Mass spectrometry based metabolic profiling of six-row barley (*Hordeum vulgare* L.) genotypes varying in resistance to *Fusarium graminearum*

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To My Mother

ABSTRACT

Fusarium head blight (FHB) of barley (*Hordeum vulgare* L.) is a devastating disease, caused by *Fusarium graminearum* (teleomorph: *Gibberella zea*), resulting in reduced yield and quality of grain by producing mycotoxins. The resistance in barley to FHB is quantitative and controlled by several genes, thus making it difficult to breed for resistance. In wheat (*Triticum aestivum* L.) and barley more than 100 quantitative trait loci (QTL) for resistance have been reported against FHB, but the mechanisms of resistance controlled by these QTL are unknown.

Metabolic profiling technology was applied to better understand the mechanisms of resistance and to phenotype resistance in barley genotypes against FHB. The current study aimed to: 1) identified the resistance related (RR) metabolites by comparing resistance in barley cultivars Chevron and Stander against FHB, and determined antimicrobial properties of selected RR metabolites under *in vitro* conditions; 2) determined the effects of selected RR metabolites on inhibition of trichothecene biosynthesis by *F. graminearum* under *in vitro* conditions; 3) identified biomarker metabolites, in six barley genotypes ('Chevron', H5277-44, H5277-164, M92-513, M122, and 'Stander') varying in resistance to FHB, for potential biomarker selection to screen barley genotypes for resistance.

Barley genotypes were mock-inoculated or pathogen-inoculated under greenhouse conditions; metabolites were extracted using aqueous methanol and analyzed using LC-ESI-LTQ-Orbitrap. XCMS and CAMERA algorithms were used to process the LC/MS output. Significant metabolites were classified as RR constitutive, and RR induced based on their greater abundance in resistant genotypes. Deoxynivalenol (DON) and its detoxified metabolite DON-3-*O*-glucoside (D3G), designated here as resistance indicator metabolites, were detected in both resistant and susceptible genotypes. The resistant cultivar Chevron had the least DON accumulation and high level of DON conversion to D3G. The selected RR metabolites varied in their ability to inhibit mycelial biomass and trichothecene synthesis by *F. graminearum in vitro*. The major potential biomarkers selected were: *p*-coumaric acid, sinapic acid, naringenin, naringenin-glucoside, kaempferol-glucosides, jasmonic acid, methyl jasmonate, and linolenic acid. In

conclusion, we have demonstrated here that the mass spectrometry tool can be used to better understand the mechanisms of quantitative resistance in barley against biotic stress and to select potential biomarkers to screen for FHB resistance.

RÉSUMÉ

La fusariose de l'épi (FE) de l'orge est une maladie dévastatrice causée par *Fusarium graminearum* (Gibberella zea) et résultant en pertes de rendement et de qualité du grain dû à la production de mycotoxines. La résistance à la FE chez l'orge peut être quantifiée et est généralement contrôlée par plusieurs gènes, ce qui limite l'amélioration de ce trait par de simples croisements. Plus de 100 loci de caractères quantitatifs (LCQ) de résistance contre la FE ont été rapportés chez le blé et l'orge, mais les mécanismes de résistance contrôlés par ces LCQ sont inconnus.

La technologie de profilage métabolique a été appliquée afin de mieux comprendre les mécanismes de résistance contre la FE et de 'phénotyper' la résistance de certains génotypes d'orge. Les objectifs de cette étude sont : 1) d'identifier les métabolites reliés à la résistance (RR) en comparant la résistance contre la FE des cultivars Chevron et Stander et de déterminer les propriétés antimicrobiennes des métabolites RR sélectionnés *in vitro*; 2) de déterminer l'effet des métabolites RR sélectionnés sur l'inhibition de la biosynthèse du trichothécène par *F. graminearum in vitro*; et 3) d'identifier des métabolites biomarqueurs chez six génotypes ('Chevron', H5277-44, H5277-164, M92-513, 'M122' et 'Stander') avec une résistance différente à la FE, afin de sélectionner des biomarqueurs permettant d'évaluer la résistance chez les génotypes d'orge.

Les génotypes d'orge ont été inoculés avec de l'eau ou un pathogène en conditions de serre. Les métabolites ont été extraits avec du méthanol aqueux et analysés avec LC-ESI-LTQ-Orbitrap. Les algorithmes XCMS et CAMERA ont été utilisés pour traiter le produit LC/MS. Des métabolites significatifs ont été classifiés en fonction de leur lien avec la résistance constitutive et exprimés en fonction de leur abondance qui est plus importante chez les génotypes résistants. Le déoxynivalénol (DON) et le glucoside DON-3-O, son métabolite détoxifié (D3G), désignés ici comme des métabolites indicateurs la résistance, ont été détectés dans les génotypes résistants et sensibles. Le cultivar résistant Chevron a produit le plus bas niveau de DON total et la plus grande proportion de DON converti en D3G. Les métabolites RR sélectionnés ont varié dans leur habileté à inhibiter la biomasse de mycélium et la synthèse du trichothécène par *G. zeae in vitro*. Les biomarqueurs potentiels qui ont été sélectionnés sont : l'acide *p*-coumarique,

l'acide sinapique, la naringinine, le glucoside de naringinine, les glucosides de kaempférol, l'acide jasmonique, le jasmonate de méthyl et l'acide linolénique. En conclusion, nous avons démontré que la spectrométrie de masse peut être utilisée afin de mieux comprendre les mécanismes de résistance quantitative chez l'orge contre le stress biotique et pour sélectionner des biomarqueurs potentiels permettant d'évaluer la résistance FE.

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LIST OF ABBREVIATIONS

15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivalenol
AME	Accurate mass error
AUDPC	area under disease progress curve
CAN score	Canonical score
CDA	Canonical discriminant analysis
D3G	DON-3-*O*-glucoside
DON	deoxynivalenol
dpi	days post inoculation
ESI	electrospray ionization
FHB	fusarium head blight
GC-MS	gas chromatography mass spectrometry
gs	growth stages
h	hours
hai	hours after inoculation
hpi	hours post inoculation
JA	jasmonic acid
LC-MS	liquid chromatography mass spectrometry
LD_{50}	leathal dose 50
LTQ	linear ion trap quadrupole
m/z ratio	mass to charge ratio
MeJA	methyl jasmonic acid
MS	mass spectrometry
NIV	nivalenol
NMR	nuclear magnetic resonance
PDC	proportion of DON conversion
PR	pathogenesis related
PR-Protein	pathogenesis related protein
PRr	pathogenesis related resistant

PRs	pathogenesis related susceptible
PSD	proportion of spikelets diseased
QTL	quantitative trait loci
RE	resistance equivalence
RM	resistant mock inoculated
RP	resistant pathogen inoculated
RR	resistance related
RRC	resistance related constitutive metabolite
RRI	resistance related induced metabolite
RT	retention time
SM	susceptible mock inoculated
SNA	synthetic nutrient-poor agar
SP	susceptible pathogen inoculated
TDP	total DON produced
UDP	uridine diphosphate glucose
UGT	UDP-glucosyltransferase
ZEN	zearalenone

List of genotypes used in the studies

Cultivars: Chevron, Stander, and M122

Breeding lines: H5277-44, H5277-164, and M92-513

CONTRIBUTIONS OF AUTHORS

This thesis has been written in the form of manuscripts. This format has been approved by the Faculty of Graduate Studies, at McGill University, as described in "Guidelines for Thesis Preparation and Submission". This research was designed by me in cooperation with Dr. Ajjamada. C. Kushalappa, thesis supervisor and a co-author of all the manuscripts. I conducted the greenhouse and laboratory work, analyzed the data, wrote the manuscripts, and the thesis under the supervision of Dr. Ajjamada C. Kushalappa. The current thesis is composed of seven chapters. The first and second chapters are Introduction and Literature Review, respectively. Chapters 3, 4, and 5 represent the greenhouse and laboratory experiments and were written in the form of manuscripts either published or to be submitted for publication. Chapters 6 and 7 are Summary Conclusions and suggestions for future research, and Contributions to Knowledge, respectively.

The first manuscript (Chapter 3) was co-authored by Dr. Ajjamada C. Kushalappa, who helped in planning the study, revised successive versions of the manuscripts, thesis, and provided valuable suggestions at all stages of this work. Mr. Kenchappa Kumaraswamy helped in handling the greenhouse experiments. Dr. Thin M Choo provided the six-row barley genotypes and their background information. Dr. Denis Faubert analyzed the samples based on LC/MS. Dr. Habiballah Hamzehzarghani helped me in statistical analysis. Mr. Yves Dion and Dr. Sylvie Rioux critically read the written manuscripts and gave valuable suggestions.

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CHAPTER 1

INTRODUCTION

Barley (*Hordeum* v*ulgare* L.) is one of the most important cultivated crops in North America. Barley grows better in cooler seasons with annual mean temperature of 22 ± 2 ^{0}C and requires annual rain fall of 390-430 mm (Alberta, Agriculture and Rural Development, 2007). The total barley production of Canada during the year 2009 was 9, 517,000 tonnes (Statistics Canada 2009a). Globally Canada is one of the largest commercial producers of barley with its area under barley production of 3, 505,000 ha during 2009 (Statistics Canada 2009b).

One of the most important diseases of barley in Canada is fusarium head blight (FHB) and the major causal organism of FHB is *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* schwein (Petch.)) (Sutton 1982; Parry et al. 1995; Bai and Shaner 2004). The general symptoms of FHB are whitening of infected spikelets, and in humid conditions the infected spikelet turns salmon pink to orange due to the presence of pathogen mycelia (Leonard and Bushnell 2003).

The yield losses due to FHB are very high, reaching a maximum during epidemic years (Nganje et al. 2004). In addition to yield loss, the grain quality is also reduced by the production of trichothecene toxins. More than 15 toxins have been reported from different *Fusarium* species, and the most common being: deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) (Mirocha et al. 1994). Deoxynivalenol inhibits protein synthesis and cell division, leading to eventual death of affected plant parts (Feinberg and McLaughlin 1998). The toxin also affects animal and human health; swine are the most affected (Trenholm et al. 1984). Accordingly, there are differential tolerance limits for toxin content stipulated for marketing barley grains and feed for human and animal consumption.

The six-row cultivars are more prone to FHB than the two-row barley cultivars (Buerstmayr et al. 2004; Yoshida et al. 2005). Chinese and Japanese scientists have screened more than 25,000 barley accessions and approximately 30,000 wheat cultivars in order to find the most resistant ones (Choo 2006). Though none had high resistance, those with partial resistance were released for commercial cultivation. The general

consensus is that resistance is the key to any integrated FHB management program (Gilbert and Tekauz 2000).

The resistance in wheat against FHB has been classified into two main types, type I and type II (Schroder and Christinsen 1963), though up to five types have been proposed (Mesterházy 1995). Type I, is resistance to initial infection and is generally evaluated based on spray inoculation. Type II, is resistance to spread of blight symptoms within a spike, generally evaluated based on single floret/spikelet inoculation. Type III, is resistance to kernel infection. Type IV, is tolerance (tolerant cultivars show the same level of disease severity but vary in grain yield). Type V, is resistance to mycotoxin (DON) accumulation. Type I and II, along with DON assessment in grains (type V), are most commonly used in wheat breeding programs. However, in barley the FHB symptoms usually do not spread internally from initially infected spikelet to adjacent spikelets (Bai and Shaner 1994) and accordingly the barley breeding programs are mainly based on type I resistance. The type II resistance significantly varies among wheat lines, and accordingly, it has been extensively studied (Steffenson 2003). Type II resistance in wheat and barley is quite stable and not much influenced by the environment, unlike type I resistance (Bai and Shaner 1994).

Resistance in wheat and barley to FHB is complex and is inherited quantitatively. More than 100 quantitative trait loci (QTL) have been identified and related to FHB resistance, especially in wheat, for both type I and II resistance, reflecting its polygenic nature. Resistance is found in diverse germplasm and frequently at different chromosomal locations (Shen et al. 2003; Paillard et al. 2004; Oliver et al. 2005; Buerstmayr et al. 2009). FHB resistance QTL have been reported on all seven chromosomes, particularly in six-row barley. However, often these were associated with agronomic and morphological traits, such as closed florets, heading date, spike density, plant height, etc. characters (Steffenson 2003). The evaluation based on type I and V varies a lot with environment, especially under field conditions due to polygenic nature of resistance. A cross between ‘Chevron’ and M69 yielded several minor QTL for FHB severity, kernel discolouration, low DON content, and a major QTL on chromosome 2H for FHB severity (de la Pena et al. 1999). The QTL-2H has been reported to be one of the major QTL in different studies (de la Pena et al. 1999; Ma et al. 2000; Dahleen et al.

2003; Horsley et al. 2006) but this also coincides with heading date (Horsley et al. 2006; Nduulu et al. 2007). Similar results were also observed for QTL on 3H (de la Pena et al. 1999; Zhu et al. 1999; Ma et al. 2000; Dahleen et al. 2003). A unique QTL, Fhb-2 independent of morphological characters was reported from barley genotype PI 643302 (Yu et al. 2010). The QTL identification is mainly based on disease severity and DON content. These are highly variable under field conditions because of variation in amount of inoculum, time of inoculation, opening and closing of florets during the day time, surface wetness and temperature during inoculation, etc. Accordingly, these are insufficient to characterize more than 100 QTL present in wheat and barley against FHB.

Application of new technologies involving genomics, transcriptomics, proteomics and metabolomics can reveal the mechanisms of resistance in Triticeae to FHB (Schena et al. 1995; Kagnoff and Eckmann, 2001). Molecular and biochemical basis of FHB resistance mechanisms are largely unknown, but there have been several attempts to elucidate the resistance mechanisms in wheat and barley (Chen et al. 1993; Bai et al. 2001b; Pritsch et al. 2001; Bernardo et al. 2007; Walter et al. 2010). Use of transcriptomics and proteomics to identify pathogenesis related proteins and genes for FHB resistance have been reported (Boddu et al. 2006, 2007; Golkari et al. 2007; Geddes et al. 2008). For example pathogenesis related proteins (PR1 –PR5) have been detected upon point inoculation in wheat (Pritsch et al. 2001). These PR proteins were expressed within 6 to 12 hours after pathogen infection and reached the peak after 36 to 48 h (Pritsch et al. 1999). Some of these PR proteins were shown to have *in vitro* antifungal activities (Yun et al. 1997). Greater abundances of chitinases (PR3) and thaumatin like protein (PR5) were detected in resistant barley genotypes (Geddes et al. 2008). Eight different PR proteins (PR1 to PR5 and PR8, 9 and 14) had antifungal activities for FHB (Anand et al. 2003; Edreva 2005).

Metabolomics has emerged as one of the functional genomics tools that contribute to our understanding of complex molecular interactions in biological systems (Fiehn 2002; Hall et al. 2002; Guy et al. 2008; Shulaev et al. 2008). The study of whole genome sequencing in different organisms like *Arabidopsis*, *Brachypodium distachyon*, rice (Oryza sativa), etc. have opened new horizons in genomics research. Metabolic profiling of whole plant or organism will complement to better understand functions of genes and

also their contribution to disease resistance. However, because of the occurrence of metabolites belonging to different chemical groups several extraction protocols, including different solvents are needed to detected metabolites. In addition, the chemical nature and concentrations of metabolites are highly variable so, require several metabolite detection platforms including gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography and mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS) and nuclear magnetic resonance (NMR) (Schauer et al. 2006; Dettmer et al. 2007; Allwood and Goodacre 2010). GC-MS is highly sensitive and cheap but is predominantly used to detect highly polar or nonpolar metabolites that are volatile, whereas liquid chromatography detects the compounds that are semi-polar (Lahaye et al. 2004; Roessner 2008). The preferred method for analyzing semi-polar metabolites is by using LC-MS with a soft ionization method, such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), resulting in positive mode (protonated) or negative mode (deprotonated) molecular masses (de Vos et al. 2007; Allwood and Goodacre 2010).

Wheat cultivars varying in resistance to FHB have been profiled and several resistance related (RR) metabolites have been identified (Hamzehzarghani et al. 2005, 2008a; Paranidharan et al. 2008). Many of the identified RR metabolites belonged to the phenylpropanoid, flavonoid, fatty acid, or terpenoid metabolic pathways. Several of these RR metabolites were known for antimicrobial, signaling, and cell wall enforcement functions. Phenolic acids especially, ferulic acid, *p*-coumaric acid, and sinapic acids are known to have antioxidant properties and also lead to lignin synthesis (Dixon and Paiva 1995). Metabolites with antioxidant and pro-oxidant properties could inhibit the toxin production in *Fusarium* spp. (Boutigny et al. 2008). Ferulic acid, a phenolic acid significantly inhibited trichothecene production by *F. culmorum* at 1 mM concentration under *in vitro* conditions (Boutigny et al. 2009). Natural phenolic acids from wheat bran inhibited trichothecene production under *in vitro* conditions (Boutigny et al. 2010). Phenolic acids inhibit trichothecene biosynthesis at the level of transcription of the *Tri* genes (Boutigny et al. 2009, 2010). Disruption of the *Tri5* gene, a major gene involved in trichothecene synthesis, lead to trichothecene nonproducing mutants of *F. graminearum* (Proctor et al. 1995).

Metabolomics of barley against FHB should be globally similar to that in wheat. Since GC-MS detects only volatile metabolites a metabolomics study based on LC-MS should be able to better explain the mechanisms of type I resistance in barley. Such a study should also complement mechanisms of resistance in wheat and other Triticeae against FHB.

1.1. General Hypothesis

It is hypothesized that barley cultivars varying in resistance to FHB also should vary in their metabolite profiles. Metabolites related to resistance are linked in their metabolic pathways and also to genes that regulate them, which in turn can be transferred to elite genotypes to increase resistance against FHB.

1.2. General objectives

1. To identify RR metabolites in barley against FHB based on metabolic profiling.
2. To study the effect of RR metabolites on *F. graminearum* biomass production and type B trichothecene biosynthesis.
3. To identify RR metabolites, in several barley genotypes varying in resistance against FHB, for potential use as biomarkers to screen breeding lines.

CHAPTER 2

GENERAL REVIEW OF THE LITERATURE

2.1. Barley

Barley (*Hordeum* v*ulgare* L.) is one of the most important world-wide cereal crops, and ranks fourth in its world cereal production (FAO). Barley is well adapted to most adverse climatic conditions through its evolution (Zhou 2010). Barley is a diploid (2n=14), with a 5000 Mbp genome and is a predominantly self pollinating species. The cultivated barley show a close relationship with that of wild and weedy barley i.e. *H. vulgare* L. subsp. *spontaneum* C Koch (Zhou 2010).

2.1.1. Barley types

Barley cultivars are classified as six-row and two-row, based on the spikelet arrangement. In six-row all the 3 spikelets on either side are fertile whereas in the case of two-row barley only the central spikelet is fertile and the lateral ones are reduced or absent. The difference in spike morphology is controlled primarily by a single gene (*vrs1)* with the two-row type being dominant (Fregeau-Reid et al. 2001). Two-row barley is known for greater yield than six-row barley. Two-row barley lines are often more resistant to FHB, mainly because of closed florets (Mesfin et al. 2003; Bai and Shaner 2004 and Sato et al. 2008). However, six-row barley contains more protein and is also preferred in North America for brewing as it contains a more diastase enzyme, which helps in the conversion of protein and starch into fermentable sugars. In the last decade, production of six-row malting barley cultivars has increased in western Canada and most of these cultivars are susceptible to FHB (Tekauz et al. 2000).

Barley can also be classified as hulled and hulless based on the presence or absence of pericarp (husk) adhered to the testal layers of the seed (grain) (Pourkheirandish and Komatsuda 2007). The hulled and hullessness feature is governed by *nud1* locus on chromosome 7HL (Franckowiack and Konishi 1997). Barley is also classified more often as yellow, purple and black based on the colour of the pericarp (Fregeau-Reid et al. 2001). Barley is widely consumed because of its ready availability and its dietary health benefits, including dietary fiber, β-glucan, and antioxidants (Ames

et al. 2006; Keenan et al. 2007). Barley is well known for its industrial use, especially in the malting and brewing industry, and is also used as animal feed (Choo 2006).

2.2. Fusarium head blight of barley

One of the major diseases of barley in Canada is FHB, also known as scab or ear blight. Fusarium head blight is caused by a range of *Fusarium* spp. but *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) (Petch.), *F. culmorum,* and *F. avenaceum* are considered as the predominant causal agents of FHB worldwide (Parry et al. 1995). Durum, spring wheat, and barley are most affected by this disease, but the disease is not confined to wheat and barley. FHB can affect other small grain crops, including corn, oats, rye, triticale, canaryseed, and some forage grasses (Parry et al. 1995).

Initially in the infected spikelets of barley water soaked spots appear and later they turn to dark brown colour and eventually spikelets are killed. Under humid conditions entire spikelet becomes blighted as salmon-pink to orange spore (macroconidia) masses develop on the glumes, lemma, and palea (Bai and Shaner 2004). Infected spikelets fail to produce any kernel and massive losses were observed under severe FHB epidemics. (Steffenson 1998). In addition to yield losses, *F. graminearum* also produces several mycotoxins in barley kernels (Bai and Shaner 2004; Choo 2006). FHB is an internationally important disease as the disease prevails in most parts of the world (Parry et al. 1995), especially in North America. Though, around 23 *Fusarium* species have been isolated from barley kernels, *F. graminearum* is the most prevalent species in North America and the Far East (Choo 2006). Various wheat genotypes showed similar reactions to different *F. graminearum* isolates; this is a very important observation as resistance is not related to any particular isolate of *F. graminearum* (Toth et al. 2008). Two environmental factors, temperature and moisture, have important influence on fusarium head blight epidemics. The optimum conditions for infection of heads by *F. graminearum* are 25 ^{0}C and 100% humidity for at least 24 h, and in such conditions FHB is capable of destroying a wheat farm in a few weeks (McMullen et al. 1997).

2.2.1. *Fusarium* trichothecene mycotoxins

Fusarium trichothecenes are sesquiterpenoid mycotoxins produced by *Fusarium* spp. and known to add virulence to the disease (Bai and shaner 2004; Foroud and Eudes 2009). On a global note, DON, NIV, and ZEN are the most commonly found *Fusarium* toxins in barley in greater quantities followed by 3-ADON, 15-ADON, T-2, and H-2 are few of the toxins present in minor quantities. Among these mycotoxins produced by *Fusarium*, deoxynivalenol (DON) is the major toxin, which has raised much concern because the tolerance levels in feed recommended by the Canadian Food Inspection Agency for DON is <1 mg kg^{-1} for swine, young calves, and lactating dairy animals. This level is slightly greater for poultry and cattle (<5 mg/kg) (Choo 2006). Studies show that DON level is as high as 15 mg kg^{-1} (Yoshihizawa and Jin 1995) where as the maximum permissible limit is less than 2 mg kg^{-1} (Dexter and Nowicki 2003; Shaner 2003). Barley samples are frequently contaminated by two or more types of *Fusarium* toxins, the survey conducted in eastern Canada showed that almost 30% of the samples were contaminated with 2 to 4 types of toxins (Campbell et al. 2002).

It is alarming that the *Fusarium* toxins can survive food processing or brewing, and toxins like DON, NIV, and ZEN have been detected in barley-based beer samples and in infant cereals (Choo 2006). Although the levels of many toxins produced by *Fusarium* species are relatively low they could have additive and synergistic effects on human and animal health. Toxins produced by *Fusarium* can remain in animal tissues, milk, and eggs (Yiannikouris and Jouany 2002) and also the toxins can be carried over into animal derived food products. So the amount of DON accumulation in barley and wheat determine how the grain will be used in industries. Correlation between greater amounts of DON and high disease severity has not been observed (Bai et al. 2001b; Bai and Shaner 2004). Severely infected spikelets are shriveled and shrunken and are blown away during threshing and cleaning processes, so only less severely infected kernels will remain and this can erroneously lead to detection of low levels of DON from such samples.

2.2.2. FHB management

The currently available disease management strategies include use of resistant cultivars, foliar spray of fungicides, and other cultural practices to minimize the incidence of FHB. But none of the above said measures can give foolproof resistance to FHB since none of the screened barley cultivars are immune. Fungicides, like Js399-19, or tebuconazole, are effective against FHB and their application resulted in lower DON accumulation in wheat (Zhang et al. 2009). Carbendazim is one of the major FHB fungicides used in China but recent studies showed the emergence of resistant isolates of *F. graminearum* under field conditions (Chen and Zhou 2009). Several disease forecasting methods have been developed to optimize fungicide application (Paul et al. 2010). Fungicides can minimize the yield losses but may not reduce the mycotoxin contamination to the tolerable limit for human and animal consumption (Bai and Shaner 2004).

2.3. Resistance of barley genotypes to FHB

More than 25,000 accessions from China have been screened for FHB resistance but none seem to be immune to *F. graminearum* (Choo 2006). However those accessions which are less susceptible to FHB are named as resistant accessions because the frequency of FHB resistance was less than 0.1%. Seemingly, many of the resistant genotypes identified have not been used extensively in barley resistance breeding programs apart from ‘Mimai 114’, ‘Gobernadora’ and ‘Clho4196’. ‘Clho4196’ is a two-row cultivar from China and it is reported as one of the most resistant cultivars to *F. graminearum* (Heta and Hiura 1963; Urrea et al. 2002; Buerstmayr et al. 2004) and it has been used extensively as one of the parents in six-row barley breeding programs in North Dakota (Urrea et al. 2002).

Till now ‘Chevron’, a six-row cultivar is the best known resistant cultivar to FHB. Though there have been several attempts to identify and transfer the resistance from wild species to cultivated barley, some wild species that appeared to be resistant to *F. graminearum* were strongly associated with very poor agronomic characteristics (Choo 2006). Development of resistant six-row barley cultivars from two-row resistant barley is

a difficult task because of the very close linkage between the two-row *vrs1* trait and a major FHB resistant QTL on chromosome 2HL (Horsley et al. 2006).

