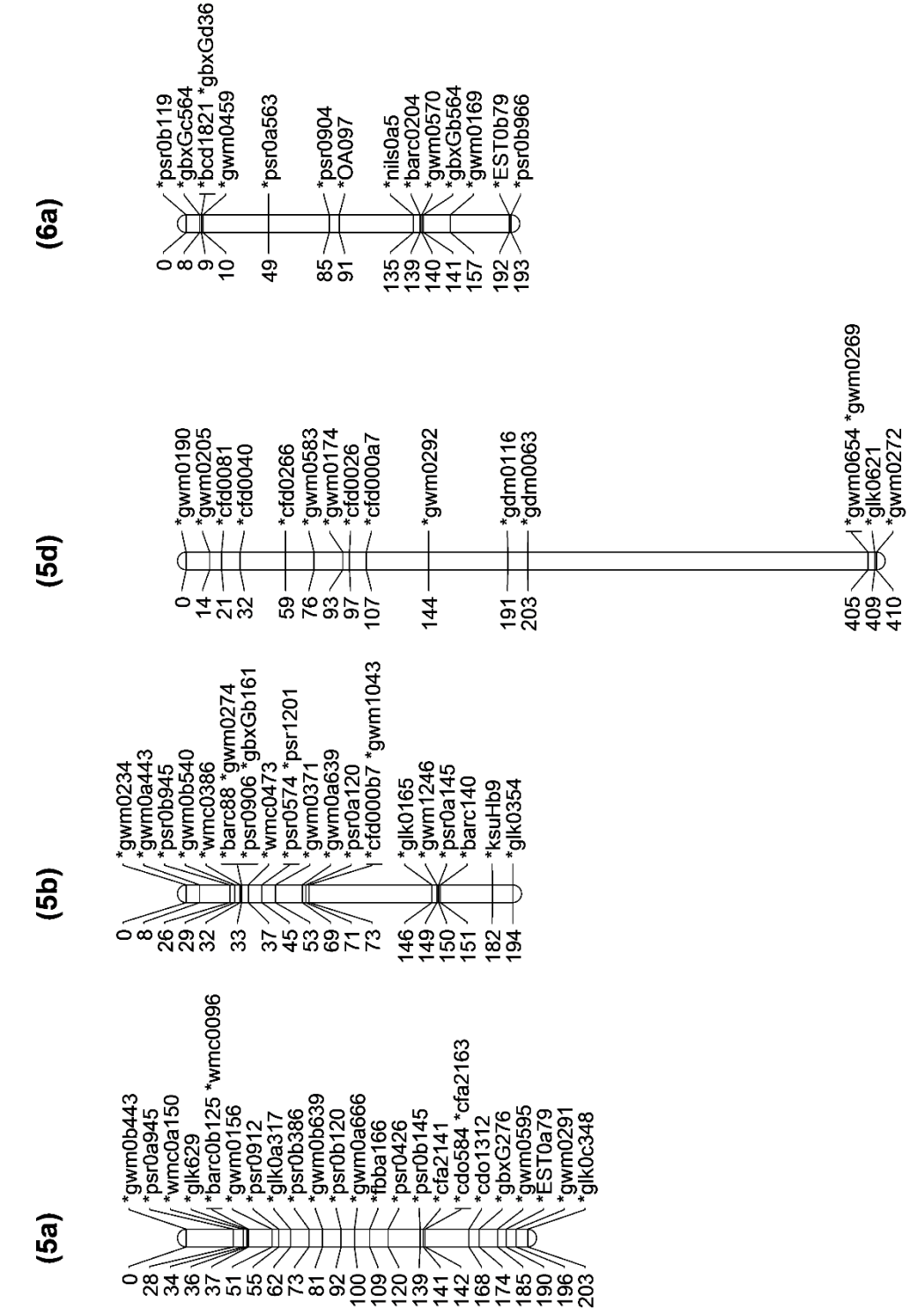
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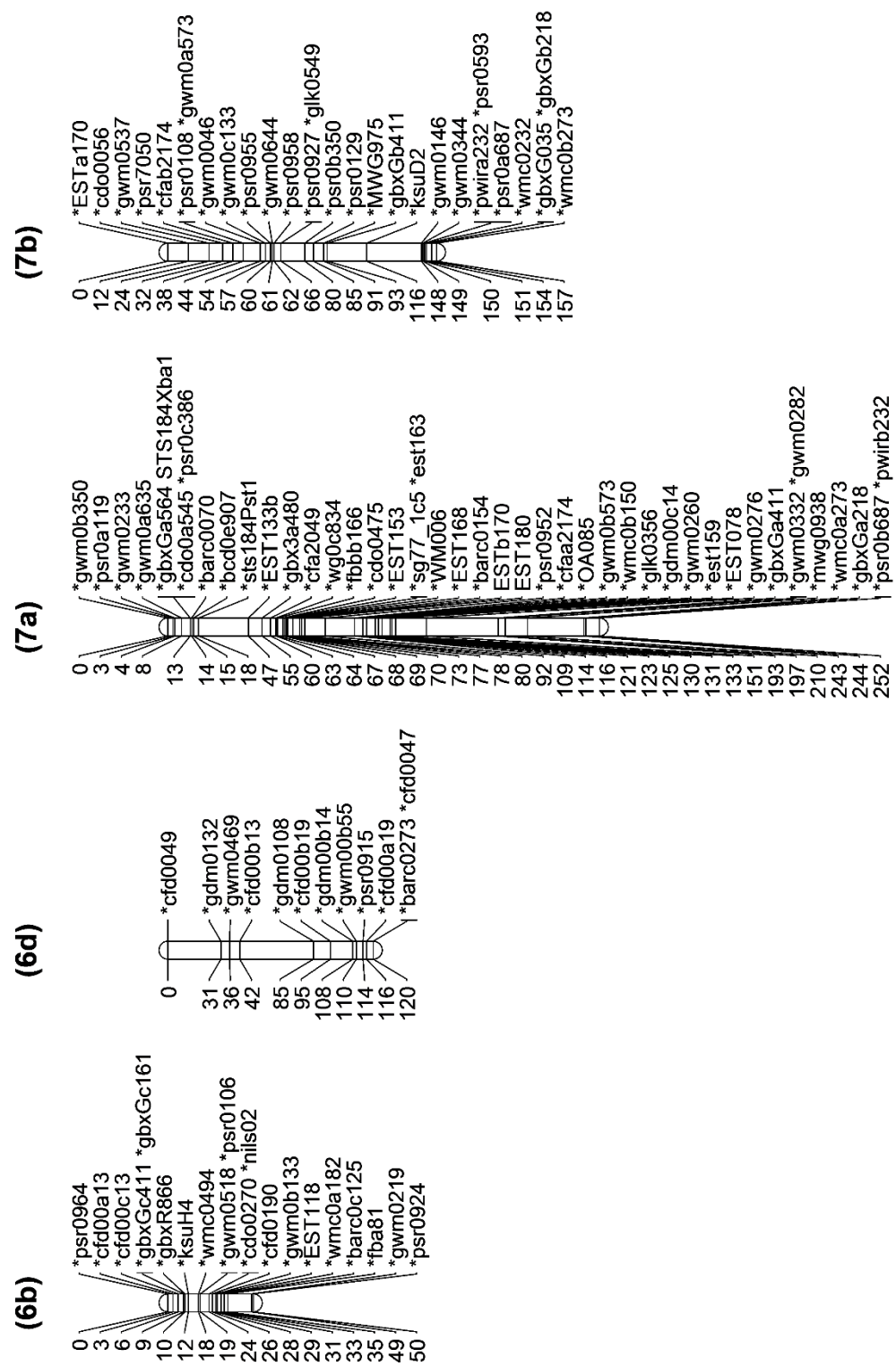
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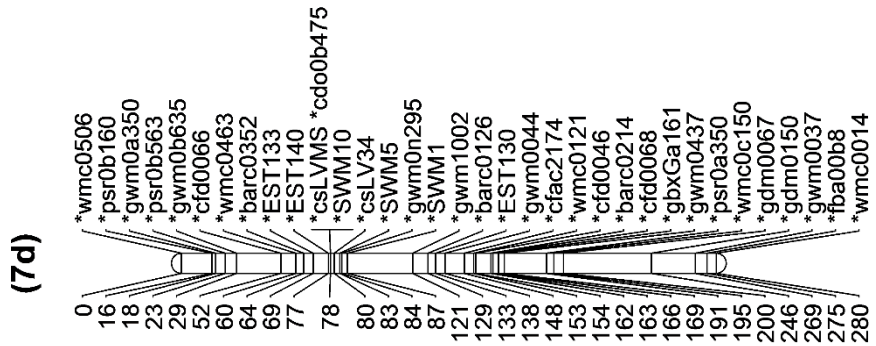
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121
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148
153
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163
166
169
191
195
200
246
269
275
280

# ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| Avr | Avirulence |
| Bgt | *Blumeria graminis* f.sp. *tritici* |
| cDNA | Complementary DNA |
| Cq | Quantitative cycle |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| Dpi | Days post inoculation |
| ET | Ethylene |
| ETI | Effector triggered immunity |
| GC | Gas chromatography |
| $H_2O_2$ | Hydrogen peroxide |
| HR | Hypersensitive response |
| JA | Jasmonic acid |
| LB | Lal Bahadur |
| Lr | Leaf rust |
| MAMP | Microbial–associated molecular patterns |
| MANOVA | Multivariate analysis of variance |
| MAPK | Mitogen-activated protein kinases |
| mL | Millilitre |
| Mm | Millimetre |
| mM | Millimolar |
| MNE | Mean normalized expression |
| MS | Mass spectrometry |
| NB-LRR | Nucleotide binding Leucine-rich repeats |
| NIL | Near Isogenic Line |
| PAMP | Pathogen–associated molecular patterns |
| PC | Principal component |
| PCA | Principal component analysis |
| PCD | Programmed cell death |
| PCR | Polymerase chain reaction |
| PM | Powdery mildew |
| PR | Pathogenesis related |
| PTI | PAMP triggered immunity |
| qPCR | Quantitative polymerase chain reaction |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| QTL | Quantitative trait locus |
| RLS | Ramularia leaf spot |
| RNA | Ribonucleic acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| SA | Salicylic acid |
| SAG | Senescence associated gene |
| SAR | Systemic acquired resistance |
| STB | Septoria tritici blotch |
| UV | Ultraviolet |
| Yr | Yellow rust |
| µg | Microgram |
| µL | Microlitre |
| µM | Micromolar |

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