2.3.1. Apparent resistance to FHB

Interestingly, there are some morphological characteristics which are showing apparent resistance to FHB in two-row barley such as, spike density, lax spike, closed flowering, and early heading date. Higher spikelet density in the six-row barley types which can retain longer duration of wetness so increasing infection levels. Closed (cleistogamous) flowering in the two-row types which avoid direct deposition of spores on ovary and thereby more resistant. Most of the six-row types have open (chasmogamous) florets and so are more susceptible (Yoshida et al. 2005: Kubo et al. 2010). It is well established that especially in wheat with open florets the spikelets are most susceptible at the anthesis stage (Zhai 1981; Vivar et al. 1997). Also, the extruding anthers support spore germination by producing choline and betaine (Strange et al. 1978; Browne and Brindle 2007). The early heading barley types are less susceptible as they avoid cumulative build-up of inoculum which progress through the season (Heta and Hiura 1963; Vivar et al. 1997).

2.3.2. True resistance to FHB

Several biochemical characters were associated with true resistance. Coloured barley appears to be a source of resistance to FHB. Black barley genotypes were more resistant (Zhou and Chao 1991) as they contained more lignin, and its precursors such as ferulic acid, and *p*-coumaric acid. Purple coloured lemma, and pericarp make barley less susceptible to FHB (Choo et al. 2004) since these contain considerable amount of the anthocyanins, delphinidin and cyanidin (Mullick et al. 1958).

Breeders use five types of resistance to screen breeding lines, though only the first two are common: type I is resistance to initial infection assessed based on spray inoculation; type II is resistance to spread of disease within spike; type III is resistance to kernel discolouration; type IV is tolerance to FHB; type V is resistance to mycotoxin accumulation (Schroeder and Christensen 1963; Mesterházy 1995). Resistance by constitutive and/or induced metabolites was linked to type I resistance (Doohan et al.

2000; Mohammadi and Kazemi, 2002; Siranidou et al. 2002; Hamzehzarghani et al. 2005 and 2008a). DON as virulence factor was linked to the type II mechanism of resistance as DON non-producing strains contained to the only inoculated spikelet in wheat and barley (Proctor et al. 1995; Desjardins et al. 1996; Bai et al. 2001b). As in the case of wheat, resistance to FHB in barley is quantitatively inherited, and the estimates for heritability of both resistance to FHB and DON accumulation are low to moderate. The literature suggests that environment and genotype interactions are important to the expression of resistance to FHB and DON accumulation (Bai and Shaner 2004).

2.4. Resistance to DON accumulation

More than 15 *Fusarium* toxins have been detected in infected barley kernels (Choo 2006). Among all the toxins produced by *Fusarium* DON is of prime importance because it reduces the quality of grain and for malting and brewing purposes the tolerance is zero. Infected plants reduce DON accumulation by converting the DON to less toxic conjugates. The mechanisms by which DON is detoxified are through glycosylation, acetylation and de-epoxidisation.

Though chemical transformation involves conjugation of toxic compound to polar substances like sugars, amino acids and sulphates, so far only the conjugation of *Fusarium* toxin DON into sugars is known in plants (Berthiller et al. 2005). Glycosylation of DON seems to be a very effective method of chemical transformation by plant enzymes into stable and non toxic storage forms of *Fusarium* toxin. Identification of UPD glycosyletransferase in *Arabidopsis thaliana* (Poppenberger et al. 2003) was break through research in understanding the mechanism of chemical transformation of DON into glycosylated conjugate. Glycosylated DON had significantly reduced capacity for inhibition of protein synthesis (Poppenberger et al. 2003). Natural occurrence of DON-*O*-glucoside in *Fusarium* infected wheat was identified and linked to Qfhs.ndsu-3BS (QTL) contributing to DON resistance (Lemmens et al. 2005). Formation of glycosylated conjugates is not limited only to DON. Schneweis et al. (2002) identified the presence of zearalenone-4-*β*-D-glucopyranoside in *Fusarium* infected wheat samples, showing the importance of study in other *Fusarium* toxin conjugates formed in plants.

Fusarium protects itself from self destruction by trichothecenes through sequestration, effluxing toxins to the host, and by self-acetylation. Acetylation of DON at the C-3 position leads to less toxic DON derivatives and is an important phenomenon (Alexander, 2008). This lead to the identification of a DON acetylation gene in *Fusarium* spp. Kimura et al. (1998 a, b) discovered the *Tri101* gene which encodes a 3-*O*-acetyltransferase enzyme for DON acetylation in *F. graminearum*. Expression of *Tri101* gene from *F. sporotrichioides* in wheat and barley showed reduced FHB incidence and DON accumulation under greenhouse conditions, but the field studies failed to confirm the findings; interestingly, they found slightly greater levels of DON accumulation in barley transgenic lines (Muhitch et al. 2000; Manoharan et al. 2006). It is possible that plants could have de-acetylases (Boutigny et al. 2008), though it requires an experimental proof of reconversion of 3ADON into DON. De-epoxide forms of DON were less toxic than the epoxide forms. There are no de-epoxide forms reported in plants (Boutigny et al. 2008). However, the re-conversion of these conjugated non-toxins into toxic compounds during food/feed processing and during digestion by animals is dangerous (Bethiller et al. 2005; Alexander 2008; He et al. 2010).

Greater levels of resistance were associated with lesser levels of DON (Mesterházy et al. 1995). Yu et al. (2008) reported lowest and highest mean DON content for resistant and susceptible wheat cultivars, respectively. Though low DON content has been proposed as a type V resistance to FHB, the correlation between disease severity and DON content in grains was not always positive (Mesterházy et al. 1999; Miedaner et al. 2001; Lemmens et al. 2005). Variability reported for DON resistance is mainly due to variation in the host, pathogen chemotypes, and environmental factors. Studies involving controlled conditions could provide valuable sources of information as it minimizes the evenivronmental factors.

2.5. Genetics of barley resistance to FHB

More than 100 QTL have been identified in wheat against FHB but the numbers reported from barley are very few (Choo 2006; Buerstmayr et al. 2009). QTL have been reported from all the seven chromosomes, except for 7D, but most of these are unstable (de la Pena et al. 1999; Mesfin et al. 2003; Choo 2006; Buerstmayr et al. 2009). Generally

these QTL contain several genes and often some are linked to pleiotropic effects, making them unsuitable for further breeding. In barley, among the identified QTL, QTL-1H for low FHB severity and DON accumulation was found in different studies (de la Pena et al. 1999; Dahleen et al. 2003; Mesfin et al. 2003) and this QTL was found to be associated with heading date (Mesfin et al. 2003). Molecular mapping of six-row cultivars 'Chevron' and M69 identified 3 major QTL *Fhb*1, *Fhb*2, and *Fhb*3 and were associated with FHB severity, kernel discolouration, and low DON content on chromosome 2H, respectively (de la Pena et al. 1999; Ma et al. 2000; Mesfin et al. 2003; Dahleen et al. 2003; Horsley et al. 2006). However, these were also associated with heading date, *vrs1* locus (row type) and plant height (Horsley et al. 2006). Chromosome 5H carried a major QTL for FHB resistance and DON accumulation and minor QTL for heading date and plant height (de la Pena et al. 1999; Dahleen et al. 2003; Mesfin et al. 2003). In similar studies it was shown that chromosome 3H, 4H, and 6H also carried QTL for FHB and DON accumulation. In an attempt to detect QTL for FHB resistance from five top cross progeny of two-row and six-row barley identified 32 QTL but only 10 QTL were consistent among years. Among the identified QTL none of them were associated with the *vrs1* locus, indicating pleiotropic effect of the *vrs1* locus in earlier studies. These QTL were associated with flowering type (*cly1*) on chromosome 2H (Sato et al. 2008). QTL for FHB resistance was reported near the *vrs1* locus in a two-row and six-row barley cross, showing further evidence for pleiotropic effects of two-row types on FHB resistance (Hori et al. 2005). Mutational breeding approach to convert the two-row 'Clho4196' into a six-row phenotype resulted in undesirable characters like, tall stature and late ripening (Boyd et al. 2008). However, the coincidence of association of QTL and morphological characters could also be due to tight linkage. However, the brewers prefer six-row associated characteristics. Attempts have been made in wheat to fine map the QTL (Buerstmayr et al. 2008, 2009). Still the functions of these QTL are not known. It is crucial to know the functions of these genes for future stability of the breeding program, as often these QTL are associated with pleiotropic effects. Other technologies like transcriptomics, proteomics, and metabolomics can be used to better understand the mechanisms of resistance.

2.6. Biochemical resistance

Biochemicals, proteins and metabolites, have been used to better understand the mechanisms of FHB resistance.

2.6.1. FHB resistance through proteins

Pathogenesis related proteins are well established for plant disease resistance as antimicrobial compounds interfering with invading pathogen. So far eighteen families of PR proteins have been identified (Edreva 2005; Liu and Ekramoddoullah 2006). Several PR proteins have been detected in wheat and associated with FHB resistance; different groups of PR (PR1 to PR5) proteins have been shown to have antifungal activity. PR-2 (β-1-3 glucanase) and PR-3 (chitinase) proteins inhibit fungal growth by degrading the fungal cell wall (Anand et al. 2003). PR5 (thaumatin-like protein) exhibits sequence homology to thaumatin of rice protein and showed that PR5 binds to β-1-3 glucan of fungal cell walls and alters the plasma membrane (Trudel et al. 1998; Chen et al. 1999;Selitrennikoff 2001). Transgenic wheat lines overexpressing class II chitinases showed enhanced resistance to FHB and lower levels of DON (Shin et al. 2008). Expression of an antibody fusion protein (recombinant antibody) derived from chicken and an antifungal peptide from *Aspergillus giganteus* in wheat transgenic lines showed increased level of type I and II resistance than non transgenic controls (Li and Yen 2008). Several PR proteins associated with FHB resistance were identified in resistant and moderately resistant barley cultivars (Geddes et al. 2008). Greter abundances of PR-3 and PR-5 proteins were associated with resistance, and decrease in abundance of these proteins observed in the susceptible cultivar, 'Stander' (Geddes et al. 2008). Antioxidant, jasmonic acid signaling, and PR-proteins were up-regulated following *F. graminearum*-wheat infection (Zhou et al. 2006).

2.6.2. FHB resistance through metabolites

Plants adapt to biotic and abiotic stresses through production of low molecular mass natural products, known as secondary metabolites. These secondary metabolites provide resistance to plants during oxidative or pathogenic stress conditions, and arise through different metabolite pathways such as phenylpropanoid, fatty acid, flavonoid,

alkaloid, and isoprenoid pathways. These metabolites may include phytoalexins as they are synthesized *de novo* upon pathogen infection (Pedras et al. 2007). Upon pathogen infection, antimicrobial compounds are induced by signal transduction pathways through receptors by host resistance genes (Dangl and Jones 2001). Different metabolites belong to phenylpropanoid, fatty acid, flavonoid, alkaloid, and isoprenoid pathway groups may have antioxidant, signaling and cell wall fortification functions (Dixon 1986; Dixon and Paiva 1995; Dixon 2001). Phenylpropanoid derivatives and other metabolites are used for defensive functions by different plant species, and most of the plant natural products have broad spectrum antimicrobial properties (Naoumkina et al. 2010).

Phenolic acids may play a significant role in plant disease resistance against pathogens and these can be both constitutive and induced upon pathogen infection (Goodman et al. 1986). Upon pathogen infection plants may accumulate massive amounts of phenolic compounds at the site of infection (Matern and Kneusel 1988; Nicholson et al. 2008). Resistant wheat cultivar 'Frontana' produced high levels of free phenolic acids in the lemma and palea following FHB infection (Siranidou et al. 2002). Ferulic acid and *p*-coumaric acids, precursors of lignin, were the most predominant phenolic compounds present in the outer layers of barley kernels (Nordkvist et al. 1984) and these inhibited *F. graminearum* and *F. culmorum* growth (McKeehen et al. 1999). The presence of free and bound phenolic acids and their polymerization in plant cell walls are the first step in plant defense against invading pathogens (Matern and Kneusel 1988). Association of cell walls development and high proportion of *p*-coumaric acid esterification with lignin in wheat straw has been observed (Sun et al. 1998). Cell wall lignification in wheat and barley leads to protection from fungal penetration (Bechinger 1999) and stops the spread of *F. graminearum* (Jansen et al. 2005).

Phenolic acids have very strong antioxidant properties (Rice-Evans et al. 1996). Production of plant secondary metabolites in kernels with antioxidant properties, especially phenolic acids were effective in reducing the mycotoxin accumulation in kernels (Bily 2003; Chen et al. 2006). Ferulic acid, showed a significant inhibition of mycelial growth at 2.5 mM concentration under *in vitro* studies (Boutigny et al. 2008). Trichothecene/DON produced by *F. graminearum* and *F. culmorum* was completely inhibited at 1 mM concentration; this could be due to antioxidant property of phenolic

acids (Boutigny et al. 2009). Natural phenolic acids extracted from wheat bran and also reconstituted mixtures of phenolic acids showed significant inhibition of DON biosynthesis by *F. culmorum* under *in vitro* conditions (Boutigny et al. 2010). Quantitative trait loci mapping for these antioxidant, signaling and cell walls fortification components could be a useful tool for enhancing resistance to FHB in breeding programs.

Flavonoids are also known for their antioxidant properties (Rice-Evans et al. 1996; Pietta 2000). Flavonoids are synthesized downstream of the phenylpropanoid pathway; 4-coumaryl CoA being the precursor for flavonoid synthesis (Naoumkina et al. 2010). Sakuranetin, a flavanone was induced from ultraviolet-irradiated leaves of rice and showed antimicrobial activity for rice blast disease caused by *Pyricularia oryzae* infection (Dixon 2001). Flavonoids and proanthocyanidins have antimicrobial, radical scavenging activities, in addition to affinity towards proteins and enzymes, preventing the enzymatic activity (Santos-Buelga and Scalbert 2000). Naringenin from barley genotypes acts as an antimicrobial compound by inhibiting spore germination of *Pyricularia oryze in vitro* (Mizutani et al. 1996). Naringenin exhibited greater antioxidant capacity through radical scavenging activity *in vitro* (Cavia-Saiz et al. 2010). Conjugated forms of flavonoids are much more efficient in protecting plants against pathogens. For example, glycosilated forms of flavonoids from carnation (Dianthus caryophyllus) generally have increased activities against *F. oxysporum* (Galeotti et al. 2008). Catechin is one of the most commonly described flavonoids present in barley and was up-regulated significantly during FHB infection in barley; the authors postulated that catechin could act as a marker for FHB resistance in barley (Eggert et al. 2010). *In vitro* inhibition of growth of *F. graminearum* and other *Fusarium* spp. was caused by flavonoids and flavones (Silva et al. 1998). Lack of flavonoid biosynthesis in barley seed testa was related to increased penetration of *F. graminearum, F.culmorum,* and *F. poae* (Skadhauge et al. 1997).

Fatty acids, both saturated and polyunsaturated, are known to have antimicrobial, antioxidant, and pro-oxidant properties (Henry et al. 2002; Blokhina et al. 2003; Erasto et al. 2007). Polyunsaturated fatty acids like linolenic acid oxidize to oxylipins *viz*., jasmonic acid (JA), and in turn the JA converts to methyl jasmonate (MeJA) (Blee 2002). Oxylipins are known for plant defense signaling and induction of PR proteins (Schweizer et al. 1997; Panstruga et al. 2009). Jasmonic acid and MeJA are important lipid-driven

hormone signaling molecules for plant defense and stress responses (Balbi and Devoto 2007; Farmer 2007;Zhang and Xing 2008; Panstruga et al. 2009). Jasmonic acid acts as a signaling molecule for necrotrophic pathogens like *F. graminearum,* whereas salicylic acid has been reported as a signaling molecule for biotrophs (Panstruga et al. 2009). Jasmonate and ethylene signaling genes were up-regulated during FHB infection in resistant wheat cultivar, 'Sumai3' and these were associated with down-regulation of salicylic acid pathway genes (Li and Yen 2008), indicating the importance of JA pathway signaling in FHB resistance.

Enzymes like cutinases and lipases produced by pathogens degrade the cuticular wax, thereby facilitating the penetration of hyphae. Subsequently release of cutin monomers by *Fusarium* cutinase complexes with nonspecific lipid transfer proteins from the host and lead to signaling cascade for cutin repair (Blein et al. 2002). Fatty acid signaling also leads to the production of oxylipins, like JA and its conjugates (Walter et al. 2010). Plant cuticular wax is a mixture of different fatty acids along with suberin and glycerol (Heredia 2003; Franke et al. 2005). Waxy surfaces of spikelets could drastically reduce the moisture availability, thereby affecting the spore germination, contributing to type-I resistance (Yoshida et al. 2005). Increased deposition of cuticular wax on the adaxial surface of transgenic rice leaves was observed when overexpression of transcription factor OsWRKY89 In turn; this transcription factor was induced by MeJA and lead to disease resistance in rice against rice blast fungi (Wang et al. 2007).

Over-expression of JA methyltransferase, a key enzyme in conversion of JA to MeJA in *Arabidopsis,* caused enhanced resistance to necrotrophic fungi, *Botrytis cinerea* (Seo 2001). DON induced defense-related genes similar to *F. pseudograminearum* in wheat suggesting DON probably induced oxylipin production and defense (Desmond et al. 2008). When wheat cultivars were treated with MeJA, many of the defense related (anti-microbial, oxidative stress, signaling molecules) genes were up-regulated (Desmond et al. 2008). Defense-related genes were up-regulated upon treatment with DON in wheat cultivars (Desmond et al. 2008) and several RR metabolites were produced (Paranidharan et al. 2008).

Many of the phenylpropanoid, flavonoid and signaling pathway metabolites are known to have a role in FHB resistance. Profiling of these pathway metabolites upon *F.*

graminearum infection could lead to identification of many of the induced metabolites and study of their function.

2.7. Metabolomics applications to study resistance to FHB

The plant kingdom has an enormous biochemical diversity and its metabolite number could exceed 200,000 (Dixon and Strack 2003; Goodacre et al. 2004; Oksman-Caldentey and Saito 2005). It has been estimated that *Arabidopsis* has 5,000 different primary and secondary metabolites in leaves, but so far, only 10 % of the metabolites have been annotated using current technologies available (Bino et al. 2004). Metabolic diversity in kinds of chemicals, amounts, and multiple extraction protocols suggestes that different analytical platforms are needed (Sumner et al. 2003; Dettmer et al. 2007; Hegeman 2010).

Metabolomics has emerged as one of the functional genomics tools that contribute to our understanding of the complex molecular interactions in biological systems (Fiehn 2002; Hall et al. 2002; Bino et al. 2004), and it is rightly defined as an integral part of systems biology (Weckwerth 2010). This further enables us to infer relevant associations between metabolites and phenotypes of the organism (Bino 2004). As Weckwerth (2004) reported, biochemical phenotypes of an organism are the final result of interaction between the genotype and the environment. One of the important steps in metabolomics is identification and quantification of variation in metabolites in a given situation or organism to study the dynamics of the metabolome and to analyze the metabolomics pathways to represent the functional role of each metabolite following stimulus/stress for better understanding of the phenotype (Fiehn 2002; Guy et al. 2008).

Tomato fruit metabolites have been identified and incorporated into the MoTo (Metabolome Tomato) database by using reverse phase liquid chromatography coupled to quadrupole time of flight mass spectrometry (Moco et al. 2006). The genetic basis of natural variation was studied using LC-QTOF-MS, which uncovered many qualitative and quantitative differences in RILs obtained from two divergent *Arabidopsis* accessions, which enabled the identification of QTL for 75% of the mass signals identified (Kurentjes et al. 2006). Genetic differences of two distinct barley cultivars for boron toxicity were elucidated using a GC-MS-based metabolomics approach (Roessner et al.

2006). Using a mass spectrophotometry based metabolic approach, 157 metabolic QTL (mQTL) were identified for biomass and metabolic content in *Arabidopsis* recombinant inbred lines and introgression lines (Lisec et al. 2006). LC-Q ion trap mass spectrometry was used to identify the significant difference of defense metabolites (flavonoids) in wheat varieties (Loset et al. 2007). Very few metabolites were identified based on NMR, but choline and betain were associated with susceptibility (Browne and Brindle 2007). GC-MS metabolic profiling of wheat and barley tissues 72 h after *F. graminearum* infection was distinctly different from control tissues (Skadsen et al. 2007).

2.7.1. Technological platform for metabolomics application to FHB

Metabolic profiling is used to detect and quantify all the metabolites in a sample. Multiple analytical platforms are being used for metabolic profiling (Sumner et al. 2003; Shulaev 2006; Roessner 2008; Allwood and Goodacre 2010). These platforms include NMR, GC-MS, LC-MS, Capillary electrophoresis-MS and Fourier transform infra red spectroscopy (Shulaev et al. 2008). GC-MS is a widely used analytical technique (Roessner 2008; Shulaev et al. 2008). Though GC-MS can detect hundreds of compounds it can only detects either extremely polar or non-polar volatile compounds and also requires chemical derivatization (Shulaev 2006; Shulaev et al. 2008). The main advantage of GC is its large available fragmentation libraries (Roessner et al. 2000; Sumner et al. 2003). Where as in case LC-MS, limited availability of commercial fragmentation libraries and ion suppression are a major problems but now by using nano electrospray they have overcome the ion suppression problem (Scholz et al. 2004; Dettmer et al. 2007; Allwood and Goodacre 2010). Clearly no single analytical technique can detect thousands of plant metabolites in a cell or tissue, so combination of extraction methods and analytical platforms should be used for comprehensive analysis.

2.7.2. Bioinformatics tools for metabolomics data processing

Metabolite identification, Challenges and Libraries available: Identification of metabolites is the most challenging part of metabolomics. Like genomics, transcriptomics, and proteomics, metabolomics too generates huge data sets. Handling such large data sets is an overwhelming task and demands specialized statistical and

bioinformatical tools. Metabolomics data processing is most time consuming, as there are different steps in the pipeline of metabolite identification, *viz*. reduction of noise (S/N ratio), deconvolution of spectra, peak picking, chromatogram alignment, identification, and quantification of compounds (Shulaev 2006 ; Dettmer et al. 2007). Seven golden rules were proposed for structure elucidation of unknown compounds in mass spectrometry based metabolomics (Kind and Fiehn 2007). Study of isotopic pattern, adducts, and neutral loss removal is an important step in compound identification to minimize the false positives (Kind and Fiehn 2007; Kuhl et al. 2009). At present, identification of metabolites in LC-MS based metabolomics is by using accurate mass though it's possible to identify compounds with an *m/z* up to 1000 with error of ± 5ppm, but this is not sufficient (Fiehn and kind 2006) since there are more possible metabolites with the same mass. Further use of isotope pattern studies and MS/MS spectral fragmentation studies will enhance compound identification (Matsuda et al. 2009).

Standards for the annotation and handling of plant metabolomics data are still under development. Presently plant metabolomics thrives on the recommendations of the metabolomics standards initiative (Sumner et al. 2007; Fiehn et al. 2008). Most of the available commercial data processing software tools are vendor platform-dependent, vendor independent data processing softwares will boost the data processing in metabolomics. XCMS, XCMS2, and metaXCMS (Smith et al. 2006; Benton et al. 2008; Tautenhahn et al. 2010), MZ mine (Katajamaa et al. 2006), MetAlign (Lommen 2009), and MZedDB (Draper et al. 2009) are a few vendor platform-independent data processing tools in metabolomics. There are several databases to identify compounds based on accurate mass such as the XCMS linked to METLIN, PubChem, CAS, KNApSAcK (plant based database), HMDB, Fiehn lab, MoTo, etc. (Tohge and Fernie 2009). For metabolomics database and pathway analysis/view, MetaCyc (http://metacyc.org/), KEGG (http://www.genome.ad.jp/kegg/), AraCyc (http://www.Arabidopsis.org/tools/aracyc/), and PlantCyc (http://www.plantcyc.org/). Plantmetabolomics.org is a web-based plant metabolomics experiments portal (Bais et al. 2010), and MetPA, metabolomics pathway analysis is another web-based tool for pathway analysis and visualization (Xia and Wishart 2010).

CHAPTER 3

Mass spectrometry-based metabolomics application to identify quantitative resistance-related metabolites in barley against Fusarium head blight

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CONNECTING STATEMENT FOR CHAPTER 3

The content of Chapter 3 is derived from a published article (reformatted here to fit the thesis), co-authored by Bollina V, Kumaraswamy GK, Kushalappa AC, Choo TM, Dion Y, Rioux S, Faubert D. and Hamzehzarghani H. entitled "Mass spectrometry based metabolomics application to identify quantitative resistance-related metabolites in barley against *Fusarium* head blight". The results of this study, in parts, authored by myself, Kushalappa, AC, Choo, TM, Dion, Y, and Rioux, S. were also presented as poster with abstract at the Metabolomics society's 5th annual international conference, held at Edmonton Canada during August 30 to September 2, 2009. This article was published in the journal *Molecular Plant Pathology* in 2010, Volume 11, Pages 769-782.

Hypothetically, any changes at plant cellular level could lead to a detectable change in the plant cell metabolite profile. Existing literature shows a great deal of use of metabolic profiling to phenotype plants against different stresses. Metabolites associated with fungal disease resistance in grapes were identified using GC/MS (Batovska et al. 2008). Wheat cultivars have been discriminated based on metabolic profiling, and resistant related metabolites were identified for FHB resistance using GC-MS technology (Hamzehzarghani et al. 2005; 2008a, b; Paranidharan et al. 2008). However, GC-MS has limitation to detect only volatile metabolites. Application of LC-MS technology for both biotic and abiotic stresses has been reviewed (Dettmer et al. 2007; Shulaev et al. 2008; Allwood and Goodacre 2010; Hegeman 2010) and protocols have been established for various plant traits (Moco et al. 2006; de Vos et al. 2007). It was hypothesized that the barley cultivars, resistant and susceptible to *F. graminearum* also vary in their metabolic profiles and liquid chromatography and hybrid mass spectrometry (LC-MS) tool cab be used to phenotype resistance. Consequently the objective of this study was to explore the LC-MS based comprehensive metabolic profiling to identify RR metabolites to distinguish susceptible and resistant barley cultivars.

3.1. Abstract

Quantitative resistance is generally controlled by several genes. More than 100 resistance QTL have been identified in wheat and barley against FHB, caused by *F. graminearum*, implying the possible occurrence of several resistance mechanisms. The objective of this study was to apply metabolomics to identify the metabolites in barley that are related to resistance against FHB. Barley cultivars, Chevron and Stander, were mock-inoculated or pathogen-inoculated during the anthesis stage. The disease severity was assessed as the proportion of spikelets diseased. The 'Chevron' (0.33) was found to have a higher level of quantitative resistance than 'Stander' (0.88). Spikelet samples were harvested at 48 h post-inoculation; metabolites were extracted and analysed using an LC-ESI-LTQ-Orbitrap (Thermo Fisher, Waltham, MA, USA). The output was imported to an XCMS 1.12.1 platform, the peaks were deconvoluted and adducts were sieved. Of the 1826 peaks retained, a *t*-test identified 496 treatment significant metabolites. Among these, 194 were RR constitutive metabolites, whose abundance was greater in resistant mock-inoculated than in susceptible mock-inoculated genotypes. Fifty metabolites were assigned putative names on the basis of accurate mass, fragmentation pattern, and number of carbons in the molecule. The RR metabolites mainly belonged to the phenylpropanoid, flavonoid, fatty acid, or terpenoid metabolic pathways. Selected RR metabolites were assayed *in vitro* for antifungal activity on the basis of fungal biomass production. The application of these RR metabolites as potential biomarkers for screening and the potential of mass spectrometry-based metabolomics for the identification of gene functions are discussed.

3.2. Introduction

Quantitative resistance in plants against pathogen stress is generally controlled by several genes. Unlike monogenic traits, polygenic traits are difficult to identify and also to transfer to elite cultivars. Quantitative resistance mechanisms, in addition to structural mechanisms, generally involve both metabolites and proteins (Agrios 2005). Several metabolites in plants have been identified to have antimicrobial, signaling, cell wall enforcement, etc., properties. In this study, we explore a comprehensive metabolomics approach for the visualization of an array of metabolites and the detection of potential RR

metabolites, using barley and a necrotrophic pathogen *Gibberella zeae*, causal agent of FHB, as a model system.

Fusarium head blight is one of the most destructive and devastating diseases of barley, as well as wheat, and Triticale. Fusarium head blight not only causes a loss in grain yield, but also a deterioration in grain quality, by producing several trichothecene toxins that are detrimental to human and animal health (Sutton 1982; Bai and Shaner 2004; Choo 2006). Breeding for resistance is the most economical and environmentally safe way to manage the disease (Bai and Shaner 2004). Complete resistance to FHB in barley was not detected in more than 25,000 barley accessions screened (Choo 2006). The breeding lines are generally screened for two types of resistance: type I, resistance to initial infection based on spray inoculation; type II, resistance to the spread of disease within the spike on single spikelet inoculation (Schroeder and Christensen 1963). Barley genotypes, in general, have high type II resistance, unlike wheat genotypes and, accordingly, the screening for resistance in barley against FHB is mainly focused on type I resistance. In addition, the amount of DON, a virulence factor (Jansen et al. 2005; Ilgen et al. 2009), has also been quantified to rank cultivar resistance. However, the genotypes ranking based on quantitative resistance (type I) has been highly variable among locations and years. More than 100 QTL for FHB resistance have been identified on all seven chromosomes of wheat and barley, but only about 25% are relatively stable (Buerstmayr et al. 2009; Foroud and Eudes 2009); only the function of QTL on chromosome 3BS has been partially explained to be caused by the detoxification of DON to DON-3-*O*-glucoside in wheat (Poppenberger et al. 2003; Lemmens et al. 2005;). However, the latter mechanism is partially associated with type II resistance, as DON-negative mutants are unable to spread within the wheat spike (Jansen et al. 2005; Ilgen et al. 2009). Barley already has high type II resistance, although the mechanisms involved have not been explored. The variation in DON accumulation in spikelets and the occurrence of several FHB resistant QTL in barley, as in wheat, indicate the existence of several mechanisms of resistance. Thus, it is inadequate to perform resistance evaluation and QTL identification on the basis of the type of resistance and amount of DON only. Molecular breeders have attempted to fine map the QTL locations by further segregation, but have often found no resistance (Lulin et al. 2010). It is possible that a trait, such as

quantitative resistance, can be controlled by genes in more than one locus (Keurentjes et al. 2006). In addition, quantitative resistance is strongly influenced by the environment. An uncontrolled environment, under field conditions, leads to large experimental variations, resulting in inconsistent results over years and locations. Thus, the evaluation of breeding lines under multiple environments, locations, and years is quite expensive, time consuming, and leads to inconsistent genotype ranking. Accordingly, both conventional and molecular breeders are looking for better screening tools that not only discriminate the levels of resistance, but also explain the mechanisms of resistance or have a direct link to resistance genes.

Functional genomics approaches, such as transcriptomics, proteomics, and metabolomics, can reveal the biochemical mechanisms of resistance (Fiehn et al. 2000). Several PR proteins have been identified in wheat and barley against FHB (Zhou et al. 2005; Geddes et al. 2008; Shin et al. 2008). Host enzymes that detoxify the major virulence factor, DON, have been identified (Poppenberger et al. 2003; Lemmens et al. 2005; Lulin et al. 2010). The metabolomics approach has been used in wheat, and several RR metabolites, whose abundances are greater in resistant than in susceptible genotypes, such as cinnamic acid, myo-inositol, D-fructose, *O*-methyloxime, *p*-coumaric acid, benzoic acid, and ferulic acid, have been identified (Hamzehzarghani 2005, 2008a, b; Paranidharan et al. 2008). Metabolic profiling based on NMR identified glutamine, glutamate, alanine, and *trans*-aconitate metabolites in wheat resistant to FHB (Browne and Brindle 2007). Most of the RR metabolites identified in wheat mainly belonged to three major metabolic pathways: the phenylpropanoid, fatty acid, and polyamine. These studies, however, were based on GC-MS, which can detect only volatile metabolites. Several RR metabolites, such as flavonoids, glucosinolates, and terpenoids, are nonvolatiles (Vorst et al. 2005). In this study, LC-MS was explored for comprehensive metabolic profiling and to identify RR metabolites. For selected metabolites, the antimicrobial properties were determined. A modelling approach was used to combine the intensities of RR metabolites with their antimicrobial properties to derive resistance equivalence (RE).

3.3. Experimental procedures

3.3.1. Plant and fungus production

Six-row barley cultivars, Chevron (R = resistant) and Stander (S = susceptible), varying in quantitative resistance to FHB, were used in this study. Plants were produced under greenhouse conditions. Seeds were sown in pots containing pasteurized soil and pro-mix (50:50). Plants were fertilized once every 2 weeks with 200 mL of a 0.3% solution of Plant-Prod (20–20–20 NPK + trace elements; Plant Products Co Ltd., Brampton, ON, Canada) (Hamzehzarghani et al. 2005). The greenhouse conditions were maintained at 22 ± 3 °C, 70 ± 10% relative humidity and 16 h photoperiod throughout the growing period. At each 2-week interval, plants were thinned to retain one tiller in addition to the main stem. *Gibberella zeae* (isolate 15–35) was obtained from the Centre de Recherche sur les Grains Inc. (CEROM, Saint-Mathieu-de-Beloeil, QC, Canada) and maintained on potato dextrose agar (PDA). Fresh cultures were produced using synthetic nutrient-poor agar (SNA) medium (Nirenberg 1981). Seven-day-old cultures were flooded with sterile water, the surface of the medium was gently scraped with a sterile glass slide to dislodge macroconidia, and these were filtered through four layers of cheesecloth. A conidial suspension of 1.5×10^5 macroconidia/mL in an aqueous solution of 0.02% Tween 80 was produced using a haemocytometer. Fresh inoculum was prepared for each inoculation.

3.3.2. Inoculation and incubation

Spikes were inoculated between mid-anthesis to the early milk growth stage (GS = 65–73) (Zadoks et al. 1974). If spikes were still enclosed within a sheath, they were gently pulled before inoculation. The spikelets were either mock-inoculated (M) (sterile water containing 0.02% Tween 80) or pathogen-inoculated (P) with *G. zeae* macroconidial spore suspension until run-off, using an airbrush (Model Badger-200.3, Deluxe set™, Badger Air Brush Co., Franklin Park, IL, USA). To assess type II resistance, two opposite mid-spikelets were individually inoculated using a syringe by dispensing about 10 µL of spore suspension. Immediately after inoculation, plants were covered with transparent plastic bags sprayed inside with sterile water to maintain high moisture to facilitate infection. Bags were removed at 48 h post-inoculation (hpi).

3.3.3. Disease severity assessment

In the spray-inoculated spikelets, the number of spikelets diseased was recorded at each 2-day interval until two consecutive readings were the same: 14 days post-inoculation (dpi). From the number of spikelets infected per spike, the following monocyclic process parameters were calculated: proportion of spikelets diseased out of mid ten spikelets per spike (PSD) at 14 dpi, and the area under the disease progress curve based on PSD (AUDPC) (Hamzehzarghani et al. 2005). For the individual spikelet-inoculated plants, the number of spikelets diseased was assessed at 14 dpi. These data were used to determine the disease spread beyond the inoculated spikelet (type II resistance). Each of the above two experiments were designed as randomized complete blocks with two cultivars, spray or individual spikelet inoculation, and five replicates over time of about 3-5 days. The experimental units consisted of 10-12 spikes for the spray inoculation, to assess type I resistance, and five spikes for the individual spikelet inoculation of two spikelets, to assess type II resistance.

3.3.4. Sampling and metabolite extraction

Ten spikelets, in the mid-region of the spray-inoculated spikes, were harvested at 48 hpi using a pair of forceps, sliced longitudinally using a sterile blade and reproductive structures were removed to retain only the lemma, palea, rechilla node, and spikelet glumes in the sample. The samples were placed in labelled tubes and liquid nitrogen was poured after sampling each spikelet. The tubes were stored for a maximum of 1 month at −80 °C for further analysis.

Metabolites were extracted from samples 1–5 days ahead of their analysis. The samples were crushed in liquid nitrogen using a mortar and pestle which was cleaned using methanol and precooled with liquid nitrogen. The metabolites were extracted according to de Vos et al. (2007) with some modifications. One hundred milligrams of the powdered sample were placed in a 2.2 mL microcentrifuge tube that was washed using methanol and precooled with liquid nitrogen; 400 µL of 100 % cold methanol was added and finally, the methanol concentration in the sample was adjusted to 65% using high-performance liquid chromatography (HPLC) grade water. To this an internal standard, genistein (210 pg/µL), was added for abundance correction and the mixture was

stirred using a vortex stirrer. Each sample was sonicated for 15 min at 40 kHz in a water bath at room temperature. Sample extracts were centrifuged for 10 min at 20,000 x***g*** at room temperature. The supernatant was filtered through a 0.22 µm poly vinylidene difluoride (PVDF) membrane filter (Millipore Corporation, Bedford, MA, USA) and centrifuged at 2,520 x***g*** for 10 min. The filtrate was placed in labeled sampling glass vials and stored at −20 °C.

3.3.5. Metabolite analysis

The metabolites were analyzed using LC-MS, with electrospray ionization, a quadrupole linear ion trap capable of MS^n and an Orbitrap electrostatic fourier transform mass spectrometer capable of high mass accuracy and resolution (LC-ESI-LTQ-Orbitrap). The Orbitrap was externally calibrated every day. A 5 µL sample extract was injected automatically using a 96-well autosampler maintained at 20 °C. For chromatographic separation of the compounds, a capillary C-18 reversed-phase column, with an internal diameter of 500 µm, length of 10 cm and packed with a Jupiter stationary phase of 5 µm particle, 300 Å pore, reversed-phase material (Phenomenex, Torrance, CA, USA), was used. This column was installed on the LC-2D system (Eksigent, Dublin, CA, USA) and coupled to the LTQ-Orbitrap. The column was maintained at 25 °C and the mobile phase was adjusted to a flow rate of 800 nL/min and eluted with 2.5 mM ammonium acetate (buffer A) and 100% methanol (buffer B). During the first 10 min, a 5 µL sample was loaded onto the column with a flow rate of 8 µL/min and, subsequently, the gradient was shifted from 10% to 90% buffer B in 30 min and then back to 10% buffer B for 10 min. Electrospray, capillary and tube lens voltages were set to −3.5 kV, −37 V and −110 V, respectively. The capillary temperature was set to 275 °C. The MS and MS/MS data acquisitions were accomplished using a four-scan event cycle comprising a full-scan MS for scan event one acquired in the Orbitrap, which enabled high resolution and high mass accuracy analysis. The mass resolution for MS was set at 60,000 (at *m/z* 400) and used to trigger the three additional MS/MS events acquired in parallel in the linear ion trap for the top three most intense ions. The mass over charge ratio range was 70–1000 for MS scanning, with a target value of 500,000 charges, and from approximately one-third of the parent *m/z* ratio to 2,000 for MS/MS scanning, with

a target value of 20,000 charges. Data were recorded in centroid mode. For all scan events, the maximum ion fill time was set to 100 ms and the number of microscans to unity. For the MS/MS mode, the normalized collision energy was maintained at 35 eV, the activation *q* was set to 0.25 and the activation time to 30 ms. Target ions already selected for MS/MS were dynamically excluded for 15 s.

3.3.6. Peak deconvolution

The raw output files from the LTQ-Orbitrap were converted into mzData format using Bioworks (Thermo Fisher Scientific, San Jose, CA, USA), keeping only MS1, the parent ion. The mzData files were later imported to the XCMS 1.12.1 platform (Smith et al. 2006). Baseline was corrected and the peaks were deconvoluted and aligned across samples (treatments and replicates) using default program settings, except for the s/n threshold of 5:1 and bandwidth (bw) of 10 s. A frame width of *m/z*= 0.001 and a retention time RT = 10 s were used for peak alignment. The aligned output for each sample consisted of accurate masses (70–1000 *m/z*), retention times (RT = 1–3,600 s) and abundances (ion current counts) of each peak. CAMERA (Kuhl et al. 2009) a bioinformatics tool based on the R platform and XCMS, was used to identify adducts, isotopes and neutral losses, which were multiple peaks of the same compound found at a given retention time. The output from XCMS was imported to MS-EXCEL. Multiple peaks with adducts, isotopes and neutral losses were excluded from the total peak list. The retained peak abundances were subjected to statistical analyses.

3.3.7. Experimental design and statistical analysis

The experiment on metabolic profiling was a randomized complete block design with two cultivars, Chevron resistant (R) and Stander susceptible (S) to FHB, and two inoculations mock-inoculated (M) and pathogen-inoculated (P), with five blocks, conducted over a time interval of 3–5 days. Each experimental unit consisted of 60 spikelets harvested from six spikes produced by three plants in one pot. The data on the accurate masses of peaks and their abundances (ion current count) for all samples were subjected to a *t*-test using SAS version 9.2 (Johnson 1998), and those with significant treatment effects at $P < 0.05$ were retained, and designated as metabolites. Four different

treatment combinations were compared (RM vs. SM, RP vs. RM, SP vs. SM and RP vs. SP, where R = resistant, S = susceptible, P = pathogen-inoculated and M = mock-inoculated) to assess treatment effects.

The abundances of 496 metabolites with significant treatment effects at the $P < 0.05$ level were subjected to canonical discriminant analysis and hierarchical cluster analysis to classify the treatments using the CANDISC procedure of SAS version 9.2 (Johanson 1998). The data dimension was reduced by a non-supervised principal component analysis, and the principal components were subjected to supervised discriminant analysis to classify the treatments. The CAN scores were used to develop a scatter plot which discriminated the treatments. The metabolite loadings that contributed to the CAN scores were used to explain the resistance function (Hamzehzarghani et al. 2008a). Hierarchical cluster analysis was performed using principal components to further classify the treatments. The Euclidean distances between different groups were used to construct the dendrogram to visualize the clustering pattern of different treatments and replicates.

3.3.8. Identification of PR and RR metabolites

The *t*-test was also used to identify (to better explain the plant–pathogen interaction) the RR metabolites (whose abundances were significantly greater in the resistant than susceptible to FHB cultivar) and PR metabolites (whose abundances were significantly greater in pathogen- than in mock-inoculated plants) (Hamzehzarghani et al. 2008a). Within the RR metabolites, RR constitutive metabolites (mock-inoculated) (RRC = RM > SM) and RR-induced metabolites (pathogen and mock-inoculated) (RRI = RP > RM and RP > SP) were identified. In addition, the induced metabolites were further grouped into PR metabolites in resistant (PRr = RP > RM) and susceptible (PRs = SP > SM) forms.

3.3.8.1. Assignment of putative names of identity to metabolites

The RR metabolites identified above were assigned putative names of identity on the basis of three criteria.

1. Accurate mass match: the accurate masses were automatically searched, using XCMS linked to METLIN (http://metlin.scripps.edu/metabo_search.php) and other libraries (Tohge and Fernie 2009), including PubChem (http://pubchem.ncbi.nlm.nih.gov/), KNApSAcK (http://kanaya.naist.jp/KNApSAcK/), HMDB (http://hmdb.ca/) and MoTo (http://appliedbioinformatics.wur.nl). For all the metabolites, the accurate mass error [AME = (observed − exact mass)/ (exact mass)] was calculated and, if AME > 5 p.p.m., the compound was considered to be unidentified.

2. Mass fragmentation pattern: the mass fragments were obtained using InteliXtract version 12 (ACDlabs, Toronto, ON, Canada) and the fragmentation patterns were searched in the above databases, if available. In addition, the chemical structure was manually verified for a given fragment using the ChemSketch function of InteliXtract. A few RR metabolite standards were spiked under similar LC/MS conditions and fragmentation patterns were compared to identify a given metabolite.

3. Number of carbon atoms in the molecule: InteliXtract was also used to calculate the number of carbon atoms in the peak if isotope abundances were available. The isotope abundances that passed the criteria of containing only ^{13}C based on InteliXtract were further used to calculate the possible number of carbon atoms from the relative intensity of the ^{12}C and ^{13}C peaks [(Intensities of $^{13}C/^{12}C \times 100\%)/1.1\%$, where 1.1% is the natural abundance of ^{13}C]. The predicted number of carbon atoms in the putatively identified metabolite was used to reduce false annotations.

The RR metabolites putatively identified here were searched in metabolic pathways, such as the Plant Metabolic Network (http://www.plantcyc.org) and KEGG (http://www.genome.jp/kegg-bin/get_htext?br08003.keg), and the linkage was used to explain the mechanisms of resistance: precursors of antimicrobial, signaling, or cell walls-enforcing compounds.

3.3.9. Antifungal activity and RE for RR metabolites

Selected RR metabolites were used to evaluate the antimicrobial properties: pyroglutamic acid, *p*-coumaric acid, capric acid, quinic acid, D-gluconate, lauric acid, sinapate, and ferulic acid (the latter was used as a positive check). In this study, naringenin and kaempferol were used instead of their detected glucosidal forms, as they

were unavailable. Antifungal studies were performed in a liquid culture medium containing 5 mL of PDA. *Gibberella zeae* macroconidial suspension was inoculated to medium to contain 10^4 spores/mL. The macroconidia were harvested from SNA medium and washed twice with sterile water by centrifugation. The RR metabolites at mock final concentrations of 0, 1, 2 and 4 mM were individually inoculated to the culture tubes containing medium and spores. For capric acid, mock final concentrations of 0, 0.01, 0.05 and 0.1 mM were used, as no growth was observed at higher concentrations. As many of the compounds are hydrophobic, all were dissolved in methanol. The pH of the medium was adjusted using NaOH and, for the entire study, the pH was in the range 6.35–6.45. Liquid cultures were incubated at 25 °C in the dark on an orbital shaker at 120 r.p.m. After 5 days of incubation, mycelia were separated by centrifugation, lyophilized and the biomass was quantified. The amount of fungal biomass was expressed as the proportion of the control. The data for different concentrations were subjected to probit analysis to derive leathal dose 50 (LD_{50}) values for each compound using SAS. The LD_{50} values were subjected to analysis of variance (ANOVA) and Duncan's multiple range test using SAS. Resistance equivalence for a metabolite was calculated using $RE = (AR/AS)/LD_{50}$, where AR is the abundance in the resistant cultivar, AS is the abundance in the susceptible cultivar and LD_{50} is the millimolar concentration. A higher RE value of a metabolite indicates a higher level of resistance, which may be caused by the greater abundance of the compound in the cultivar or a lower LD_{50} value.

3.4. Results

3.4.1. Disease severity

The barley cultivars, Chevron and Stander, varied significantly in their resistance to FHB, with PSD of 0.37 and 0.88, and AUDPC of 3.34 and 8.83, respectively, indicating that ‘Chevron’ had a higher level of type I resistance than the ‘Stander’. Interestingly, all the individual spikelets inoculated were diseased, but there was no further spread of disease within a spike beyond the inoculated spikelets, indicating a very high level of type II resistance in both the cultivars. The six-row barley has six spikelets per node and the disease failed to spread from the inoculated spikelet to nearby spikelets, even within the same rachis node.

3.4.2. Comparative analysis of metabolic profiles

A total of 1970 peaks, with a signal-to-noise ratio (s/n) of > 5:1 was detected in this study. Following the sieving of adducts, isotopes, and neutral losses, a total of 1826 peaks was retained. The abundances of these were corrected for the variation in extraction by dividing the abundance of each metabolite with that of the internal standard, genistein. The abundances of 1826 peaks were subjected to a *t*-test. A total of 496 peaks showed significant treatment effects, in either of the pairs, and were designated as metabolites: RM vsSM = 289; RP vsSP = 130; RP vsRM = 55; SP vsSM = 22, where R = resistant, S = susceptible, P = pathogen-inoculated and M = mock-inoculated. These metabolites may have greater abundance in either of the genotypes, and those with greater abundance in the resistant cultivar were designated as RR metabolites. Thus, among these, only 194 were RR metabolites. In addition, 26 were PR metabolites, including 5 PRr and 21 PRs metabolites (Spplementary Table 3.1).

3.4.3. Classification of observations and treatments using canonical discriminant analysis

Four hundred and ninety-six metabolites, significant at the $P < 0.05$ level from the *t*-test, were subjected to canonical discriminant analysis and hierarchical cluster analysis to better understand the relationship among treatments. The CAN1 vector explained 70.4% of the variance, discriminating the resistant from the susceptible cultivar, whereas the CAN2 vector explained 22.6% of the variance, discriminating the pathogen from mock-inoculation (Fig. 3.1 and 3.2). A total of 134 metabolites had high positive loading ($L > 0.9$) to CAN1 that explained the constitutive resistance function in ‘Chevron’, and two metabolites had high positive loading ($L > 0.9$) to CAN2 that explained the pathogenesis function (the metabolite loadings of these, when are also RRC or PR (217), are presented in Spplementary Table 3.1).

3.4.4. PR and RR metabolites

The 496 metabolites with significant treatment effects ($P < 0.05$) were further classified on the basis of significance between specific combinations of treatments into different PR and RR metabolite groups. Two hundred and seventeen metabolites were RR or PR (Supplementary Table 3.1). Fifty metabolites were assigned putative names on

the basis of accurate mass, fragmentation pattern (Fig. 3.3), and isotope pattern (Table 3.1). The median accurate mass error of the internal standard, genistein (*m/z* 270.0528), was 0.3 p.p.m. for the entire study.

3.4.4.1. RR constitutive metabolites (RRC = RM > SM)

Among the 194 RRC metabolites (Supplementary Table 3.1), 47 were assigned putative names of identity (Table 3.1). These metabolites belonged to different chemical groups: *amino acids*: aspartic acid, arginine, aminoadepic acid, and pyroglutamic acid; *fatty acids*: capric acid, methyl dodeconic acid (fatty acid ester), lauric acid, undecanoic acid, and omega-hydroxydodecanoic acid; *alkaloids*: murranimbine and 3-methylxanthine; *lignans*: dihydrocubebin; *phenolics: trans-p*-ferulyl alcohol 4-*O*-[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside, *p*-coumaric acid, and sinapate; *flavonoids*: kaempferol 3-*O*-rhamnoside, naringenin 7-glucoside, kaempferol 3-rhamnoside-7-glucoside, kaempferide 3-glucoside-7-rhamnoside, and kaempferol 3-sophoroside-7-rhamnoside; *organic acids*: pyruvic acid, malonic acid, D-gluconate, and citric acid; *terpenes*: astragaloside III and juanislamin.

3.4.4.2. PR metabolites (PRr = RP > RM; PRs = SP > SM)

Among the 26 PR metabolites, only five were PRr metabolites; the remaining 21 were PRs metabolites. Of the PRr metabolites, only one was assigned a putative name of identity: *diterpenoid*: 16-diacetoxy-7α-hydroxy-18-malonyloxy-ent-cleroda-3-enehas. Among PRs metabolites, only three were assigned putative names of identity: *phenols*: quinic acid; *terpenes*: 3β-hydroxy cinnamolide; *fatty acid*: lauric acid.

3.4.4.3. Identification of DON detoxification product

The virulence factor, DON (*m/z*= 296.1259), and its detoxified product, DON-3-*O*-glucoside (*m/z*= 458.1788) (Fig. 3.3), were detected in the resistant but not susceptible cultivar. This is the first report of DON-3-*O*-glucoside in barley.

3.4.5. Relative antifungal activity and RE of RR metabolites

Seven RR metabolites (pyroglutamic acid, *p*-coumaric acid, capric acid, quinic acid, D-gluconate, lauric acid, and sinapate), naringenin (parent compound of naringenin-

7-glucoside which was detected) and kaempferol (parent compound of kaempferol glucoside which was detected) were used for fungal biomass inhibition studies. Ferulic acid, a phenolic compound, was used as a positive check as it has been reported to reduce the biomass of *G. zeae* (Boutigny et al. 2009). Except for kaempferol, all other compounds reduced significantly (P <0.01) *G. zeae* biomass production (Fig. 3.4). When kaempferol was added to the liquid culture medium, it precipitated and failed to dissolve completely. Capric acid inhibited biomass completely at a dose of >0.5 mM, and so lower concentrations were evaluated; significant biomass was observed at 0.1 mM. In addition, both *p*-coumaric acid and naringenin showed greater biomass inhibition than that of ferulic acid, which was equal to that of sinapic acid.

Resistance equivalence for the RR metabolites detected here in the resistant cultivar Chevron was derived as RE =[(AR/AS)/LD_{50}], where AR is the abundance of the metabolite in the resistant cultivar, AS is the abundance of the metabolite in the susceptible cultivar and LD_{50} is the concentration (mM) of metabolite that inhibited 50% of the biomass of *G. zeae*. Resistance equivalence ranged from zero for ferulic acid, as we did not detect this metabolite in our study, to 12.16 for capric acid and 3.01 for *p*-coumaric acid (Table 3.2). Higher the RE value, higher is the potential resistance. Highest RE for capric acid was mainly a result of its lowest LD_{50}.

3.5. Discussion

The disease severity differed significantly between the two barley cultivars used in this study. The 'Chevron' had a greater quantitative resistance (type I) than the'Stander' (PSD = 0.37 and 0.88, and AUDPC = 3.34 and 8.83, respectively). Both cultivars had very high levels of type II resistance, confirming the earlier findings (Choo et al. 2004; Ma et al. 2000).

In the present study, 1970 peaks were detected by LC-ESI-LTQ-Orbitrap (Thermo Fisher, Waltham, MA, USA). Of these, 496 metabolites had significant treatment effects (P <0.05). A canonical discriminant analysis of these metabolites identified constitutive resistance, but failed to identify induced resistance; however, it explained PR function. One hundred and thirty-four metabolites had high positive loading to CAN1, which mainly explained constitutive resistance, and two metabolites had high

positive loading to CAN2, which explained pathogenesis function. More specific plant–pathogen interaction was further explored using univariate analysis. A *t*-test identified 194 RR metabolites, where all were RRC metabolites and none were RRI metabolites, as observed by canonical discriminant analysis. Of these, 50 were assigned putative names, and these metabolites belonged mainly to four chemical groups: the phenylpropanoids, flavonoids, fatty acids, and terpenoids. However, our previous studies using GC-MS detected only phenylpropanoids and fatty acids (Hamzehzarghani et al. 2005; 2008a, b; Paranidharan et al. 2008), but not flavonoids and terpenoids, making LC-MS a more comprehensive MS-based metabolomics tool to study biotic stress (Vorst et al. 2005). Furthermore, we report here, for the first time, the occurrence of the DON detoxified product, DON-3-*O*-glucoside, in barley (Fig. 3.3). However, the fragmentation pattern library for metabolites based on LC-MS is rather limited, when compared with GC-MS; accordingly, not many compounds were assigned putative names. The RR metabolites that were not assigned putative names are still useful markers, and may be identified in future with progress in metabolomics databases (Tohge and Fernie 2009). This is the first study to report RR metabolites in barley against FHB following a metabolomics approach, which enabled the visualization of several metabolites of the plant–pathogen interaction. The RR metabolites reported here have several known mechanisms of resistance, and, in addition, the relative antimicrobial properties of some of these were also demonstrated.

We detected a resistance indicator metabolite, DON-3-*O*-glucoside, the detoxified product of DON to the less toxic glucoside, through enzymatic activity in the resistant cultivar (Lemmens et al. 2005; Poppenberger et al. 2003). Although we detected DON and its detoxified product, they were not detected in all replicates. This is because, in this study, we used a nonpolar column; use of a polar column should better detect trichothecenes (Berthiller et al. 2007). Interestingly, the accumulation of a small amount of DON in the 'Stander' allele at chromosome 3 has been reported, even though it was used as a susceptible parent in the production of a recombinant inbred line population (Smith et al. 2004). Although both cultivars used in this study accumulate a small amount of DON, the mechanisms may be different, either by inhibition of synthesis through

antioxidants (Boutigny et al. 2009) or by conversion of already produced DON through enzymatic action (Poppenberger et al. 2003; Lemmens et al. 2005;).

The metabolites identified here were interlinked in a satellite metabolic pathway (Fig. 3.5) to better understand the role of metabolites in plant defense function. The RR metabolites identified here belong to different metabolic pathways, in particular flavonoid, phenylpropanoid, fatty acid, and terpenoid. The putative mechanisms of resistance of RR metabolites are discussed below

3.5.1. Phenylpropanoid pathway

In our study, *p*-coumaric acid and sinapate were identified as RR metabolites. The accumulation of phenolic compounds at the site of pathogen inoculation has been reported (Bily et al. 2003; Chen et al. 2006; Boutigny et al. 2008). Phenolics act not only as antimicrobial agents, but also inhibit the synthesis of DON, a virulence factor of *G. zeae*, through their antioxidant properties (Boutigny et al. 2009). Further, these metabolites are the precursors of lignin, which acts as a general barrier for pathogen advancement (Humphreys and Chapple 2002).

Significant amounts of *p*-coumaroyl-hydroxyagmatine in barley near-isogenic lines were observed following *Erysiphe graminis hordei* inoculation, and this was corroborated with *in vitro* and *in vivo* antifungal activity (von Ropenack et al. 1998). In our study, both *p*-coumaric acid and sinapate had low LD_{50} values, and the former had the second highest RE. These metabolites are also known for cell walls lignification (Jansen et al. 2005), but their conversion to lignomonomers (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) was not significant in the study. Quinic acid was detected here as a PRs not PRr metabolite, and this also had a high LD_{50} value. It is possible that quinic acid is used in resistance to enhance the cell walls. The incorporation of radiolabelled quinic acid into a resistant tomato genotype against *Fusarium oxysporum* degraded most of the quinic acid, and converted it to lignin, whereas it was accumulated in the susceptible plant (Dixon and Paiva 1995; Fuchs and Vries 1969). Flux analysis of this pathway can reveal the role of quinic acid against FHB.

3.5.2. Flavonoid pathway

This is a downstream the phenylpropanoid pathway. In our study, 16 flavonoids and isoflavonoids were identified as RR metabolites. The flavonol kaempferol and its glucosylated forms, identified here as RR metabolites, were linked to the flavonol biosynthesis pathway (Fig. 3.6). The flavonoids identified in this study have been reported previously from plants, and the naturally occurring flavonoids and flavonoid coumarins inhibited the biosynthesis of trichothecene in *F. sporotrichioides* (Desjardins 1988). The flavonoids, kaempferol-*O*-rutinoside and kaempferol-3-*O*-β-D-glucopyranosyl isolated from carnation, showed significant inhibition of *F. oxysporum* growth (Galeotti et al. 2008). Flavonoid glucosides have excellent antioxidant activities (Ko et al. 2005), and thus it is quite possible that they also inhibit DON synthesis, as in ferulic acid (Boutigny et al. 2009). Some flavonoid monomers from barley testa were potent inhibitors of *Fusarium* spp. (Skadhauge et al. 1997).

3.5.3. Fatty acid pathway

In this study, five fatty acids were detected. Capric acid and lauric acid, identified here as RR metabolites, inhibited *G. zeae* mycelial biomass significantly (Table 3.2). These also have antifungal activity against *Pseudomonas aeruginosa, Candida albicans* (Kabara 1984), and *Fusarium* spp. (Liu et al. 2008). Among the RR metabolites tested here, capric acid had the highest mycelial inhibition and RE.

3.5.4. Other RR metabolites

In the present study, we identified six amino acids, four of which were RR metabolites (pyroglutamic acid, aspartate, 2-aminoadipic acid, and arginine), and four organic acids (pyruvic acid, melonic acid, citrate, and gluconate). Pyroglutamic acid, a nonprotein amino acid, showed antimicrobial activity against *Bacillus subtilis* and *Pseudomonas putida* (Huttunen et al. 1995). Arginine, identified here as an RR metabolite ($P < 0.01$), acts as a precursor for the biosynthesis of a polyamine, putrescine (Nakada and Itoh 2003). Polyamines have been reported from wheat (Paranidharan et al. 2008), and these are involved in a variety of stress responses (Bajaj et al. 1999). Gluconic acid, an organic acid, identified here as an RRC metabolite, had an LD_{50} value of 2.8 mM

for mycelial inhibition. Enhanced disease resistance was observed following the overexpression of glucose oxidase, an enzyme that converts glucose to gluconic acid, in cabbage and tobacco against *Xanthomonas campestris* pv. *campestris* (Lee et al. 2002), and in rice against *Magnaporthe grisea* and *X. oryzae* pv. *oryzae* (Kachroo et al. 2003). This indicates that many such diverse compounds are involved in a complex network of disease suppression and plant resistance to biotic and abiotic stresses.

The 'Chevron' was most resistant than the 'Stander'. This is in accordance with several other studies that reported a higher level of resistance in 'Chevron' relative to several other genotypes (Urrea et al. 2002; Capettini et al. 2003; Choo 2006). 'Chevron' has been used in molecular breeding programmes and the crosses 'Chevron' × M69 (de la Pena et al. 1999) and 'Chevron' × 'Stander' (Ma et al. 2000) have been used to identify several QTL. Although 'Stander' is a susceptible relative to 'Chevron', resistance QTL has been reported from crosses with Fredrickson (Mesfin et al. 2003). A recombinant inbred line with 'Stander' QTL at chromosome 3 produced less DON than that with alternating QTL (Smith et al. 2004).

The goal of this study was to explore the potential of comprehensive metabolomics approach to identify RR metabolites. Accordingly, we used the cultivar Chevron, as it has been proposed to possess several mechanisms and QTL for resistance. Such an exploratory step at the outset is not possible using near-isogenic lines as they are expected to possess a specific mechanism. However, metabolites that are significantly different between cultivars may also be a result of cultivar background effects. Accordingly, for selected RR metabolites, we established their antifungal effects and also derived their RE to obtain a combined parameter to better discriminate resistance. In addition, we used information in the literature to obtain the defensive role of RR metabolites. Metabolic fluxes are also important, as resistant cultivar can use a given RR metabolite as a precursor to produce a metabolite with greater RE, whereas a susceptible cultivar will accumulate the metabolite (Dixon and Paiva 1995; Fuchs and Vries 1969). Our study indicates that the resistance in barley to FHB, as in wheat, is controlled by several RR metabolites. Various combinations of these RR metabolites can be accumulated through breeding in a cultivar to achieve greater levels of quantitative resistance (Hamzehzarghani et al. 2008a). In addition, the knowledge base on the

occurrence of several RR metabolites in different metabolic pathways can be used to overexpress certain important metabolites through metabolic engineering. Alternatively, individuals or mixtures of these RR metabolites can be exploited as biofungicides, in particular capric acid, which had the lowest LD_{50}, applied to spikes to manage FHB. The RR metabolites identified in this study have shed some light onto the different resistance mechanisms against FHB involved in barley. However, we have not identified all possible RR metabolites in barley, and improvement of the metabolomics protocol for the detection of more polar metabolites and the analysis of other cultivars, including genotypes, recombinant inbred and near-isogenic lines, may reveal other important metabolites that might explain more mechanisms of resistance.

Table 3.1 Resistance-related (RR) metabolites, with putative names of identity detected in six-row barley genotypes inoculated with mock-inoculation or pathogen.

Exp. mass (M), median	Exp. RT, median	Theor. mass (M)	AME (p.p.m.)	Putative name of identity	Compound group	Molecular formula	RR metabolites	MS/MS fragments	*P* <0.05	Fold change	Database
88.01635	2.41	88.01604	3.5	Pyruvic acid	OA	$C_3H_4O_3$	RRC	**87.01**, 58.78	0.0116	32.19	A,B,C,D
104.0112	2.53	104.0109	2	Malonic acid	OA	$C_3H_4O_4$	RRC†	**103, 59**, 84.99, 74.96	0.007	1.57	A,B,C,D
129.04265	2.42	129.0426	0.39	Pyroglutamic acid	AA	$C_5H_7NO_3$	RRC	128.14, 110.26, 100.11, 84.25	0.0348	2.33	A,B,C,D
133.03765	2.31	133.0375	1.1	Aspartic acid	AA	$C_4H_7NO_4$	RRC	115.19, 114.19, 88.19, 89.1	0.0249	1.38	A,C
161.06865	2.42	161.0688	0.92	2-Aminoadipic acid	AA	$C_6H_{11}NO_4$	RRC		0.0440	2.08	A,B,C,D
164.04735	34.43	164.0473	0.31	*p*-Coumaric acid	PA	$C_9H_8O_3$	RRC*	**119.05**, **163**, **145.20**, 134.96	0.0076	3.48	A,B,C,D
166.04977	2.35	166.0491	4	3-Methylxanthine	ALK	$C_6H_6N_4O_2$	RRC	128.93, 75.00, **96.68**, 105.08	0.0311	1.33	A,B,C,D
172.14575	40.63	172.1463	3.1	Capric acid	FA	$C_{10}H_{20}O_2$	RRC	**171.25, 153.22**, 127.26, 148.23	0.0296	1.22	A,B,C,D
174.11165	2.27	174.1116	0.29	L-Arginine	AA	$C_6H_{14}N_4O_2$	RRC*	**129.05**, 155.04 142.93, 154.93	0.0095	2.13	A,B,C,D
186.16165	41.94	186.162	1.8	Undecanoic acid	FA	$C_{11}H_{22}O_2$	RRC	141.19, **167.15**,	0.0452	1.31	A,C

								185.11, **80.17**			
192.02715	2.45	192.027	0.78	Citric acid	OA	$C_6H_8O_7$	RRC†	**110.99**, **172.99**, **84.92**, **126.96**	0.0002	1.74	A,B,C,D
192.06335	2.34	192.0638	2.3	Quinic acid	PP	$C_7H_{12}O_6$	PRs	**173.06**, 111.11, 127.19, 85.17	0.0394	1.78	A,B,C,D
196.05825	2.32	196.0583	0.24	D-Gluconate	OA	$C_6H_{12}O_7$	RRC†		0.0007	1.58	A,B,C,D
200.17755	43.21	200.1776	0.24	Lauric acid	FA	$C_{12}H_{24}O_2$	RRC*, PRs	181.12, 167.13, 155.09, 135.28	0.0139	1.31	A,B,C,D
216.17235	36.04	216.1725	0.68	Omega-Hydroxydodecanoic acid	FA	$C_{12}H_{24}O_3$	RRC		0.0497	1.50	A
224.06865	34.53	224.0684	1.1	Sinapate	PA	$C_{11}H_{12}O_5$	RRC	208.13,179.11,164.16	0.0245	1.19	A,C
250.15665	37.82	250.1569	0.99	3β-Hydroxycinnamolide	ST	$C_{15}H_{22}O_3$	PRs	205.17, 231.09, 184.29, 164.09	0.0□87	1.06	A
288.22975	39.28	288.2301	1.2	10,16-Dihydroxy-hexadecanoate	FA	$C_{16}H_{32}O_4$	RRC*	243.17, 269.22, 189.19, 259.17, 227.12	0.0021	2.1	A
316.07915	2.49	316.0794	0.78	Quinovic acid	SAP	$C_{13}H_{16}O_9$	RRC†	153.04, 305.64, 165.13, 296.92	0.0007	17.39	A
328.13075	26.02	328.1311	1	Seselinol isovalerate	COU	$C_{19}H_{20}O_5$	RRC	309.19, 291.22, 229.17, 185.09	0.0289	1.74	A
332.07395	2.38	332.0743	1	β-Glucogallin	Tannin	$C_{13}H_{16}O_{10}$	RRC†		0.002	4.0	A,C
340.13065	26.02	□40.131	1.3	6-Prenylnaringenin	FLA	$C_{20}H_{20}O_5$	RRC	289.18, 183.04, 307.20, 321.20	0.00□9	1.79	A,C
354.10995	24.68	354.1103	0.98	Licoisoflavone A	ISF	$C_{20}H_{18}O_6$	RRC	235.21, 320.31, 255.26	0.0446	1.65	A,C

358.10645	2.27	358.1052	3.4	5-Hydroxy-3,6,7,4'-tetramethoxyflavone	FLA	$C_{19}H_{18}O_7$	RRC*		0.003	2.37	A
358.14095	26.02	358.1416	1.8	(–)-Dihydrocubebin	LIG	$C_{20}H_{22}O_6$	RRC		0.0255	1.9	A
418.08895	27.09	418.0899	2.2	Isoscutellarein 7-xyloside		$C_{20}H_{18}O_{10}$	RRC	180.92, 237.15, 310.47, 313.03	0.0217	1.43	A
428.16775	23.71	428.1682	1	*trans-p*-Ferulyl alcohol 4-*O*-[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside	PA	$C_{20}H_{28}O_{10}$	RRC*		0.0077	2.14	A
432.10485	23.54	432.1056	1.7	Kaempferol 3-*O*-rhamnoside	FLA	$C_{21}H_{20}O_{10}$	RRC*	153.06, 171.03, 399.24, 385.17	0.0091	3.03	A
432.17785	35.41	432.1784	1.2	Juanislamin	ST	$C_{23}H_{28}O_8$	RRC		0.0424	1.64	A
434.12055	31.01	434.1212	1.4	Naringenin 7-glucoside	FLA	$C_{21}H_{22}O_{10}$	RRC	313.19, 253.33, 310.97, 231.08	0.0333	1.62	A
462.11535	24.04	462.1162	1.8	Scoparin	COU	$C_{22}H_{22}O_{11}$	RRC†	299.13, 300.19, 155.09, 307.07	0.0012	3.6	A,C
484.24265	32.43	484.2434	1.5	Segetalin B		$C_{24}H_{32}N_6O_5$	RRC		0.0298	2.34	A
510.28125	38.13	510.2828	3	16-Diacetoxy-7α-hydroxy-18-malonyloxy-ent-cleroda-3-ene	DT	$C_{27}H_{42}O_9$	PRr*	281.26, 227.12, 153.14	0.017	3.44	A
526.26145	□1.07	526.262	1	Murranimbine	AL	$C_{36}H_{34}N_2O_2$	RRC*		0.0104	2.93	A
536.18865	16.95	536.1893	1.2	7-*O*-(4-Methoxycinnamoyl) tecomoside	PP	$C_{26}H_{32}O_{12}$	RRC	235.31, 299.30, 192.11, 161.01	0.0233	3.46	A
548.15265	2.44	548.1529	4.5	Hemsleyanoside	ISF	$C_{26}H_{28}O_{13}$	RRC		0.0407	2.57	A

582.20875	28.927	582.2101	2.3	Auriculatin 4'-*O*-glucoside	ISF	$C_{31}H_{34}O_{11}$	RRC*		0.0193	1.79	A
584.20945	18.45	584.2105	1.7	Sylvestroside III		$C_{27}H_{36}O_{14}$	RRC	195.05, 282.88, 179.43, 165.20	0.0201	1.57	A
594.15755	23.86	594.1584	1.4	Kaempferol 3-rhamnoside-7-glucoside	FLA	$C_{27}H_{30}O_{15}$	RRC†		0.0006	5.45	A
608.17255	25.79	608.1741	2.5	Kaempferide 3-glucoside-7-rhamnoside	FLA	$C_{28}H_{32}O_{15}$	RRC*	299.01, 284.07, 300.49, 269.12	0.004	2.31	A
637.23545	36.90	637.2371	2.5	6'-O-α-D-Xylopyranosylalangiside	TER	$C_{30}H_{39}NO_{14}$	RRC*	310.06, 20□.99, 507.23, 618.19, 363.2	0.0168	1.66	A
654.17775	25.77	654.1795	2.6	Syringetin 3-rutinoside	PA	$C_{29}H_{34}O_{17}$	RRC†		0.0012	2.37	A
710.20435	22.80	710.2058	2	Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside	FLA	$C_{32}H_{38}O_{18}$	RRC†	401.08, 311.15, 283.11, 341.12	0.0003	6.48	A
740.21435	21.40	740.2163	2.6	Kaempferol 3-rhamninoside	FLA	$C_{33}H_{40}O_{19}$	RRC†		0.0009	8.94	A
740.21465	24.93	740.2163	2.2	Kaempferol 3-rhamnoside-7-glucosyl-(1->2)-rhamnoside	FLA	$C_{33}H_{40}O_{19}$	RRC*		0.0152	1.61	A
756.20965	19.57	756.2112	2	Kaempferol 3-gentiobioside-7-rhamnoside	FLA	$C_{33}H_{40}O_{20}$	RRC†		0.0004	5.15	A
756.20965	22.81	756.2112	2	Kaempferol 3-sophoroside-7-rhamnoside	FLA	$C_{33}H_{40}O_{20}$	RRC†		0.0003	6.12	A
770.22485	21.72	770.2269	2	Rhamnetin 3-	FLA	$C_{34}H_{42}O_{20}$	RRC†		0.0004	7.67	A

				rhamninoside							
784.45935	42.53	784.4609	1.9	Astragaloside III	TT	$C_{41}H_{68}O_{14}$	RRC		0.0473	3.62	A,C
786.22015	21.41	786.2218	2	Isorhamnetin 3-rutinoside-7-glucoside	FLA	$C_{34}H_{42}O_{21}$	RRC†		0.0003	9.94	A

Databases used for metabolites identified: A, KNApSAcK; B, METLIN; C, KEGG; D, CAS. MS/MS fragmentation in bold indicates the actual match of the fragment in the database.

AA, amino acid; ALK, alkaloid; AME, accurate mass error (p.p.m.) was calculated using the formula [(Measured accurate mass—Theoretical mass)/(Theoretical mass)]; BQ, benzoquinone; COU, coumarin; DT, diterpenoid; FA, fatty acid; FLA, flavonoid; ISF, isoflavonoid; LIG, lignan; OA, organic acid; PA, phenolic acid; PP, phenylpropanoid; PRr, pathogenesis-related resistant; PRs, pathogenesis-related susceptible; RRC, resistant-related constitutive; RT, retention time; SAP, saponine; ST, sesqueterpenoid; TER, terpenoid; TT, triterpenoid.

*Significant at $P < 0.01$.

†Significant at $P < 0.001$.

Table 3.2 Relative abundance, LD_{50} value and resistance equivalence of resistance-related (RR) metabolites identified in barley cultivar Chevron.

RR metabolites	Relative abundance of RR metabolites	LD_{50} value of RR metabolites (mM)	Resistance equivalence*
Pyroglutamic acid	2.33	3.172	0.73
p-Coumaric acid	3.48	1.154	3.01
Capric acid	1.22	0.1003	12.16
Quinic acid	1.78	3.155	0.56
Ferulic acid	0	1.766	0
D-Gluconate	1.58	2.65	0.59
Lauric acid	1.31	2.142	0.61
Sinapate	1.19	1.747	0.68
Naringenin	1.43	1.58	0.9
Kaempferol	1.24	4.768	0.25

*Resistance equivalence (RE) =[(AR/AS)/LD_{50}], where AR is the abundance of the metabolite in the resistant cultivar, AS is the abundance of the metabolite in the susceptible cultivar and LD_{50} is the concentration (mM) of metabolite that inhibited 50% of the biomass of *Gibberella zeae*. Higher the RE value, higher the potential of resistance.

Fig. 3.1 Scatter plot of canonical discriminant analysis based on the abundances of 496 significant metabolites (P <0.05) from barley spikelets of resistant (R) and susceptible (S) cultivars, mock –inoculated (M) or pathogen-inoculated(P). CAN1 separated the cultivars and mainly identified constitutive resistance, whereas CAN2 separated pathogen- from mock-inoculated and mainly explained pathogenesis function.

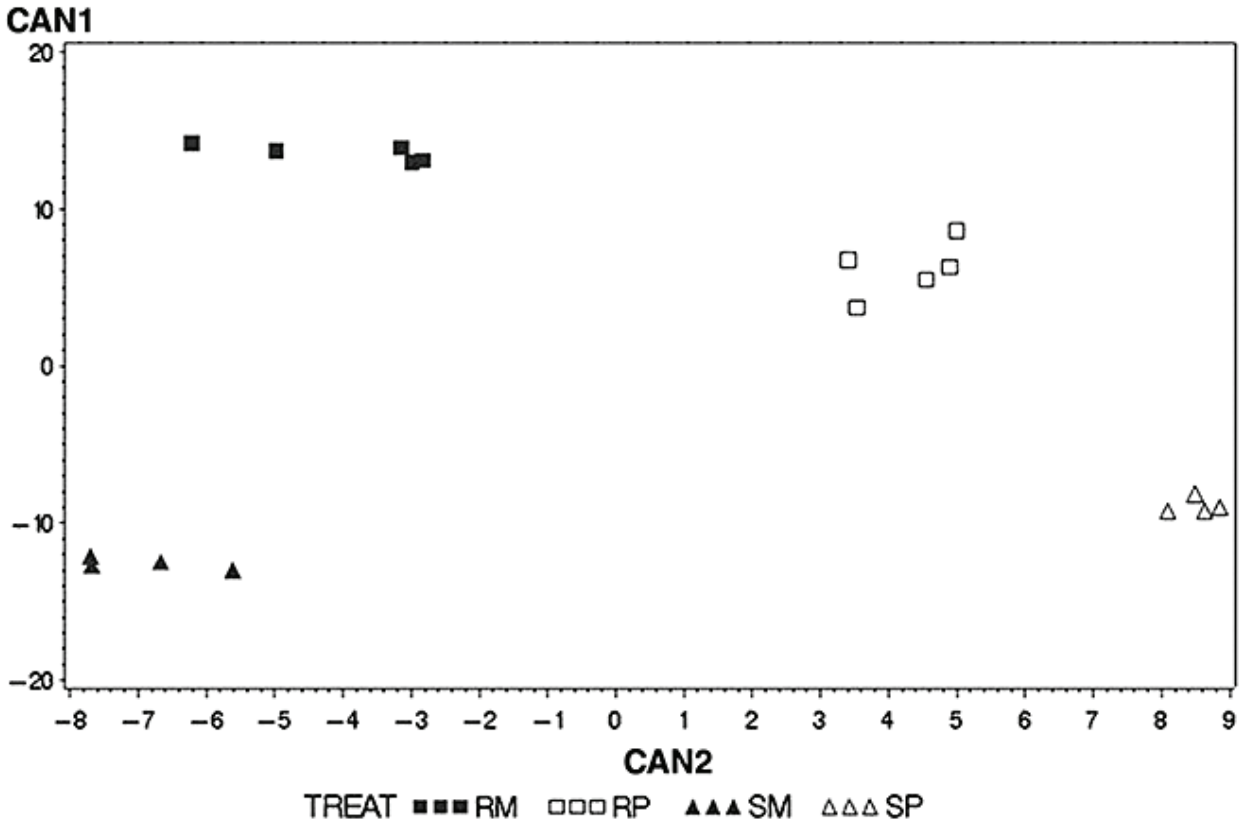


Fig. 3.2 Dendrogram based on hierarchical cluster analysis (HCA) of principal components of abundances of 496 metabolites (P <0.05). The treatments are: S, susceptible ('Stander'); R, resistant ('Chevron'); M, mock-inoculation; P, pathogen-inoculation. Each line represents one replicate.

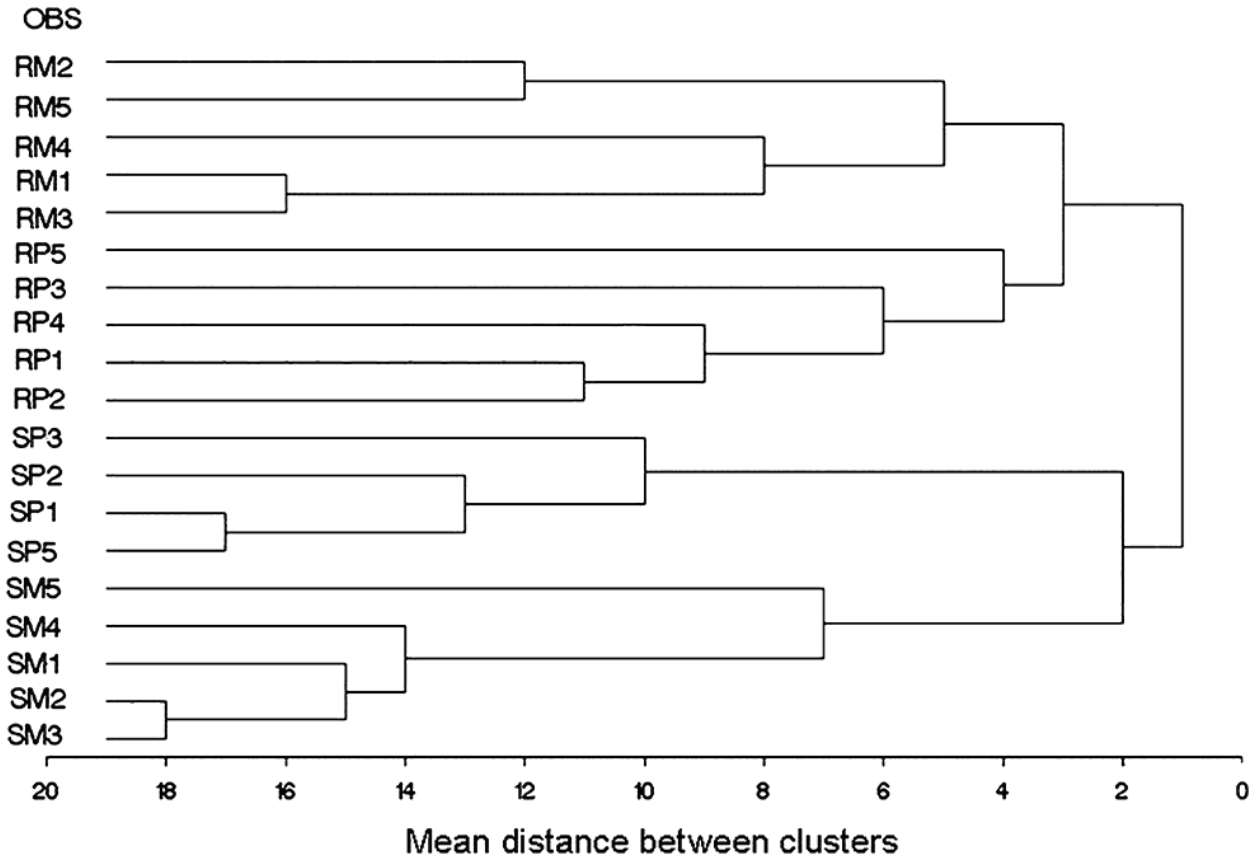


Fig. 3.3 The fragmentation pattern, MS/MS spectra, in negative ionization mode $[M - H]^-$ of deoxynivalenol-3-*O*-glucoside (*m/z*= 458.1788), a detoxification product of deoxynivalenol (DON) (*m/z*= 296.1259).

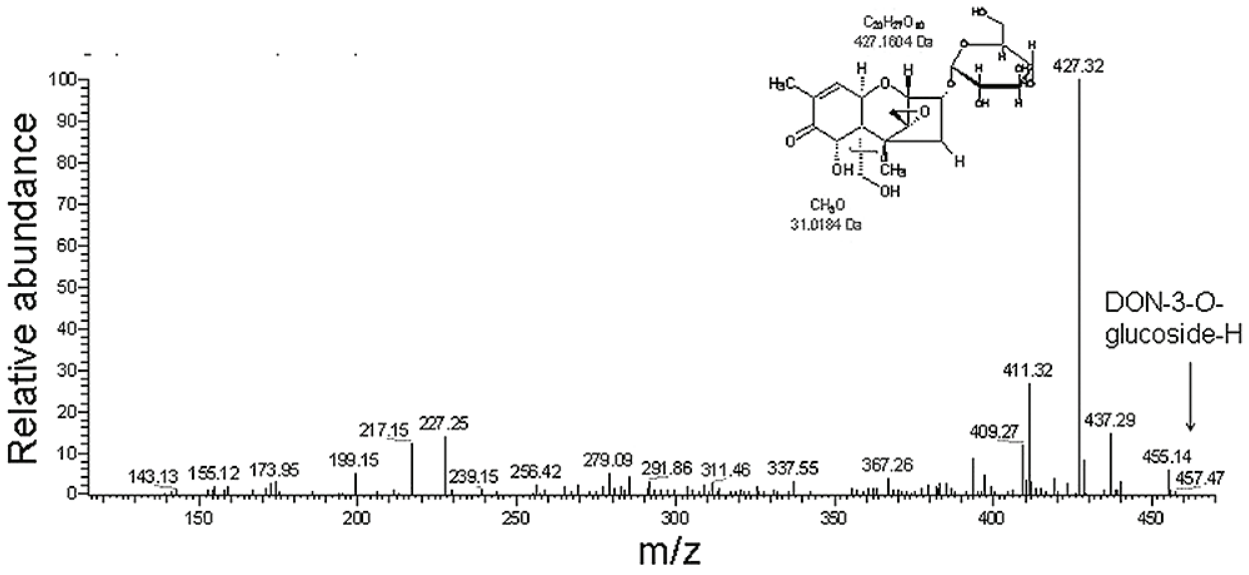


Fig. 3.4 Antimicrobial properties of resistance-related (RR) metabolites assessed *in vitro* against *Gibberella zeae*. The metabolite concentrations in mM for 50% inhibition (LD_{50} values) of the mycelial biomass by 10 RR metabolites; the letters A–D indicate the Duncan rankings of RR metabolites at $P < 0.01$.

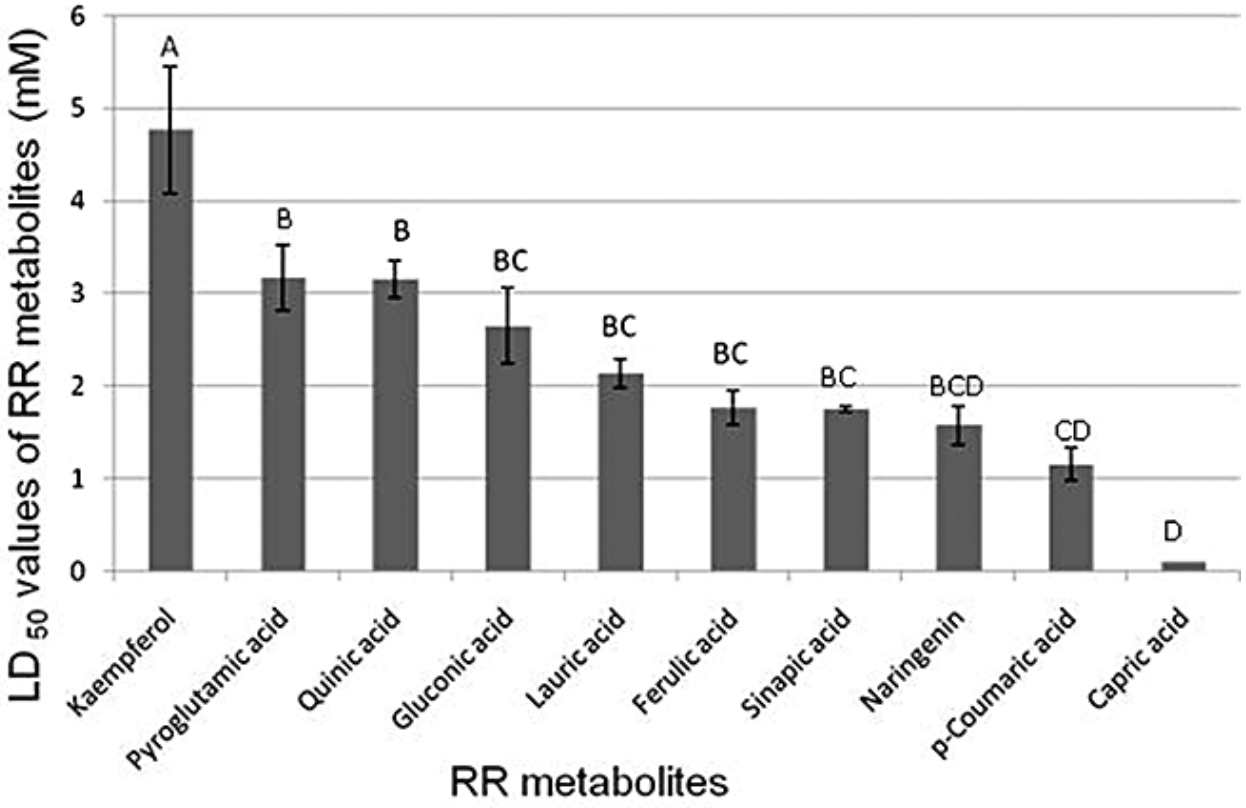


Fig. 3.5 Satellite metabolic pathway of barley. The resistance-related (RR) metabolites detected in mock-inoculated or pathogen-inoculated barley cultivars: bold, significant (at P <0.05); italic, not significant, but identified; regular font, not identified in this study.

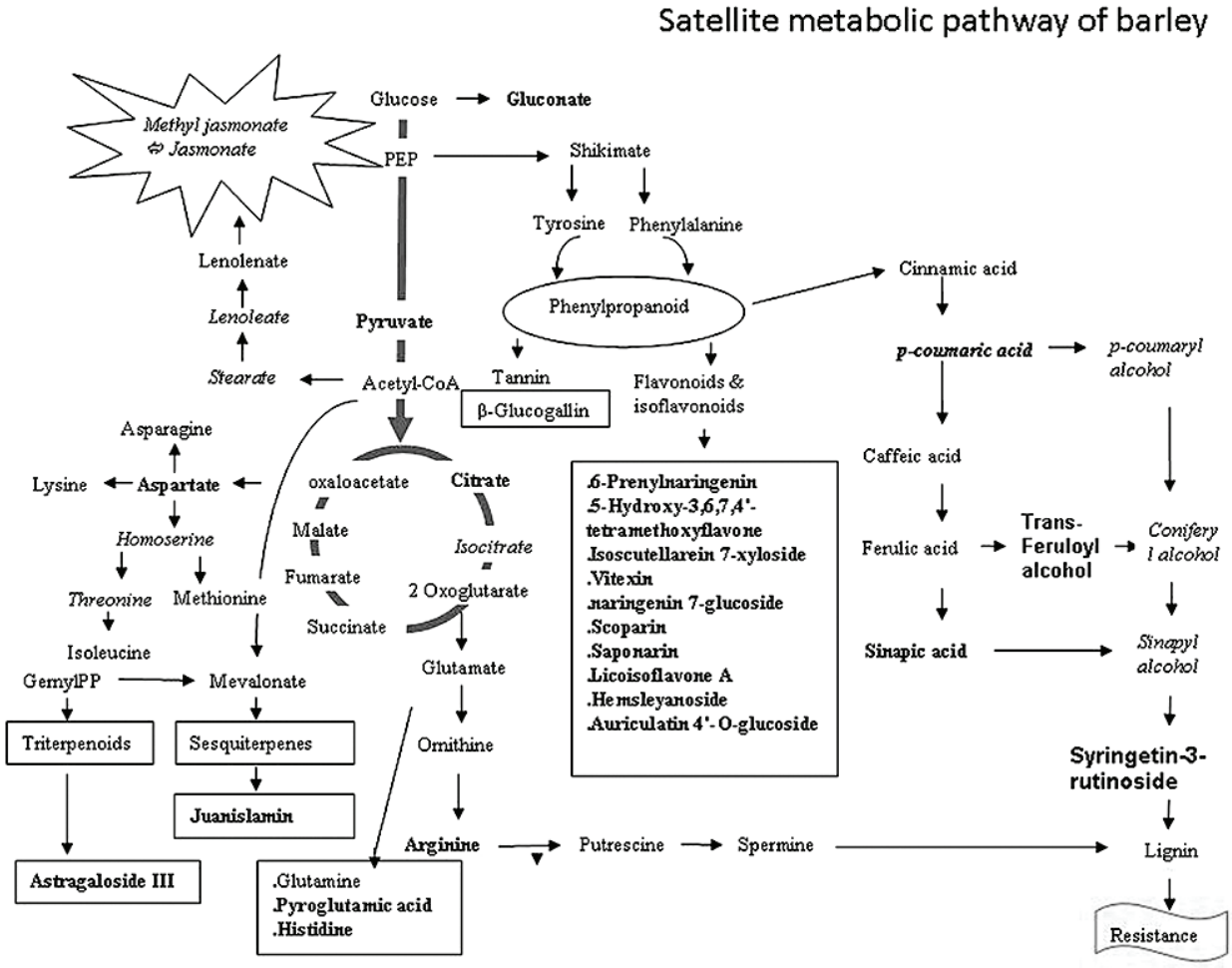
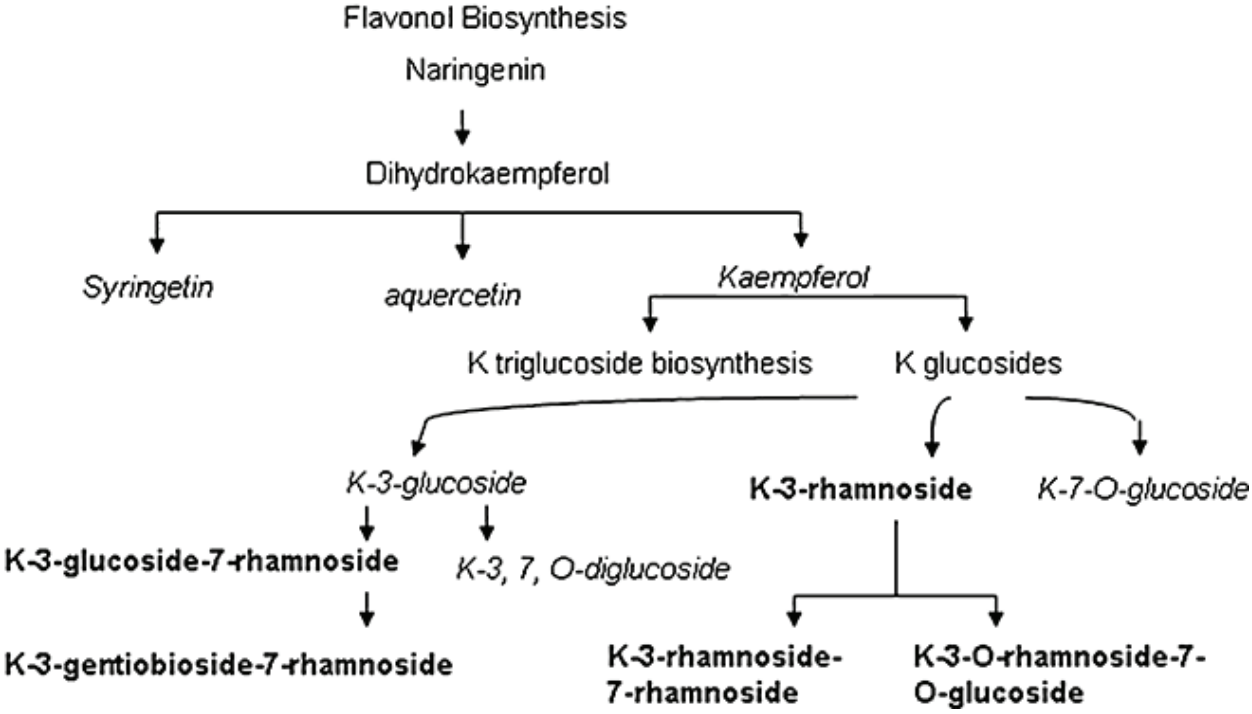


Fig. 3.6 Schematic diagram of part of the flavonoid biosynthesis pathway leading to kaempferol production inmock-inoculated or pathogen-inoculated barley cultivars: bold, significant (at P <0.05); italic, not significant at P <0.05, but identified with AME < 5 p.p.m.; K, kaempferol.



CHAPTER 4

In vitro inhibition of trichothecene biosynthesis in *Fusarium graminearum*, by resistance related endogenous metabolites identified in barley

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CONNECTING STATEMENT FOR CHAPTER 4

Chapter 4 consists of a manuscript prepared by Bollina, V and Kushalappa, AC, entitled "*In vitro* inhibition of trichothecene biosynthesis in *Fusarium graminearum*, by resistance related endogenous metabolites identified in barley". This manuscript will be submitted for publication.

In Chapter 3 we identified several RR metabolites in six-row barley cultivars. In addition, the biomass inhibition was demonstrated for selected RR metabolites. Similarly, Kumaraswamy et al. (2011) also demonstrated biomass inhibition by RR metabolites identified in two-row barley. The present study (Chapter 4) was designed to determine the effect of selected RR metabolites on levels of inhibition of type B trichothecene biosynthesis.

Trichothecenes are the mycotoxins produced by many *Fusarium* spp.; more than 15 different mycotoxins were detected from FHB infected barley grains (Choo 2006). Trichothecenes are dangerous to human and animal health as the minimum acceptable levels trichothecenes have been set for different products. Deoxynivalnol is a major trichothecene toxin and also has been reported as virulence factor (Jansen et al. 2005). The increase in resistance in barley was associated with decrease in DON production. However, the decrease in DON can be due to reduced synthesis or conversion of DON to D3G (Lemmens et al. 2005). Extracts from wheat bran consisting major phenolic compounds showed complete inhibition of type B trichothecene biosynthesis by *Fusarium* spp. (Boutigny et al. 2010). Ferulic acid a major phenolic acid content in wheat and barley spikelets showed a significant inhibition of type B trichothecene biosynthesis under *in vitro* conditions (Boutigny et al. 2009). In the present study we hypothesize that plant endogenous compounds interfere in the type B trichothecene biosynthesis under *in vitro* conditions. In the present study, *Phenolic acids*: *p*-coumaric, sinapic, ferulic, and caffeic acid; *flavonoids*: quercetin and naringenin, and *fatty acids*: capric, lauric acid, and methyl jasmonate were selected to study the effect on biosynthesis of trichothecene by *F. graminearum*.

4.1. Abstract

Trichothecenes are sesquiterpene mycotoxins produced in grains of wheat and barley by *Fusarium* spp. and they cause serious health problems in humans and animals. A trichothecene, DON, produced by *F. graminearum*, the causal agent of FHB in wheat and barley, has been reported as a virulence factor. In this study the effects of ten selected RR endogenous metabolites, previously demonstrated to have antimicrobial property, on trichothecene biosynthesis was studied *in vitro*. These RR metabolites belong to phenolic, flavonoid, and fatty acid chemical groups. Basal liquid medium inoculated with *F. graminearum* and with or without RR metabolites at LD_{50} molar concentrations were incubated for 5 days. Dry fungal biomass was determined. The trichothecenes were extracted from spent media and analyzed using LC-MS-ESI-LTQ-Orbitap. Trichothecene production was completely inhibited by lauric, *p*-coumaric, sinapic, ferulic, naringenin, quercetin, and methyl jasmonate at LD_{50} molar concentrations. Trichothecene biosynthesis inhibition, relative to control, was partial by capric, quinic, and caffeic acid. The cumulative effects of these RR metabolites, as both antimicrobials and trichothecene inhibitors, and their potential application as biomarker metabolites are discussed.

4.2. Introduction

Fusarium head blight caused by *G. zeae* is a predominant disease of barley, wheat, and other Triticeae, worldwide. Severe infection leads to tremendous yield losses, and also indirectly it affects the quality of grains by producing trichothecene toxins. Deoxynivalenol,, 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), nivalenol (NIV), zearalenone (ZEN), and fusaric acid are the major trichothecene contaminants of grains (Bai and Shaner 2004; Choo 2006). The level of the DON present in grains is highly important for industries like brewing and malting. The industrial standards set for mycotoxin contamination is only 0.5 ppm or less of DON in grains (Wolf-Hall 2007). However, DON content in other finished products of wheat and barley are allowed to a permissible level of 2 ppm kg^{-1} (Dexter and Nowicki 2003; Shaner 2003). The most economic way to reduce yield loss and amount of trichothecenes in grains is through breeding for resistance.

Resistance in wheat and barley is assessed mainly based on type I, resistance to initial infection, and type II, resistance to spread within spike (Schroeder and Christensen 1963). Type II, resistance to spread within spike, in barley is very high as compared to wheat, and accordingly the breeding programs on barley are mainly based on type I resistance. Type V, resistance to DON and other trichothecenes has been reported in wheat (Miller and Young 1985; Mesterházy 2002). DON is a virulence factor in FHB disease development (Proctor et al. 1995). Trichothecene non-producing mutant failed to spread to other spikelets through the rachis in wheat (Jansen et al. 2005). Even though pathogen fails to spread through rachis, DON was able to spread to other spikelets of barley (Boddu et al. 2006). DON can be detoxified by an enzyme DON-glucosyltransferase to D3G (Poppenberger et al. 2003). Barley 2H QTL bin 10 analyses identified the UDP-glucosyltransferage gene which converts DON to D3G (Boyd et al. 2010). A QTL on chromosome 3BS has been associated with detoxification of DON to D3G (Lemmens et al. 2005). Resistance in the plants to DON production, in addition to detoxification (type V-1), can also be due to reduced synthesis of DON (type V-2) (Boutigny et al. 2008). Reduction of DON synthesis is possible due to inhibition of toxin synthesis by induced and constitutive endogenous metabolites in plants and this is considered to be due to pro-oxidant and antioxidant properties of these compounds (Boutigny et al. 2008). Ferulic acid and other natural phenolic acids from wheat bran can inhibit the biosynthesis of trichothecenes (Boutigny et al. 2009, 2010). Wheat and barley plants produce several phenolics and flavonoids which also have antioxidant properties (Hamzehzarghani et al. 2008a; Bollina et al. 2010; Kumaraswamy et al. 2011).

The biosynthesis of trichothecenes involves a complex isoprenoid metabolism pathway involving geranyl pyrophosphate (Desjardins et al. 1993). Genes involved in trichothecene biosynthesis (*Tri*) have been identified and 10 such *Tri* genes were known in *F. graminearum* (Brown et al. 2004). Among all *Tri* genes *Tri5* gene is very important in trichothecene biosynthesis, as it encodes the key enzyme trichodine synthase, and this enzyme catalyses the first step in trichothecene biosynthesis (Hohn and Desjardins 1992). Transcriptional level of *Tri* genes involved in inhibition of trichothecene biosynthesis by ferulic acid has been demonstrated (Boutigny et al. 2009). The decrease in trichothecene biosynthesis by ferulic acid was related to lesser level of expression of *Tri* genes

(Boutigny et al. 2009). Disruption of *Tri5* gene, a major gene involved in trichothecene production led to trichothecene nonproducing mutants of *F. graminearum* (Proctor et al. 1995). The phenolic acids extracted from wheat bran led to significant decrease in the expression of *Tri* genes (Boutigny et al. 2010).

The mechanisms of resistance in wheat and barley to FHB can be apparent or true (Kushalappa et al. 2010). The two-row barley has high levels of apparent resistance because of the closed florets, and relatively the open floret, six-row barley and also wheat are more susceptible to FHB. The mechanisms of true resistance can be structural or biochemical. Based on mass spectrometry several RR metabolites have been identified in wheat (Hamzehzarghani et al. 2008a) and in barley (Bollina et al. 2010; Kumraswamy et al. 2011). Deoxynivalenol is a virulence factor (Jansen et al. 2005) and inoculation of DON also produced several RR metabolites (Paranidharan et al. 2008). Pathogenesis related proteins also have been identified in barley (Geddes et al. 2008). The RR metabolites and PR proteins are reported to have antimicrobial, signaling, and cell walls enforcement properties which reduce the progress of the pathogen in resistant hosts. The RR metabolites varied in their ability to reduce *F. graminearum* biomass, *in vitro* (Bollina et al. 2010; Kumaraswamy et al. 2011). Ferulic acid not only reduces the biomass but also inhibits the synthesis of trichothecenes, the mechanism of which has been considered to be due to its antioxidant property (Boutigny et al. 2010). Accordingly, the objective of this study was to evaluate other phenolic flavonoid, and fatty acids, that have been identified in our previous studies as RR metabolites in barley and also reduced biomass *in vitro*, for their ability to inhibit synthesis of trichothecenes in *F. graminearum in vitro*.

4.3. Materials and methods

4.3.1. Selection of RR metabolites

Ten compounds, previously identified as RR metabolites in barley against *F. graminearum* and also shown to inhibit *F. graminearum* biomass, were selected for trichothecene biosynthesis inhibition and these belonged to different chemical groups: *fatty acids*: capric, lauric, and methyl jasmonate; *phenolic acids*: *p*-coumaric, sinapic, quinic, ferulic, and caffeic acid; *flavonoids*: naringenin and quercetin. Commercial pure

powders of all these chemicals were purchased form Sigma-Aldrich (USA). Their effect on biomass reduction in *F. graminearum* has been established (Bollina et al. 2010; Kumaraswamy et al. 2011). The molar concentrations that inhibited 50% of biomass (LD_{50}) were evaluated for inhibition of trichothecenes (Table 4.1).

4.3.2. Culture conditions

The *F. graminearum* isolate, Z3639 (obtained from Dr. R. H. Proctor) (Proctor et al. 1995) was maintained on PDA media. Spores were produced using SNA media; 10 day old cultures were flooded with sterile water and the surface of the media was gently scraped with sterile glass rod to dislodge macroconidia. These were filtered using a 4 layered cheese cloth to separate myclia; filtrate was washed with sterile water and centrifuged to separate spores. Potato dextrose broth of 5 mL in 25 mL test tubes was sterilized. All the RR metabolites were dissolved in methanol and added to the broth to make final milimolar concentrations given in Table 4.1. Same amount of methanol added to broth constituted the control sample. Finally to all test tubes, 10µL of spore suspension of *F. graminearum* containing 10^4 macroconidia mL^{-1} was added. The initial pH of the media was adjusted to 6.5 and addition of any of these metabolites did not change the pH. The test tubes were incubated on orbital shaker at 120 rpm under dark for 5 days. The entire experiment was replicated three times.

4.3.3. Extraction of trichothecenes

The 5 day old cultures were centrifuged at 3000 RPM for 15 minutes, the fungal biomass was separated from liquid media, lyophilized and the biomass was quantified. The spent media was mixed with ethyl acetate in equal volumes, organic phase was separated from media and passed through MycoSep 230 columns (Romer Labs Inc., Union, MO), to extract mainly the trichothecenes (Berthiller et al. 2005). The eluent was evaporated to dryness in a speedvac at room temperature and the residue was dissolved in 400 µL of 50% aqueous methanol (methanol: water, v/v) and stored at -20 ^{0}C until analysis.

4.3.4. Analysis using LC-MS

The trichothecenes and the RR metabolites were identified and quantified using a liquid chromatography and mass spectrometry (LC-MS) with electrospray ionization and a hybrid mass spectrometer with a linear ion trap capable of MS/MS fragmentation, and a high mass accuracy and resolution Orbitrap fourier transform mass analyzer (LC-ESI-LTQ-Orbitrap, Thermo Fisher). Kinetex column and negative ionization mode was used as explained chapter 3 (Bollina et al. 2010). The metabolites were identified based on accurate mass, fragmentation pattern and isotope ratio (Bollina et al. 2010).

4.3.5. Resistance equivalence

The proportion of DON produced (PDP), following inhibition by RR metabolites, was calculated as: $PDP = DP_m/DP_c$, where subscript m is DON produced with metabolite and c is DON produced in control. The resistance equivalence for RR metabolites (Bollina et al. 2010; Kumaraswamy et al. 2011) corrected for inhibition of DON synthesis was calculated as: RE_{DON} = (AR/AS)/PDP, where AR = abundance of endogenous RR metabolite in resistant genotype; AS = abundance of endogenous RR metabolite in susceptible genotype; $PDP = DP_m/DP_c$ is amount of DON produced in media with metabolite and in control.

4.4. Results

The amount of fungal biomass, at predetermined LD_{50} molar concentrations of RR metabolites that inhibited 50% of *F. graminearum* biomass (Bollina et al. 2010; Kumarswamy et al. 2011) were not 50% as expected in this study but they varied among RR metabolites (Table 4.2). Greater amount of biomass relative to control was detected in methyl jasmonate (66% relative to control) and caffeic acid (65%) and least amount of biomass was observed in ferulic acid (44%).

Deoxynivalenol (DON = *m/z* = 296.1259 [M]) and 3- acetyl-deoxynivalenol (3ADON = *m/z* = 338.1364 [M]) were detected in control samples. Deoxynivalenol and 3ADON were detected along with adducts, because of the use of ammonium acetate as one of the solvent. Accordingly, the abundances of pure and adducts of these metabolites were summed to obtain total abundances. The amount of both DON and 3ADON

produced by the fungus varied among treatments with different RR metabolites. The biosynthesis of both DON and 3ADON in *F. graminearum* was completely inhibited by all RR metabolites, except for capric, quinic, and caffeic acid. The amount of DON produced relative to control were: capric =11%; caffeic = 17% and quinic acids = 14%) (Table 4.2). Whereas 3ADON was observed only in quinic acid (12%) and caffeic acid (13%), and it was completely absent in capric acid.

The resistance equivalence (RE) based on trichothecene inhibition was infinity or very high for all RR metabolites, as they completely inhibited trichothecenes, except for capric = 11.09; quinic= 12.71; cafeic =12.94 (Table 4.3).

4.5. Discussion

This study reports the *in vitro* inhibition of trichothecene biosynthesis in *F. graminearum* by selected RR metabolites. A complete inhibition of biosynthesis of trichothecene was observed by lauric, *p*-coumaric acid, sinapic acid, ferulic acid, naringenin, quercetin, and methyl jasmonic acid. Relative to control, high reduction in both DON and 3ADON were observed by quinic and caffeic acid, and in addition, deoxynivalenol by capric acid.

The metabolites evaluated here for trichothecene biosynthesis inhibition have already been shown to be RR metabolites in barley against *F. graminearum* and also to inhibit mycelial biomass of *F. graminearum* under *in vitro* conditions (Bollina et al. 2010; Kumarswamy et al. 2011). However, the methods used for biomass assay varied, where the capric, lauric, *p*-coumaric, sinapic, ferulic, quinic acids, and naringenin were based on fungal biomass weight using liquid media (Bollina et al. 2010), while the methyl jasmonate, caffeic acid and ferulic acids were based on colony diameter using solid media (Kumaraswamy et al. 2011). Ferulic acid was used in both the studies as a control, and the LD_{50} molar concentration varied, 1.76 mM based on mycelial weight and 2.40 mM based on colony diameter. Thus, the biomass weight based on liquid culture was relatively more sensitive than the fungal colony diameter based on solid media culture. In this study, based on liquid media, the biomass was about 50% of control, except for MJ (66%) and capric acid (65%). These were higher because the original selection of LD_{50} was based on solid media.

The RR metabolites used in this study belonged to three different chemical groups: capric acid, lauric acid, and methyl jasmonate were fatty acids, quercetin and naringenin were flavonoids and *p*-coumaric, sinapic, ferulic, quinic, and caffeic acids were phenolic compounds. These vary in their antioxidant properties. Different antioxidant and pro-oxidant properties of the phenolic acids, flavonoids, carotenoid, and fatty acid based compounds are known to modulate biosynthesis of mycotoxins (Burow et al. 1997; Huang et al. 1997; Ponts et al. 2006).

Phenolic acids have very strong antioxidant properties (Rice-Evans et al. 1996), rapid accumulation of phenolic acids (Matern and Kneusel 1988) and release of cell wall bound esterified forms of phenolic acids was attributed upon pathogen infection (Faulds and Williamson 1995). Both cell walls bound and free phenolic acids present at the site of infection are essential in the plant defense against invading pathogen (Matern and Kneusel 1988). Dose effect of ferulic acid on biomass and trichothecene biosynthesis by *Fusarium* spp. was studied (Boutigny et al. 2009). High correlation was observed between the increase in concentration of ferulic acid and decrease in biomass and type B trichothecene biosynthesis. Though 0.1 to 1mM concentration of ferulic acid only slightly reduced the total biomass produced by *Fusarium*, it significantly inhibited the type B trichothecene biosynthesis, suggesting phenolic acids have a specific inhibitory effect on fungal secondary metabolite production. Ferulic acid was used as a positive check eventhough it was not detected as RR metabolite in our studies (Bollina et al. 2010; Kumaraswamy et al. 2011). In this study ferulic acid at LD_{50} concentration of 1.76 mM completely inhibited the type B trichothecene biosynthesis. In contrast, complete inhibition of trichothecenes was observed at 2.5 mM by Boutigny et al. (2009). Ferulic acid at concentration of 0.25 mM, 57% inhibition of trichothecene by *F. graminearum* was documented (Bily 2003). Upon addition of ferulic acid at different time points of growth of *Fusarium* spp a cumulative inhibitory effect on type B trichothecene was observed (Boutigny et al. 2009).

Other phenolic compounds like caffeic acid showed strong antioxidant property based on peroxyl radical scavenging activity whereas ferulic and *p*-coumaric acids showed moderate and low antioxidant properties, respectively (Terao et al. 1993). Phenolic acids, ferulic, syringic, vanillic, caffeic, and *p*-coumaric acids extracted from

grains of FHB resistant wheat genotypes showed significantly greater natural antioxidant and radical scavenging activity when compared to susceptible genotypes (Zhou et al. 2007). Complete inhibition of trichothecene biosynthesis by *F. culmorum* was observed when natural phenolic acids (ferulic, sinapic, *p*-coumaric, vanillic, and *p*-hydroxybenzoic acids) from wheat bran were supplemented in liquid cultures (Boutigny et al. 2010). The mechanisms by which these RR metabolites inhibit the biosynthesis of trichothecene are not clear. However, it could be due to strong antioxidant properties of these RR metabolites. And inhibition biosynthesis of trichothecenes is reported to be due to decreased expression of *Tri* genes by ferulic and other natural phenolic acids extracted from wheat bran (Boutigny 2009, 2010). Similarly in this study phenolic acids like *p*-coumaric acid, ferulic acid, and sinapic acid, showed complete inhibition of trichothecene biosynthesis. However, caffeic acid and quinic acid failed to completely inhibit trichothecene biosynthesis.

Flavonoids are also know for their antioxidant properties (Rice-Evans et al. 1996), flavonoids like saponarin and lutonarin extracted from young barley leaves showed antioxidant properties (Benedet et al. 2007). Naringenin and quercetin showed absolute inhibition of trichothecene biosynthesis in this study under *in vitro* conditions. Naringenin exhibited higher antioxidant capacity and superoxide radical scavenging efficiency under *in vitro* studies (Cavia-Saiz et al. 2010). In our study, none of the RR metabolites was detected 5 dpi, except for naringenin suggesting it was used up during the fungal growth or to inhibit type B trichothecene synthesis. This is similar to the previously reported study where ferulic acid was not detected in spent media (Boutigny et al. 2009). Upon pathogen inoculation ferulic acid concentrations decreased significantly in barley genotypes (Eggert et al. 2010). Significantly higher levels of phenolic and flavonoids were found in emmer wheat genotypes and with high levels of radical scavenging activities (Serpen et al. 2008). Increased levels of flavonoids were observed in FHB resistant maize genotype when inoculated with *F. graminearum*, and was considered to be due to their antimicrobial activity (Reid et al. 1992).

Many fatty acids are known for their antioxidant and pro-oxidant activities as well, methanolic extracts of 12 fatty acids from *Vernonia amygdalina* plants showed significant radical scavenging activities (Erasto et al. 2007). Lauric acid showed

antioxidant activity where as capric acid was inactive in the antioxidants assay (Henry et al. 2002). The saturated fatty acid, capric acid used in our study showed partial inhibition of DON biosynthesis where as lauric acid showed a complete inhibition. Contrarily, the capric acid reduced biomass production at much lower mM concentration than by lauric acid (Bollina et al. 2010). The reduced ability to inhibit toxin could be due to its poor antioxidant activity. Similarly fatty acid signaling molecule methyl jasmonate inhibited the aflatoxin production under *in vitro* conditions up to 96% by *Aspergillus flavus* (Goodrich-Tanrikulu et al. 1995).

Deoxynivalenol is a virulence factor and reduction of DON will lead to decrease in pathogen progress in plant. Resistance related metabolites not only reduce biomass but also the trichothecene synthesis, thus reducing the progress of pathogen in resistant plants. Our previous study used relative biomass reduction by different RR metabolites to derive resistance equivalence. Resistance equivalence based on biomass inhibition can effectively assess the potential of a biomarker. We have calculated RE based on DON inhibition at LD_{50} biomass inhibition. Since several RR metabolites completely inhibited DON synthesis, RE calculated based on PDI may enable more critical evaluation of RR metabolites. The use of RE for the inhibition of both mycelial and trichothecene inhibition should better evaluate RR metabolite efficiency as a potential biomarker and further use in screening for resistance in Triticeae against FHB.

Table 4.1 List of RR metabolites used in this study and their LD_{50} molar concentrations for 50% biomass inhibition of *F. graminearum*

RR metabolites[a]	LD_{50} Concentrations (mM)
Capric acid	0.10
Lauric acid	2.65
Methyl Jasmonate	1.00
p-Coumaric acid	1.15
Sinapic acid	1.74
Quinic acid	3.15
Ferulic acid	1.76
Caffeic acid	2.50
Naringenin	1.58
Quercetin[b]	2.95

[a] all the compounds were selected from Bollina et al. (2010), except for caffeic acid and methyl jasmonate which were selected from Kumaraswamy et al. (2011).

[b] LD_{50} mM concentration of quercetin was determined in this study.

Table 4.2 Proportion of biomass, DON and 3ADON produced by *F. graminearum*, relative to control, in liquid media amended with resistance related (RR) metabolites in barley.

RR metabolites	Dry fungal biomass relative yields[a]	DON produced relative to control[b]	3ADON produced relative to control[b]
Control	1.00	1.00	1.00
Capric acid	0.56	0.11	0
Lauric acid	0.62	0	0
p-Coumaric acid	0.40	0	0
Sinapic acid	0.52	0	0
Ferulic acid	0.44	0	0
Naringenin	0.61	0	0
Quinic acid	0.54	0.14	0.12
Quercetin	0.54	0	0
Methyl jasmonate	0.66	0	0
Caffeic acid	0.65	0.17	0.13

[a] Biomass in treatments as proportion of those in control; mean of 3 replicates of dry fungal biomass yields of different antifungal compounds were expressed relative to their levels in control cultures (26 ± 1.14 μg); Since these were selected based on LD_{50} values, these values should have 0.5 biomass, however, the biomass of caffeic acid and methyl jasmonate were based on solid agar studies (Kumaraswamy et al. 2011).

[b] Abundance of DON or 3ADON in treatments as proportion of those in control; mean of 3 replicates; The average abundances in control were: DON = 4.12×10^6; 3ADON = 5.13×10^6.

Table 4.3 Resistance equivalence for DON production, in media containing resistance related metabolites identified in resistant cultivars Chevron and H106-4.

RR metabolites[a]	Fold change in abundance of RR metabolites[a]	PDP[b]	Resistance equivalence[d]
Capric acid	1.22	0.11	11.09
Lauric acid	1.31	0	-
Methyl Jasmonate	2.40	0	-
p-Coumaric acid	3.48	0	-
Sinapic acid	1.19	0	-
Quinic acid	1.78	0.14	12.71
Ferulic acid	0[c]	0	-
Caffeic acid	2.20	0.17	12.94
Naringenin	1.43	0	-
Quercetin	0[c]	0	-

[a] Relative abundance of RR metabolites or fold change in abundance in resistant relative to susceptible (AR/AS, where AR = abundance of RR metabolite in resistant cultivar; AS = abundance of RR metabolite in susceptible cultivar) in 'Chevron' were based on Bollina et al. (2010) except caffeic acid and methyl jasmonate, they are reported as RR metabolites in resistant cultivar H106-4 based on Kumaraswamt et al. (2011).

[b] PDP= proportion of DON produced (DON_m/DON_c) where DON_m is DON produced with metabolite and DON_c is DON produced in control cultures.

[c] Ferulic acid and Quercetin were not identified as RR metabolites in 'Chevron', thus it was given here a zero value.

[d] Resistance equivalence for DON for cultivars Chevron and H106-4; RE_{DON} = (AR/AS)/PDP, where AR = abundance of RR metabolite in resistant cultivar ; AS = abundance of RR metabolite in susceptible cultivar (Bollina et al. 2010; Kumaraswamy et al. 2011); PDP = Poroportion of DON produced relative to control, *in vitro* at LD_{50} mM concentration of metabolite corrected for myclial biomass; Note here '-' is the infinity or the highest RE as DON production was completely inhibited.

CHAPTER 5

Identification of metabolites related to mechanisms of resistance in barley against *Fusarium graminearum* based on mass spectrometry

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CONNECTING STATEMENT FOR CHAPTER 5

Chapter 5 consists of a manuscript prepared by Bollina V, Kushalappa AC, Choo TM, Dion Y, and Rioux S. entitled "Identification of metabolites related to mechanisms of resistance in barley against *Fusarium graminearum* based on mass spectrometry". This manuscript has been submitted to a refereed journal for publication.

In our first study (Chapter 3) we used a resistant (Chevron) and susceptible (Stander) cultivars to discriminate FHB resistance based on metabolic profiling and also we established levels of *F. graminearum* biomass inhibition by selected RR metabolites. In our second study (Chapter 4) we established the effect of endogenous metabolites on inhibition of type B trichothecene biosynthesis *in vitro*. In our first study, even though we identified RRC metabolites we failed to identify any induced (RRI) metabolites, following inoculation of pathogen. In addition, the DON and the DON detoxification product DON-3-*O*-glucoside were detected in low amounts, in several samples close to noise levels. Accordingly, in the present study (Chapter 5) following modifications were included after a preliminary study: i) the samples were collected at 72 hpi instead of 48 hpi to to provide sufficient time for toxin production and detoxification; ii) we used a relatively more polar column called C-18 Kinetex.

The present study (Chapter 5) was designed to identify RRC, RRI and resitacnce indicator metabolites in several genotypes and to select some of these as potential biomarkers to screen for resistance. Six genotypes ('Chevron', H5277-44, H5277-164, M92-513, 'M122', and 'Stander') varying in FHB resistance, including a susceptible cultivar Stander, were inoculated with *F. graminearum*, and RRC, RRI and resitacnce indicator metabolites were identified. Those that were high in abundance, common to more than one genotype, with known plant defense mechanism were considered biomarker metabolites.

5.1. Abstract

Fusarium head blight (FHB) is an economically important disease of Triticeae, apart from yield reduction; it also causes quality deterioration by producing mycotoxins. Host resistance is the most promising way to control the disease. Metabolic profiling was applied to identify resistance related (RR) metabolites against *Fusarium graminearum* in six genotypes (Chevron, H5277-44, H5277-164, M92-513, M122, and Stander) varying in resistance to FHB. Disease severity was assessed in greenhouse, and the proportion of spikelets diseased (PSD) and the area under the disease progress curve (AUDPC) were used to group genotypes. Spikelets were collected at 72 h post inoculation, metabolites were extracted using aqueous solution of methanol and analyzed using LC-ESI-LTQ-Orbitrap. Abundances of metabolites were subjected to pair wise analysis, resistant vs susceptible, using *t*-test. Treatment significant metabolites were used to identify resistance related (RR) metabolites, including RR constitutive (RRC) and RR induced (RRI). Among 1430 RR metabolites, 115 were assigned putative names. These RR metabolites belonged to different chemical groups: *fatty acids*: jasmonic acid, methyl jasmonate, linolenic acid; *phenylpropanoids*: *p*-coumaric, sinapic acid; *flavonoids*: naringenin, kaempferol glucoside, catechol glucoside. In addition, resistance indicator metabolites, such as deoxynivalenol (DON) and DON-3-*O*-glucoside (D3G) were also detected. The proportion of DON conversion (PDC) to D3G was the highest in resistant genotype Chevron (PDC = 0.76). The plant defense roles of these RR and resistance indicator metabolites and their use as potential biomarkers to screen barley genotypes for FHB resistance are discussed.

5.2. Introduction

Fusarium head blight (FHB) or scab is the most destructive disease of barley and wheat in Canada. Several species cause FHB but *Fusarium graminearum* is the most common in North America (Tekauz et al. 2000; Bai and Shaner 2004). Besides yield losses, the pathogen produces several mycotoxins, such as deoxynivalenol (DON), nivalenol, zearalenone, and 3-acetyl-deoxynivalenol (3ADON) that are hazardous to human and livestock health (Choo 2006). The most promising ways of controlling FHB is through host resistance. However, among more than 25,000 barley accessions screened only a few were moderately resistant, and some of these are being widely used in breeding programs (Choo 2006).

Several FHB resistance quantitative trait loci (QTL) have been identified but often these were linked to pleiotropic effects, thus rendering them less suitable for breeding. Quantitative trait loci for FHB resistance and/or DON accumulation have been identified on all seven chromosomes of barley. A minor QTL for low FHB severity and DON accumulation was found on chromosome 1H in different studies (de la Pena et al. 1999; Dahleen et al. 2003; Mesfin et al. 2003), however, this QTL was associated with heading date (Mesfin et al. 2003). A major QTL on chromosome 2H, for FHB severity and DON accumulation (de la Pena et al. 1999; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003), was associated with heading date, *vrs1* locus (row type) and plant height. A major QTL on 5H for FHB resistance and DON accumulation was also associated with heading date and plant height (de la Pena et al. 1999; Dahleen et al. 2003; Mesfin et al. 2003). In a progeny of cross between two-row and six-row barley, 32 QTL were identified but only 10 were consistent. None of these was associated with row type locus (*vrs1)* but were associated with flowering type (*cly1*) on 2H chromosome and spike morphology (Sato et al. 2008). Two-row genotypes are generally more resistant than the six-row (Buerstmayr et al. 2004; Yoshida et al. 2005), however, a mutation of *vrs1* locus to convert two-row Clho4196 into six-row phenotype lead to undesirable phenotypes, tall stature and late ripening (Boyd et al. 2008). So, the use of disease severity and amount DON alone are inadequate to identify QTL.

There is an urgent need to better understand the mechanisms of resistance for secure pyramiding of genes or molecular breeding. Biochemical profiling can help elucidation of the mechanisms of resistance. Transcriptomics and proteomics have been used to identify PR genes and PR proteins (Boddu et al. 2006; Golkari et al. 2007; Geddes et al. 2008). Increased abundance of chitinases (PR protein 3) and thaumatin like protein (PR protein 5) were detected in resistant and moderately resistant barley genotypes, however, higher abundance of thaumatin like protein 4 was detected in susceptible genotype Stander as well (Geddes et al. 2008). Metabolic profiles have been developed for four barley genotypes and many RR metabolites have been identified (Bollina et al. 2010; Kumaraswamy et al. 2011). These were associated with antimicrobial, signaling and cell wall enforcement properties. In general, the resistant genotypes accumulated less DON and the detoxification of DON into DON-3-*O*-glucoside was reported in both wheat and barley (Lemmens et al. 2005; Bollina et al. 2010). The FHB resistant recombinant inbred lines, in general, detoxified more DON than the susceptible (Lemmens et al. 2005). Thus, metabolic profiling can be used as an excellent tool to detect several mechanisms of

resistance in barley against FHB. Here we aim to identify resistance related metabolites and project them as potential biomarker metabolites. Biomarker metabolites have been identified for resistance in grapes against fungi based on GC-MS (Batovska et al. 2008). Comprehensive metabolic profiling of rice, during various stages of development, through GC-MS studies, has revealed 21 biomarker metabolites (e. g., phenylalanine, salicylic acid, *p*-hydroxybenzoic acid, pyroglutamic acid) (Tarpley et al. 2005). Biotic and abiotic defense related genes, protein, and metabolites were proposed as defense related biomarkers for rice (Jwa et al. 2006). Similarly, comprehensive and untargeted metabolomics approach was also employed to identify biomarker metabolites for quality related traits in potato (Steinfath et al. 2010). The objective of this study was to identify resistance related metabolites, based on mass spectrometry tool, and their common occurrence in several genotypes, for potential application as biomarkers in screening for resistance.

5.3. Materials and Methods

5.3.1. Plant and fungus production

The seeds of six-row barley genotypes, 'Chevron', H5277-44, H5277-164, M92-513, 'M122', and 'Stander', which have varying levels of resistance to FHB, were sown in pots containing pasteurized soil and pro-mix (a mixture of peat moss, perlite, and vermiculite) (50:50). Greenhouse physical environmental conditions were maintained at 22 ± 3 ^{0}C, 70 ± 10% relative humidity and 16 hours of photoperiod throughout the growing period. *Fusarium graminearum* (Isolate 15-35) cultures were maintained on potato dextrose broth media and subcultured on synthetic nutrient-poor agar (SNA) media for spore production. Spore suspensions, of concentration 1.5 X 10^{5} macroconidia mL^{-1}, were prepared (Bollina et al. 2010).

5.3.2. Inoculation and incubation

For metabolic profiling, barley spikelets were inoculated between kernel watery ripe and medium milk growth stages (71 to 75) (Zadoks et al. 1974). Though six-row barley genotypes, with open florets, are most susceptible at flowering stage (growth stage=65) (Yoshida et al. 2005), in our study a later stage was selected to evaluate resistance in lemma, palea and caryopsis without endosperm, as the latter was also associated with transcripts related to resistance (Golkari et al. 2007). All the genotypes studied here are considered to be

chasmogamous flowering type, with confirmation of Chevron, M92-513, and Stander (Yoshida et al. 2005; Choo 2006). The spikelets were point inoculated either with 10 µL of pathogen or mock-inoculated (sterile water containing 0.02% Tween 80) using a repeating 500 µL Hamilton 700 series syringe (Model 7658-01) with PB600-1 dispenser (Model 83700). Immediately after inoculation, plants were covered with transparent plastic bags sprayed with water to maintain high humidity to facilitate spore germination. The covers were removed 48 h post inoculation.

5.3.3. Disease severity assessment

Spikelets were spray inoculated with macroconidial spore suspension, until run-off, using an airbrush (Model Badger-200.3). The experiment was conducted as randomized complete block design with six genotypes, 3 replicates and 30 spikes as experimental units. The number of spikelets diseased was assessed every two day intervals until two readings were the same. From the disease severity data, the proportion of spikelets diseased (PSD) at 14 days post inoculation and the area under the disease progress curve (AUDPC) were calculated (Hamzehzarghani et al. 2008).

5.3.4. Metabolite extraction and analysis using LC-LTQ-Orbitrap

The spikelets were harvested at 72 h post inoculation; endosperm was squeezed out to enable detection of low abundance metabolites avoiding highly abundant sugars (samples contained: lemma, palea, glumes, and caryopsis without endosperm), flash frozen using liquid nitrogen, and stored at -80^{0}C until metabolite extraction. The spikelets were ground in liquid nitrogen, and metabolites were extracted using aqueous methanol, within a week before analysis (Bollina et al. 2010). Briefly, samples of 100 mg were weighed into microcentrifuge tubes and immediately 400 µL of ice-cold methanol (100%) and genistein (200 pg/µL) as internal standard were added. Finally, the methanol concentration was adjusted to 50% by adding HPLC grade water. Samples were vortexed for 15 seconds, sonicated in water bath for 15 min at 40 kHz at room temperature, and centrifuged for 10 min at 20,000 xg at room temperature. The supernatant was filtered through 0.1 µm pore size PVDF (poly vinylidene difluoride) membrane filter and stored in amber coloured vials at -20 ^{0}C.

Metabolites were analyzed using a LC-hybrid-MS (LC-ESI-LTQ-Orbitrap, Thermo Fisher, Waltham, MA) as explained in Bollina et al. (2010), with some modifications. A 5 µL

sample extract was injected automatically using a 96-well auto sampler maintained at 20 °C. A capillary C-18 reversed-phase relatively polar column Kinetex (2.1 mm i.d X 10 cm, with particle size of 2.6 µm, Phenomenex) was used. The column was maintained at 25 °C and the mobile phase was adjusted to a 150 µL/min. Metabolites were eluted with 2.5 mM ammonium acetate in water (buffer A) and 100% methanol (buffer B). The mobile phase gradient was 100% A at the beginning and shifted to 70% B in 15 minutes and subsequently ramped to 90% mobile phase B over 5 minutes, followed by a 5 min ramp to 90% mobile phase A and then back to 100% buffer A with a duty cycle of 43 minutes. After every sample analysis, a blank sample consisting of water and methanol was run to minimize the carryover of metabolites. The mass resolution was set at 60,000 [FWHM] at 400 *m/z*. All the samples were first run to obtain MS 1, and in addition, a few samples were run to obtain MS/MS fragmentations using normalized collision induced dissociation energy of 35 eV, with activation q and activation time set at 0.25 and 30 msec, respectively. Data were recorded in centroid mode.

5.3.5. LC/MS output processing

The raw output was converted to mzData and imported to XCMS (V 1.24.1) to deconvolute, align and peak pick (Smith et al. 2006). Pairs of resistant and susceptible genotypes, including all the treatments and replicates were aligned together. Bioinformatics tool CAMERA linked to XCMS was used to identify isotopes, adducts and neutral losses. These outputs were exported to Microsoft EXCEL and the isotope, adduct, and neutral loss peaks were excluded from statistical analysis, as these were multiple peaks of the same compound.

5.3.6. Experimental design and statistical analysis

The experiment was designed as a randomized complete block with 6 genotypes, including five genotypes varying in resistance (R) and one (‘Stander’) susceptible (S) to FHB, two inoculations of mock-inoculated (M) and pathogen-inoculated (P), and five blocks/replicates over time. The experimental unit consisted of a pool of about 60 spikelets, ten inoculated spikelets collected from middle region of at least 6 inoculated spikes, from six different plants grown in 2 pots. Spikelets were harvested at 72 h post inoculation, endosperms were squeezed out, and the rest was analyzed.

The relative abundances of peaks (ion counts) were subjected to pair wise analysis of each of the five resistant vs the susceptible cultivar Stander, or of pathogen-inoculated vs mock-inoculated, based on *t*-test using SAS 9.2. The combinations considered were: RM vs SM, RP vs RM, SP vs SM and RP vs SP, where R = resistant, S = susceptible, P = pathogen-inoculated and M = mock-inoculated. The abundances of peaks that were significant at $P < 0.05$ were considered as treatment significant metabolites.

The abundances of treatment significant metabolites of six genotypes were also subjected to canonical discriminant analysis (CDA) using the CANDISC procedure of SAS version 9.2 to identify hidden biological functions of metabolites (Hamzehzarghani et al. 2008). The CAN vector scores classified the treatments into groups which in turn were related to levels of resistance of genotypes against FHB to identify a resistance function. The metabolites loadings to a given CAN vector were used to explain the resistance-function.

5.3.7. Identification of RRC and RRI metabolites

The treatment significant, at $P < 0.05$, metabolites were used to identify the resistance related (RR) metabolites (abundances of these metabolites were higher in resistant than in susceptible cultivar Stander). The RR metabolites based on mock-inoculations were considered constitutive metabolites (RRC = RM>SM) and pathogen-inoculations as induced metabolites (RRI = RP>RM and RP>SP) (Hamzehzarghani et al. 2008). The fold change in abundance of a metabolite in resistant relative to susceptible was calculated as: RRC = RM/SM; RRI = RP/SP. These fold changes were compared, across resistant genotypes, to identify those with higher levels of change. The RR metabolites were putatively identified based on three criteria: i) accurate mass with AME < 5ppm; ii) mass fragmentation pattern; iii) number of carbons in the formula based on isotope pattern (Bollina et al. 2010). The amount of DON, 3ADON and D3G was estimated based on external calibration curves using a trichothecene mixture (DON, nivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol) along with D3G (Sigma-Aldrich, USA), which were dissolved in methanol/water (1:1, v/v), and analyzed using similar LC-MS conditions as explained above.

5.4. Results

5.4.1. Disease severity

The disease severity in six genotypes varied from PSD = 0.34 in 'Chevron' to 0.79 in 'Stander'. The PSD in 'Stander' was significantly higher than in all the resistant genotypes, but the PSD was not significantly different among resistant genotypes. On the other hand, the AUDPC varied from 3.6 in 'Chevron' to 7.23 in 'Stander'. The AUDPC, however, significantly varied among resistant genotypes, and the Duncan's multiple range test ranking is given in Table 5.1.

5.4.2. Comparative analysis of metabolite profiles

The number of treatment significant metabolites (P <0.05) varied among different resistant genotypes: Chevron=1455, M122=1209, M92-513=1436, H5277-44=1865, and H5277-164=1237 (Table 5.2). However, the occurrence of these metabolites overlapped among genotypes.

5.4.3. Canonical discriminant analysis (CDA)

The abundances of 300 metabolites, significant in all treatment combinations, were subjected to CDA to cluster treatments. In all the treatments, the replicates were grouped together, meaning the experimental error was minimal. The CAN1 vector explained 61% of the variance, while CAN2 explained 25% of the variance (Fig. 5.1). Very high positive CAN2 scores were observed for the genotype H5277-164 inoculated with pathogen. However, the CAN vectors failed to explain any resistance-functions.

5.4.4. Resistance related constitutive metabolites (RRC)

A total of 1139 metabolites were selected as RRC across all the five resistant genotypes but the number occurring varied in different genotypes. Among these, 90 RRC metabolites were putatively identified (Table 5.3). The number of RRC metabolites identified was highest in H5277-44 and lowest in 'M122' genotypes. Among genotypes, 'Chevron' had greater fold change in abundance, relative to susceptible (RM/SM), in kaempferol-3-*O*-α-rhamnoside (7.32),

while H5277-164 had greater fold change in methyl jasmonate (7.50) and kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside (5.53).

'Chevron': In this genotype, a total of 286 metabolites were selected as RRC, of which 48 were putatively identified. High fold changes in abundances, in resistant relative to susceptible, were observed for metabolites: *phenylpropanoids*: *p*-coumaric acid (1.81), 4-methoxycinnamic acid (1.29), sinapic acid (2.56), geranyl cinnamate (2.56); *flavonoids*: naringenin (1.41), kaempferol-3-*O*-α-rhamnoside (7.32) and naringenin-7-*O*-glucoside (2.31); *fatty acids*: jasmonic acid (1.57), methyl jasmonate (1.45), linolenic acid (1.58). In general, 'Chevron' had the highest number of phenylpropanoids, followed by flavonoids and their conjugates, and fatty acids.

H5277-44: A total of 407 metabolites were selected as RRC, of which 52 were putatively identified. High fold changes in abundance were observed for: *phenylpropanoids*: *p*-coumaric acid (1.64), sinapic acid (2.10), geranyl cinnamate (1.73), lauric acid (1.46); *flavonoids*: naringenin (1.84), naringenin-7-*O*-glucoside (2.44), kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside (2.13), and rhamnetin 3-*O*-rhamninoside (1.92); *fatty acids*: jasmonic acid (1.82), methyl jasmonate (1.44), linolenic acid (2.11). This genotype had the highest number of flavonoids, followed by fatty acids.

H5277-164: A total of 157 metabolites were selected as RRC, of which 34 were putatively identified. High fold changes were observed for: *phenylpropanoids*: *p*-coumaric acid (1.41), 4-methoxycinnamic acid (1.12), sinapic acid (1.37), and geranyl cinnamate (1.53); *flavonoids:* naringenin (1.16), kaempferol 3-(2''-(Z)-*p*-coumaroyl rhamnoside) (2.79), kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside (5.53), and rhamnetin 3-*O*-rhamninoside (1.91); *fatty acids*: jasmonic acid (1.67), methyl jasmonate (7.50) and linolenic acid (1.38).

M92-513: A total of 159 metabolites were selected as RRC, of which 22 were putatively identified. High fold changes were observed for: *phenylpropanoids*: geranyl cinnamate (1.83); *flavonoids*: naringenin (1.21), quercetin 3,7-dimethyl ether(1.23), trans-*p*-sinapoyl β-D-glucopyranoside (1.87), catechin-4-ol 3-*O*-β-D-galactopyranoside (1.84), and kaempferol 3-(2''-(Z)-*p*-coumaroyl rhamnoside) (1.51); *fatty acids*: jasmonic acid (1.84), methyl jasmonate (2.12), and linolenic acid (1.36).

'M122': A total of 130 metabolites were selected as RRC, of which 15 were putatively identified. High fold changes were observed for: *phenylpropanoids*: geranyl cinnamate (1.46);

flavonoids: naringenin (1.42) quercetin 3, 7-dimethyl ether (1.51), trans-*p*-sinapoyl β-D-glucopyranoside (2.25), and kaempferol 3-(2"-(Z)-*p*-coumaroyl rhamnoside) (1.94); *fatty acids*: methyl jasmonate (1.68), and linolenic acid (1.73).

5.4.5. Resistance related induced metabolites (RRI)

A total of 291 metabolites were selected as RRI, of which 40 were putatively identified (Table 5.3). The number of RRI metabolites identified was the highest in H5277-164 and the lowest in 'Chevron' genotypes. Some of the very highly induced RRI metabolites, high fold changes in resistant relative to susceptible (RP/SP, when RP>RM) were: heptadecanoic acid (35.53) in 'M122'; (3-phenylpropionyl) glycine methyl ester (29.79) in H5277-44; kaempferol 3-(6"-caffeoylglucoside) (14.15) in Chevron and gallocatechin-4β-ol (10.54) in M122 cultivars.

'Chevron': A total of 60 metabolites were selected as RRI, of which only 5 were putatively identified. High fold changes were observed for: *phenylpropanoid*: α-cyano-4-hydroxycinnamic acid (2.26); *fatty acids*: jasmonic acid (1.57) and methyl jasmonate (1.34); *flavonoids*: kaempferol 3-(6"-caffeoylglucoside) (14.15). Jasmonic acid and methyl jasmonate were both RRC and RRI metabolites.

H5277-44: A total of 70 metabolites were selected as RRI, of which 8 were putatively identified. High fold changes were observed for: *flavonoids*: quercetin pentamethyl ether (1.50) and methionine sulfoxide (2.97); *fatty acids*: jasmonic acid (1.37), methyl jasmonate (1.36). Jasmonic acid, methyl jasmonate and quercetin pentamethyl ether were both RRC as well as RRI metabolites.

H5277-164: Out of 62 RRI metabolites selected, 19 were putatively identified. Major RRI metabolites and their fold changes were: *flavonoids*: apigenin (3.65), naringenin-7-*O*-glucoside (2.31), kaempferol 3-(2"-(Z)-*p*-coumaroyl rhamnoside) (1.29); *fatty acids*: jasmonic acid (1.45), methyl jasmonate (4.47). None of the identified metabolites belonged to phenylpropanoid. Five metabolites were both RRC and RRI, including jasmonic acid and methyl jasmonate.

M92-513: Out of 63 RRI metabolites selected, 7 were putatively identified. High fold changes were: *flavonoid*: kaempferol 3-(6"-caffeoylglucoside) (14.15); *phenylpropanoid*: pyrogallol (2.10); *fatty acid*: heptadecanoic acid (35.53).

'M122': Out of 36 RRI metabolites selected, 6 were putatively identified. High fold changes were observed for: *flavonoids*: gallocatechin-4β-ol (10.54), kaempferol-3-*O*-rhamnoside (2.88), and no major fatty acids and phenylpropanoids were induced.

5.4.6. Resistance indicator metabolites

Extra cellular metabolites produced by the pathogen that are accumulated in spikelets, especially the virulence factor, DON, and the detoxified metabolite of DON, D3G (Poppenberger et al. 2003), by enzymes produced by the plant, that are indicators of resistance, were designated as resistance indictor metabolites. No resistance indictor metabolite was detected in the mock-inoculated genotypes. Most abundant trichothecene metabolite identified was deoxynivalenol (DON, *m/z* = 295.1187), both as pure and also as its acetylated adduct (*m/z* = 355.1395). The DON detoxified product DON-3-*O*-glucoside (D3G, *m/z* = 457.1715), was also detected as pure and also as its acetate adduct (*m/z* = 517.1921). Abundances of only the pure forms of DON and D3G were used to estimate their concentrations, using standard curves. From these, the total amount of DON produced was derived as: TDP = DON + D3G; and the proportion of TDP converted to D3G was derived as: PDC = D3G/TDP. In addition, another trichothecene, 3-acetyl-deoxynivalenol (3ADON, m/z = 337.1292) was detected as both pure and it's adduct form (*m/z* = 397.1495) (Fig. 5.2a). The TDP varied from 622 mg/kg (fresh weight basis) in genotype Chevron to 1037 mg/kg in genotype M122. The PDC varied from 0.76 in genotype Chevron to 0.55 in genotype M122. Chevron, the most resistant genotype had the highest PDC = 0.76, while the moderately resistant genotypes H5277-44, H5277-164, and the susceptible Stander had moderate levels of PDC, of about 0.67. However, the PDC by M122 and M92-513 were only, 0.55 and 0.57, respectively (Fig 5.2b). Interestingly, lower the amount of DON accumulated higher was the PDC by a genotype, with a negative correlation, and a linear regression model explained 56% of the variance (Fig. 5.2c).

5.5. Discussion

This study identified resistance related constitutive, resistance related induced, and resistance indicator metabolites, and compared their fold changes in abundances relative to susceptible, across five genotypes of six-row barley varying in resistance to FHB. Both canonical discriminant analysis and *t*-tests were used to identify potential RR biomarker metabolites. The

CDA failed to explain resistance functions, either constitutive or induced resistance. In some genotypes, the pathogen and mock-inoculated observations were grouped together, while in others the inoculations were separately grouped. Interestingly, the susceptible genotype was grouped along with some resistant, indicating it also has some resistance related compounds. Since the experimental design was complex, it was difficult to associate the high loadings of metabolites with high level of resistance in a given genotype. Accordingly, the RR metabolites, distinct and common to different genotypes, were further investigated based on *t*-test. The RR metabolites varied in their abundances, and up to 7 and 35 fold changes in abundances relative to susceptible, were respectively observed for RRC and RRI metabolites. The RRC, RRI and resistance indicator metabolites were further filtered, to selected biomarkers based on three criteria: i) putatively identified; ii) fold changes in abundance relative to susceptible of >1.0, with more emphasis on higher fold changes and higher levels of significance at $P < 0.01$ and $P < 0.001$; iii) occurred in more than one genotype; iv) reported to have plant defense function, especially involving Triticeae and/or *F. graminearum* (Table 4). The highly significant and high fold change metabolites would have higher probability to be incorporated into a desired genotype in metabolic marker assisted selections.

The genotypes varied in disease severity, based on PSD, from 0.34 in 'Chevron' to 0.79 in 'Stander', and also, based on AUDPC, from 3.6 in 'Chevron' to 7.23 in 'Stander'. In this study, we have identified 90 RRC and 40 RRI metabolites, in addition to two resistance indicator metabolites, TDP and PDC. Still we have not explored all the metabolites in barley, as the protocol used here extracted only aqueous methanol soluble metabolites, and the Kinetex column used detected mainly semipolar metabolites. Using Kinetex column not only more metabolites were detected than using Jupiter column, but also we could detect DON and its conjugates (Bollina et al. 2010). Use of other experimental protocols is needed for comprehensive detection of barley metabolites. In this study, we sampled spikelets at growth stage 71-75 to include caryopsis without endosperm, as it also has resistance (Golkari et al. 2007). Also here we used individual spikelet inoculation because the use of spray inoculation increased variability, due to variation in infected tissue among replicates, and failed to identify any induced metabolites (Bollina et al. 2010). Even in spray inoculation germinated spore enters the spikelet between lemma and palea, and colonizes them from inside through stomata, similar to spores introduced into the spikelet (Yoshida et al. 2005).

The RR metabolites identified here belonged to different chemical groups, mainly, phenylpropanoids, flavonoids, fatty acids, alkaloids, and amino acids. All the putatively identified RR metabolites were previously reported from plants. Out of more than 130 RRC and RRI metabolites putatively assigned names in this study, 16 were selected as FHB resistance biomarker metabolites (Table 5.4). The metabolic pathways of synthesis of these resistance biomarker metabolites and their plausible role in plant defense are discussed below. The resistance biomarker metabolites from a specific metabolic pathway were discussed together, to emphasize the regulation of other metabolites related to a pathway, in addition to those identified here as biomarkers.

5.5.1. Pathogen virulence factor and its detoxified related metabolites

5.5.1.1. Total DON produced (TDP): The least amount of TDP was in 'Chevron' (622 mg/kg). Even though 'Stander' had the greater disease severity than M122, it accumulated lower amount of DON. Reduction in DON synthesis by *F. graminearum* has been reported in ferulic acid amended medium, and the mechanism was considered to be due to its antioxidant property, which represses the Tri gene expression of trichothecene synthesis (Boutigney et al. 2009; Boutigney et al. 2010). High amounts of phenolic acids accumulation, at the site of infection of wheat, have been reported (Nicholson et al. 2008; Matern and Kneusel 1988). Interestingly, we have identified several phenylpropanoids and flavonoids as RR metabolites which also have antioxidant properties. 'Chevron' had the greater fold change in abundances of a few flavonoids: kaempferol-3-*O*-α-rhamnoside (7.32 = RRC) and kaempferol 3-(6"-caffeoylglucoside) (14.15 = RRI) and phenylpropanoids. Whereas, the cultivar M122 had no significant phenylpropanoids even though it had high fold change abundance in a flavonoid, gallocatechin-4β-ol (10.54). More specific studies, however, are needed to clarify the mechanisms.

5.5.1.2. Proportion of DON conversion to D3G (PDC): The maximum PDC was observed for the cultivar Chevron (0.76). High ratios of DON to D3G conversion has been reported in wheat recombinant inbred line populations (Lemmens et al. 2005). Even though Stander was a susceptible cultivar, the PDC was quite high, 0.66. It is possible that the QTL at chromosome 3H of 'Stander' may be associated with this gene (Smith et al. 2004). DON is converted to D3G through activation of DON-glucosyltransferase (Poppenberger et al. 2003).

Interestingly, a decrease in the synthesis of DON (TDP) was associated with an increase in DON detoxification to D3G (PDC), and a linear regression model explained 56% of the variance (Fig. 4). It is possible that a reduced DON accumulation has increased the activity of glucosyltransferase, or vice versa, leading to a higher PDC. For example, the genotype M122 had the greater TDP and was associated with the lesser PDC.

5.5.2. Fatty acid metabolic pathway metabolites

5.5.2.1. Jasmonic acid and methyl jasmonate: Jasmonica acid and MeJA were selected as RRC metabolites in all genotypes, except for JA in 'M122', and were also selected as RRI in 'Chevron', H5277-44 and H5277-164. These were also reported as RRC in barley genotype 'H106-4', and in addition, MeJA also has significantly reduced *F. graminearum* biomass *in vitro* (Kumaraswamy et al. 2011). Jasminic acid and MeJA are important lipid-driven hormone signaling molecules for plant defense against stress response (Balbi and Devoto 2007; Panstruga et al. 2009). Jasmonic acid and its conjugates, in coordination with ethylene, reduce the infectivity of necrotrophic pathogen in plant cells (Panstruga et al. 2009). Enhanced resistance to rice blast caused by *Magnaporthe grisea* was observed upon over-expression of JA in transgenic rice (Mei et al. 2006). However, JA signaling mediated expression of disease resistance was not found in barley for powdery mildew (Kogel et al. 1995). Jasmonic acid and salicylic acid are referred to as networks of complex signaling molecules for necrotrophic and biotrophic pathogens, respectively (Panstruga et al. 2009). In this study, we didn't identify salicylic acid or its conjugates. Exogenous application of gaseous form of MeJA enhanced the disease resistance in *Arabidopsis* against necrotrophic pathogens like *Alternaria brassicicola*, *Botryris cinerea*, and *Plectosphaerella cucumerina* (Thomma et al. 2000). Over expression of JA methyltransferase, a key enzyme in the conversion of JA to MeJA in *Arabidopsis*, showed enhanced resistance to *Botrytis cinerea* (Seo 2001).

5.5.2.2. Linolenic acid: Linolenic acid, identified here as RRC in all the genotypes, is a polyunsaturated fatty acid which eventually oxidizes to oxylipins viz. jasmonic acid and its conjugates (Blee 2002; Walter et al. 2009). These oxylipins not only induce the expression of PR proteins (Schweizer et al. 1997) but also act as antimicrobial compounds (Mei et al. 2006). Linolenic acid also has been reported as RR metabolite in barley genotype H106-4 and it

inhibited the mycelial growth of *F. graminearum* under *in vitro* conditions (Kumaraswamy et al. 2011). This is released by membrane bound phospholipases and oxidized into JA under stress conditions (Li and Yen 2008). Linolenic acid is a precursor for JA biosynthesis, and up-regulation of allene oxide synthase, a major enzyme in JA biosynthesis, has been reported in FHB resistant wheat cultivar Sumai3, following inoculation with *F. graminearum* (Li and Yen 2008).

5.5.3. Phenylpropanoid pathway metabolites

***p*-Coumaric and sinapic acids**: These phenolic acids were identified as RRC metabolites in 'Chevron', H5277-44 and H5277-164. These metabolites significantly reduced *F. graminearum* biomass *in vitro* (Bollina et al. 2010; Kumaraswamy et al. 2011). A phenolic acid, ferulic acid, inhibited synthesis of trichothecenes, including a virulence factor, DON, through its antioxidant property, and other phenolics also considered to have similar effects (Boutigny et al. 2009; Boutigny et al. 2010). *p*-coumaric and ferulic acids showed significant *in vitro* inhibition of *F. graminearum* and *F. culmorum* isolates (McKeehen et al. 1999). *p*-coumaric acid and other phenylpropanoids were reported from emmer and naked barley cultivars infected by *F. graminearum* (Eggert et al. 2010). In wheat bran *p*-couamric, ferulic, and sinapic acids were abundantly present, and the reconstituted phenolic acids inhibited trichothecene biosynthesis in *in vitro* studies (Boutigny et al. 2010). *p*-coumaric acid, apart from being cell wall fortification and antimicrobial, also serves as a precursor for diverse defense related compounds, such as flavonoids, stilbenes and lignins (Whetten and Sederoff 1995).

5.5.4. Flavonoid pathway metabolites

5.5.4.1. Naringinin and its conjugates: Flavonoids are synthesized downstream of phenylpropanoid pathway. In this study, we have identified naringenin as RRC metabolite, in all the genotypes. In addition, its glucosylated conjugate, naringenin-7-*O*-glucoside, was identified as RRC in 'Chevron' and H5277-44, and as RRI in H5277-164. Naringenin also has been reported from hulled and hulless barley (Kim et al. 2007). Naringenin significantly reduced *F. graminearum* biomass *in vitro* (Bollina et al. 2010; Kumaraswamy et al. 2011).

5.5.4.2. Kaempferol glucoside conjugates: Naringenin acts as a precursor in the biosynthesis of flavones such as dihydrokaempferol, kaempferol, and kaempferol glucosides. In this study, we have identified several conjugates of kaempferol as RRC metabolites: kaempferol 3-*O*- rhamnoside, kaempferol 3-(2"-(Z)-p-coumaroyl rhamnoside), kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside, kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside, and kaempferol 3-rhamnoside-7- glucosyl-(1->2)-rhamnoside. Our previous study also identified kaempferol 3-*O*- rhamnoside, kaempferide 3-glucoside-7-rhamnoside, kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside, and kaempferol-3-gentiobioside-7-rhamnoside as RRC metabolites in Chevron cultivar (Bollina et al. 2010). However, in our previous study, the poor solubility of kaempferol in aqueous methanol resulted in least reduction in biomass, relative to control, of *F. graminearum in vitro* (Bollina et al. 2010). Kaempferol glucoside conjugates extracted from carnation inhibited the mycelial growth of *F. oxysporum* f. sp. *dianthi* (Galeotti et al. 2008).

5.5.4.3. Catechin and its conjugates: Catechin is a flavonoid and we have identified catechin conjugates like catechin 7-*O*-apiofuranoside, catechin 3-*O*-alpha L-rhamnoside, and Catechin-4-ol 3-*O*-β-D-galactopyranoside in six-row barley genotypes. Catechin was reported as RRC metabolite in two-row barley-*F. graminearum* interaction (Kumaraswamy et al. 2011). Catechin, catechin-7-*O*-β-D-apiofuranoside and catechin-7-*O*-β-D-xylopyranoside from *Ulmus davidiana* showed antioxidant properties (Jung et al. 2010), these flavonoids were detected as both constitutive and induced upon microbial infection (Grayer and Harborne, 1994). *In vitro* inhibition of *F. graminearum* biomass and other *Fusarium* spp. has been reported for flavonoids and flavones (Silva et al. 1998). In barley seeds, the penetration by *F. graminearum* and *F. culmorum* was increased, when flavonoid production was suppressed (Skadhauge et al. 1997).

In this study, we have reported several FHB resistance biomarker metabolites. Those with higher fold changes in abundance and high levels of significance, especially naringenin and further production of several forms of kaempferols, can be used in metabolic marker assisted selection. A major QTL for resistance to common bean blight was mapped in the same DNA marker region for flavonoid biosynthesis enzyme (Nodari et al. 1993). Flavonoid pathway genes have been engineered for plant disease resistance (Bovy et al. 2007). Using mass spectrometry, the amino acid sequence of enzymes catalyzing the naringinin/flavonoid pathway can be

performed and related to genome sequences of barley and other model plants to identify the genes, which in turn, can be used in DNA marker assisted selection (Hoehenwarter et al. 2008). The mass spectrometry tool standardized here also can be used to elucidate the functions of more than 100 QTL that have been identified in wheat and barley (Buerstmayr et al. 2009; Choo et al.2006). The FHB resistance biomarker metabolites, in this study, were identified based on robust greenhouse study involving blocks over time, however, still these have to be validated under different growth conditions, especially temperature. DNA marker assisted selection is an excellent tool for plant resistance improvement, as long as the effects are major, such as QTL *Fhb1* linked to DON detoxification enzyme DON-glucosyltransferase (Lemmens et al.2005). However, most of the quantitative resistance mechanisms against FHB are minor and additive. The metabolomics tool, as proposed here, can be directly used or simplified based on selected-ion monitoring to fast detect the resistance biomarker metabolites, to select cultivars with high levels of polygenic resistance to FHB. Two independent studies using genotypes Chevron and Standard, reported here and also previously by Bollina et al. (2010), show that most of the major FHB resistance biomarker metabolites are consistent in their occurrence, while others though not present in high abundances, they were associated with high abundance of their precursors. Accordingly, we have emphasized more on sets of pathway related metabolites than individuals, though these may have varying levels of plant defense effects (Bollina et al. 2010; Kumaraswamy et al. 2011).

Table 5.1 Fusarium head blight severity of 6 six-row barley genotypes, under greenhouse conditions, following spray inoculation with macroconidia of *F. graminearum*.

	PSD[a]		Duncan's classification	AUDPC		Duncan's classification
Genotypes	Mean	SE[b]		Mean	SE	
'Chevron'	0.336	0.038	B	3.609	0.161	D
M92	0.373	0.039	BA	3.968	0.170	DC
'M122'	0.395	0.084	BA	4.214	0.132	CD
DH44	0.515	0.102	BA	5.992	0.020	B
DH164	0.518	0.014	BA	5.309	0.107	B
'Stander'	0.787	0.019	A	7.239	0.274	A

[a] The PSD was scored on 14 dpi; for the AUDPC the maximum time was 14 d.

[b] Means and SE (standard error) are calculated from 3 replicate values. PSD and AUDPC are proportion of spikelets diseased and the area under disease progress curve, respectively.

Table 5.2 Total number of treatment significant peaks[a] and resistance related metabolites detected in five genotypes, of six row barley, varying in resistance to FHB.

	'Chevron'	**M92-513**	**'M122'**	**H5277-44**	**H5277-164**
Total peaks	3835	3948	3845	3842	3847
Significant at $P < 0.05$	1455	1436	1209	1865	1237
RRC[b]	286	159	130	407	157
RRI [c]	60	63	36	70	62

[a] Peaks detected were first sieved to remove adducts, and then the abundances were subjected to *t*-test, at $P < 0.05$ to select treatment significant metabolites; details in text.

[b] Resistance related constitutive

[c] Resistance related induced

Table 5.3 Putatively identified RR metabolites from barley-*Fusarium* interaction in six-row barley genotypes as compared to susceptible genotype 'Stander'.

Exp. Mass [M][a], median	**Exp RT, median**	**AME (PPM)**	**Putative name of identity**	**'Chevron'**	**H5277-44**	**H5277-164**	**M92-513**	**'M122'**
Phenylpropanoids and conjugates				Fold Change[b]	Fold Change	Fold Change	Fold Change	Fold Change
126.03222	1.54	4.96	Pyrogallol				**3.31/RRC*** 2.10/RRI*	2.51/RRC*
164.04735	2.38	0.30	*p*-coumaric acid	1.81/RRC	1.64/RRC**	1.41/RRC		
178.06321	15.68	1.18	4-Methoxycinnamic acid	1.29/RRC		1.12/RRC		
189.04287	9.58	1.42	α-cyano-4-Hydroxycinnamic acid	2.26/RRI				
205.07420	18.24	1.14	Cinnamoylglycine		1.69/RRC	1.46/RRC**		
224.06891	28.12	2.28	Sinapic acid	2.56/RRC*	2.10/RRC*	1.37/RRC*		
284.17629	2.17	-4.60	Geranyl Cinnamate	2.56/RRC	1.73/RRC	1.53/RRC	1.83/RRC**	1.46/RRC
338.10054	11.86	1.31	3-O-Caffeoylquinic acid				1.5/RRC*	
406.12571	20.04	-1.50	Astringin	2.38/RRC*				
416.14896	1.51	4.46	1-Acetoxypinoresinol		1.51/RRC*			
428.16786	16.19	-0.79	trans-p-Ferulyl alcohol 4-O-[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside	1.69/RRC*				
536.18812	15.65	-1.20	7-O-(4-Methoxycinnamoyl) tecomoside	1.44/RRC*				
580.21426	17.48	-2.10	(+)-syringaresinol O beta-D-glucoside		1.40/RRC			
328.12985	19.94	-3.80	Seselinol isovalerate	1.51/RRC	2.87/RRC*			
370.09000	1.53	-0.25	6,7-Dihydroxy-5-methoxycoumarin 6-β-D-glucopyranoside				1.95/RRC	
376.15186	15.17	-0.91	trans-Grandmarin isovalerate		1.46/RRC*			
462.11549	18.94	-1.50	Isoscoparin	**4.64/RRC**				

358.14118	17.48	-1.20	Dehydrodiconiferyl alcohol		1.48/RRC			
358.14146	19.96	-0.40	Matairesinol		2.76/RRC*		1.95/RRC	1.74/RRC*
Fatty Acids								
92.04718	29.17	-1.30	Glycerol	1.96/RRC*	2.08/RRC**	2.39=RRC*	1.44 RRC*, 2.08 RRI*	1.44/RRC*, 2.18 RRI*
162.05306	10.89	1.62	3-Hydroxy-3-methylglutaric acid				1.63/RRC*	1.63/RRC*
186.16227	27.04	1.96	Undecanoic acid		1.46/RRC*			
210.12584	1.13	1.21	Jasmonic acid	1.57/RRC, 1.73/RRI	1.82/RRC, 1.37/RRI*	1.67/RRC*, 1.45/RRI**	1.84/RRC*	
212.14151	25.19	1.40	Dihydrojasmonic acid	1.45/RRC*	1.49/RRC	3.98/RRI*		
224.14140	27.49	0.88	Methyl Jasmonate	1.45/RRC, ** 1.34/RRI*	1.44/RRC*, 1.36/RRI*	**7.50/RRC***, **4.47/RRI***	2.12/RRC*	1.68/RRC*
270.25625	30.62	1.67	Heptadecanoic acid				**35.53/RRI****	
278.22468	31.05	0.63	Linolenic acid	1.58/RRC*	2.11/RRC*	1.38/RRC	1.36/RRC	1.73/RRC
282.25629	29.61	1.74	Oleic acid		1.57/RRC*			
290.18817	26.50	-0.01	8-oxo-9,11-octadecadiynoic acid	**3.62/RRI****				
294.21947	27.88	0.23	3-Oxo-2-(2-entenyl) cyclopentaneoctanoic acid		1.61/RRI**			
308.00507	1.16	-3.70	Mevalonic acid 5-pyrophosphate				1.46/RRC	
310.21423	24.26	-0.54	13(S)-Hydroperoxy linolenic acid		2.11/RRI*			
312.22984	25.24	1.77	Octadeca-9-ene-1,18-dioic-acid				1.31/RRC	1.46/RRC*
312.30328	32.32	1.55	Arachidic acid			1.32/RRC*		
Flavonoids and Conjugates								
242.09082	9.49	-1.40	7,4'-Dihydroxyflavan			**3.11/RRI****		
270.05279	24.39	0.01	Apigenin			**3.65/RRI***		
272.06843	22.07	-0.25	Naringenin	1.41/RRC*	1.84/RRC*	1.16/RRC*	1.21/RRC*	1.42/RRC
276.15817	16.01	-1.60	cis-p-Coumaroyl agmatine			2.85/RRI		
322.06840	8.69	-1.20	Gallocatechin-4 β-ol					**10.54/RRI****
328.13071	17.46	-1.20	5-O-Methylleridol		1.53/RRC*			

330.07386	24.39	-0.12	Quercetin 3,7-dimethyl ether		1.55/RRC*		1.23/RRC	1.51/RRC*
332.11049	10.61	-0.64	1 β-(3-Hydroxy-4,5-dimethoxyphenyl)-O-glucopyranoside			2.27/RRI		
338.11508	21.01	-0.94	6-Prenylapigenin	1.68/RRC	2.45/RRC			
340.11523	15.69	-1.70	Methyl 6-O-p-trans-coumaroyl-β-D-glucopyranoside	1.47/RRC				
340.13048	19.98	-1.70	6-Prenylnaringenin		**3.02/RRC***			
340.13077	17.49	-0.88	(+-)-5-Deoxykievitone		1.50/RRC	1.98/RRC*		
372.12012	15.43	-2.10	Quercetin pentamethyl ether		1.67/RRC*, 1.5 RRI			
372.12050	20.36	-1.10	Tangeretin		1.94/RRC	1.5/RRC*		
386.12081	12.10	-1.00	trans-p-Sinapoyl β-D-glucopyranoside	1.35/RRC	2.02/RRC**		1.87/RRC	2.25/RRC*
404.11017	23.51	-1.30	5,4'-Dihydroxy-3,6,7,8,2'-pentamethoxyflavone		1.87/RRC*	2.57/RRC*, 1.41/RRI		
418.08922	19.72	-1.60	Kaempferol 3-xyloside			1.83/RRC		
422.11983	12.04	1.50	Catechin 7-O-apiofuranoside	**3.26/RRC**	**5.84/RRC***			
432.10479	17.42	-1.90	Kaempferol 3-O-α rhamnoside	**7.32/RRC***				2.88/RRI
434.12056	23.68	-1.70	Naringenin-7-O-Glucoside	2.31/RRC*	2.44/RRC*	2.31/RRI*		
436.13582	14.56	-0.11	Catechin 3-O-α-L-rhamnoside	1.57/RRC	1.81/RRC*			
446.08499	16.58	0.19	Apigenin 7-O-β-D-glucuronide		2.57/RRC**			
468.12570	12.10	-1.10	Catechin-4-ol 3-O-β-D-galactopyranoside		2.11/RRC**	1.62/RRC**	1.84/RRC*	
478.11040	20.75	-1.70	Quercetin 3-O-methyl 7-O-galactoside		1.59/RRC			
556.15682	24.15	-2.10	Epicatechin 5-O-β-D-glucopyranoside-3-benzoate		1.59/RRC			
564.18281	17.48	-2.50	Naringenin 5,7-dimethyl ether 4'-O-xylosyl-(1->4)-arabinoside		1.49/RRC		1.57/RRC	1.37/RRC**
572.16579	12.56	-4.20	Mucronulatol-(4->6) naringenin		1.83/RRC	2.07/RRC**	1.54/RRC*	
578.14159	20.32	-1.40	Kaempferol 3-(2"-(Z)-p-	1.75/RRC*	1.23/RRC	2.79/RRC**,	1.51/RRC*	1.94/RRC

			coumaroylrhamnoside)			1.29/RRI		
578.16242	20.32	-1.90	Kaempferol 7,4'-dirhamnoside	1.73/RRC				
582.20866	21.86	-1.70	Auriculatin 4'-O-glucoside	1.58/RRC	2.11/RRC**			
594.15686	17.15	-2.60	Kaempferol 3-O-glucoside 7-O-rhamnoside	1.79/RRC		2.87/RRC*		
606.17584	23.44	3.53	Skullcapflavone I 2'-(4"-E-Cinnamoylglucoside)			2.68/RRC*		
608.14993	20.40	-4.90	Vitexin 2"-O-(E)-ferulate			1.95/RRC*		
608.17274	20.42	-2.20	Kaempferide 3-glucoside-7-rhamnoside	2.05/RRC*				
610.13159	10.40	-1.00	Kaempferol 3-(6"-caffeoylglucoside)				**14.15/RRI***	
624.16730	20.89	-2.70	Isoscoparin 7-O-glucoside			**3.1/RRC****		
638.18338	20.36	-1.90	Tricin 7-rutinoside	2.09/RRC*				
710.20397	18.43	-2.60	Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside	**4.29/RRC***		2.32/RRC**	2.13/RRC*	
740.21435	19.73	-2.60	Kaempferol 3-O-rhamnosyl glucoside 7-O-rhamnoside	1.77/RRC	2.13/RRC*	**5.53/RRC***		
740.21439	17.46	-2.60	Kaempferol 3-rhamnoside-7-glucosyl-(1->2)-rhamnoside	**4.98/RRC***	1.67/RRC*	**9.80/RRC**		
770.22489	17.58	-2.60	Rhamnetin 3-O-rhamninoside	1.16/RRC	1.92/RRC**	1.91/RRC*		
Alkaloids								
197.08399	18.89	-0.02	2-Hydroxy-3-methylcarbazole			**3.47/RRI***		
237.10024	13.22	0.18	Fumariflorine			**4.04/RRI***		
239.15237	20.83	1.11	Macrophylline	**6.84/RRC**	1.71/RRC*	**4.36/RRC***		
241.16777	22.87	0.29	Valeroidine	**3.66/RRC**	**3.82/RRC***			
247.13118	27.53	-3.60	12-Cytisineacetamide	1.50/RRC*	1.59/RRC*	**3.46/RRC**		
257.06880	17.14	0.01	Cyathocaline			**7.07/RRI****		
349.22486	24.80	-1.30	Acetyllycoclavine		**19.75/RRC***			
464.26793	27.97	0.93	Psychotrine		1.40/RRC	1.67/RRC*		
554.22212	28.04	2.93	N-Methylheteropsine	1.96/RRC*				

Terpenoids								
254.18868	19.74	2.28	Isovaleroyloxylinalool					**4.56/RRI***
310.10506	12.06	-0.44	2-Oxo-6-dehydroxyneoanisatin				1.47/RRC	1.83/RRC**
334.21599	25.27	4.76	Phytocassane B				1.51/RRC*	1.71/RRC
390.15073	14.64	-4.50	Loganin				1.90/RRI	
510.28354	23.05	1.45	16-Diacetoxy-7 α-hydroxy-18-malonyloxy-ent-cleroda-3-ene	2.50/RRC			1.60/RRC	
610.26454	24.20	3.35	Briaexcavatin O	1.74/RRC		**3.11/RRC****		
644.24586	18.14	-1.50	Cineracipadesin F	1.55/RRC				
Amino acids								
129.04240	1.57	-0.74	Pyroglutamic acid	1.52/RRC				
131.09508	2.12	3.63	Isoleucine	2.47/RRC				
149.05152	1.72	2.84	Methionine		1.53/RRC*			
Others								
135.06874	2.18	2.50	Phenylacetaldoxime			1.24/RRC		
165.04654	1.47	3.29	Methionine sulfoxide		2.97/RRI*			
609.32617	26.52	-2.00	Segetalin A	1.80/RRC*, 2.23/RRI**				
203.05860	20.66	1.96	Doryanine			2.77/RRI*		
241.07407	18.89	1.11	N-Benzoylanthranilic acid			**4.43/RRI***		
264.13622	17.77	4.64	(s)-(+)-Abscisic acid					**8.31/RRI***
337.11578	15.58	-0.95	1-DL-(Indole-3-acetyl)-myo-inositol			**4.43/RRI***		
419.12094	15.39	1.06	trans-Zeatin riboside monophosphate			**5.68/RRI***		
219.11089	2.42	0.87	Pantothenic acid	**5.74/RRC****		1.89/RRC*		
400.13621	12.19	-1.70	3'-O-β-Glucopyranosyl plumbagic acid methyl ester			1.77/RRC*		
449.10674	14.71	-4.80	Cyanidin 3-O-glucoside				1.65/RRI*	
658.31566	29.06	2.30	7-Methoxygambogellic acid	1.87/RRC**		**4.01/RRC***		
312.13485	15.89	-4.00	4'-Prenyloxyresveratrol			**3.27/RRI***		

255.18361	25.14	0.83	N-decanoyl-L-Homoserine lactone	**3.05/RRC**	2.93/RRC*	1.56/RRC**		
262.14144	12.79	-0.60	(Z)-3-Hexenyl beta-D-glucopyranoside			1.54/RRI		
268.16779	14.29	1.45	Dihydro-7-hydroxymyoporone					**3.53/RRI****
283.09133	8.09	-0.94	Guanosine	1.46/RRC				
323.10048	14.87	-0.05	Indole-3-carboxylic acid β-D-glucopyranosyl ester				1.54/RRI**	
326.11411	20.98	-3.40	Sappanone a trimethyl ether		2.29/RRC			
354.11051	15.40	0.58	Triacetyl resveratrol		1.73/RRC*			
386.26643	28.58	-0.95	(+)-Rangiformic acid		1.72/RRI			
400.11588	17.47	0.19	4'-Demethylpodophyllotoxin		1.73/RRC**		1.89/RRC	1.31/RRC**
416.16755	12.99	-1.60	2-Phenylethyl O-β-D-xylopyranosyl-(1->2)-β-D-glucopyranoside		**8.23/RRC***	1.61/RRC		
566.05388	1.26	-2.00	UDP-D-glucose				1.57/RRC	
584.20934	15.20	-2.00	Sylvestroside III		1.51/RRC			
628.30546	26.56	2.97	Isogambogic acid	2.04/RRC		2.53/RRC**		
644.19368	19.16	-2.40	Piloside B	1.72/RRC				
221.10554	16.45	1.58	(3-Phenylpropionyl) glycine methyl ester		**29.79/RRI****			

[a] Observed mass is $[M-H]^-$ + H, where H mass was = 1.007276

[b] Fold change ratios in resistant relative to susceptible, ≥3.0 are indicated in bold fonts.

All the RR metabolites reported here are significant at $P < 0.05$ level; *significant at $P < 0.01$ and ** significant at $P < 0.001$.

AME = accurate mass error of the peak detected in ppm ((Observed mass – Exact mass)/Exact mass)

The fold change reported here for the genotypes are relative to susceptible 'Stander'

RRC is resistance related constitutive metabolite: RRI is resistance related induced metabolites

Table 5.4 Potential biomarker metabolites identified in six-row barley cultivars against fusarium head blight resistance.

Exp. Mass a[M], median	Potential Biomarkers	MS/MS fragmentationb	References
Resistance Related Metabolites			
164.04735	*p*-coumaric acid	**119.05,163**,145.20	McGill-MD[c]; Sanchez-Rabaneda et al. 2003
210.12584	Jasmonic acid	**165.22**, 149.21, 191.13, **209.19**	McGill-MD; METLIN
224.06891	Sinapic acid	**208.13**,179.11,**164.16**	McGill-MD; Theerasin and Baker 2009
224.14140	Methyl jasmonate	179.26, 141.13, 195.25 223.15	McGill-MD
272.06843	Naringenin	**151,119,107**, 271.29	Moco et al. 2006; Sanchez-Rabaneda et al. 2003
278.22468	Linolenic acid	**233**.31, **259**.22, 127.14, 97.12	McGill-MD, METLIN library
422.11983	Catechin 7-O-apiofuranoside	361.12, 389.33,277.18, 339.20	
432.10479	Kaempferol 3 O- rhamnoside		
434.12056	Naringenin-7-O-glucoside	**271.13, 433.12**	Sanchez-Rabaneda et al. 2003
436.13582	Catechin 3-O-α-L-rhamnoside		
468.12570	Catechin-4-ol 3-O-β-D-galactopyranoside		
578.14159	Kaempferol 3-(2"-(Z)-p-coumaroyl rhamnoside)		
594.15686	Kaempferol 3-O-glucoside 7-O-rhamnoside	**431.10**, **285.15**, 549.05, 473.11, 311.11	Moco et al. 2006; Kachlicki et al. 2008
710.20397	Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside		
740.21435	kaempferol 3-O-rhamnosyl glucoside 7-O-rhamnoside	431.09, **283.10**, 269.08, 311.14	March et al. 2004
740.21439	Kaempferol 3-rhamnoside-7- glucosyl-(1->2)-rhamnoside	431.09, 311.14, 269.08	
Resistance Indicator Metabolites			
296.1259	Total DON produced (Deoxynivalenol)	**265.28**, **247.07**, **277.24**	McGill-MD; Poppenberger et al. 2003
458.1788	Proportion of DON converted (DON-3-*O*-glucoside)	**427.33**, **277.17**, **247.22**	McGill-MD; Poppenberger et al. 2003

[a] Observed mass is $[M-H]^-$ + H, where H mass was = 1.007276

[b] Fragmentation in bold font indicates the actual match of the fragment

[c] McGill-MD (http://metabolomics.mcgill.ca/)

METLIN (http://metlin.scripps.edu/)

Fig. 5.1 Canonical discriminant analysis (CDA) of significant (P <0.05) metabolites detected in mock-inoculated and pathogen inoculated genotypes of barley.

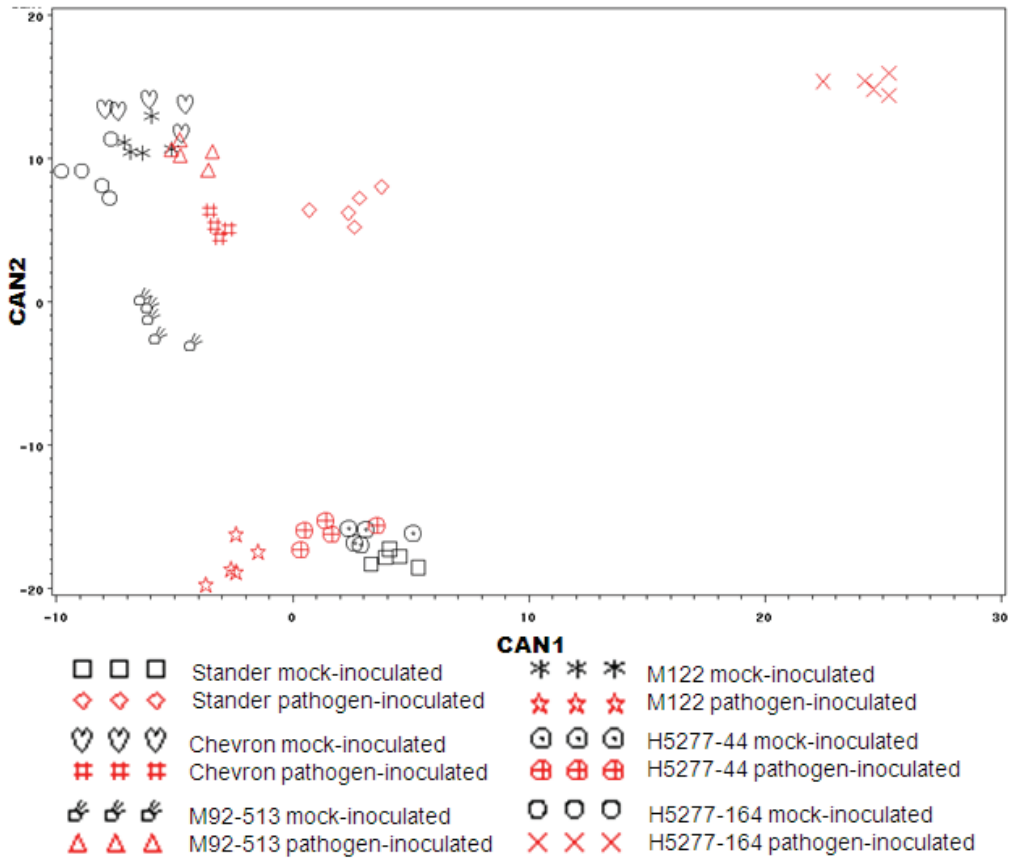

Fig. 5.2 Resistance indicator metabolites produced in six barley genotypes inoculated with *F. graminearum*: a) TDP, D3G, and 3ADON accumulated; b) The PDC to D3G in different genotypes; c) Relationship between TDP and PDC; a linear regression model to predict PDC from TDP, (R^2 = 0.56); where DON=Deoxynivalenol; D3G= DON-3-*O*-glucoside; TDP= Total DON produced; PDC = proportion of DON conversion to D3G; 3ADON= 3-acetyl-deoxynivalenol.

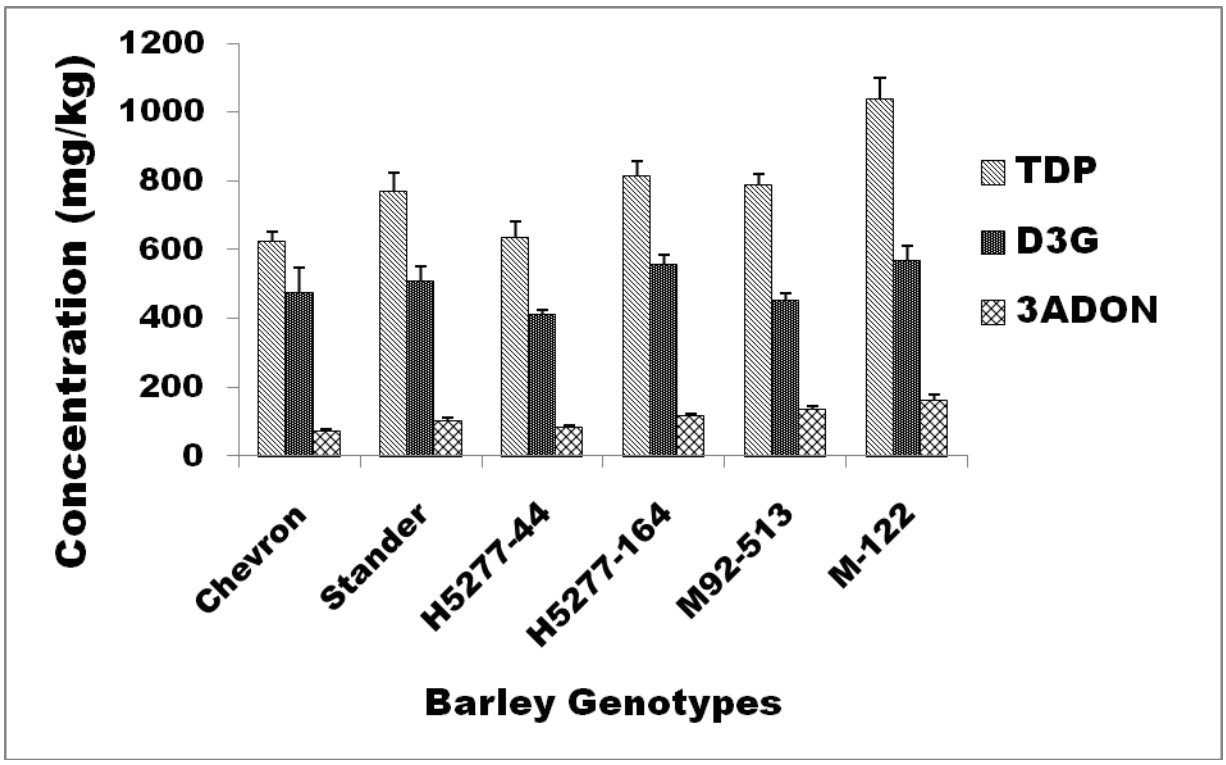

Fig. 5.2a

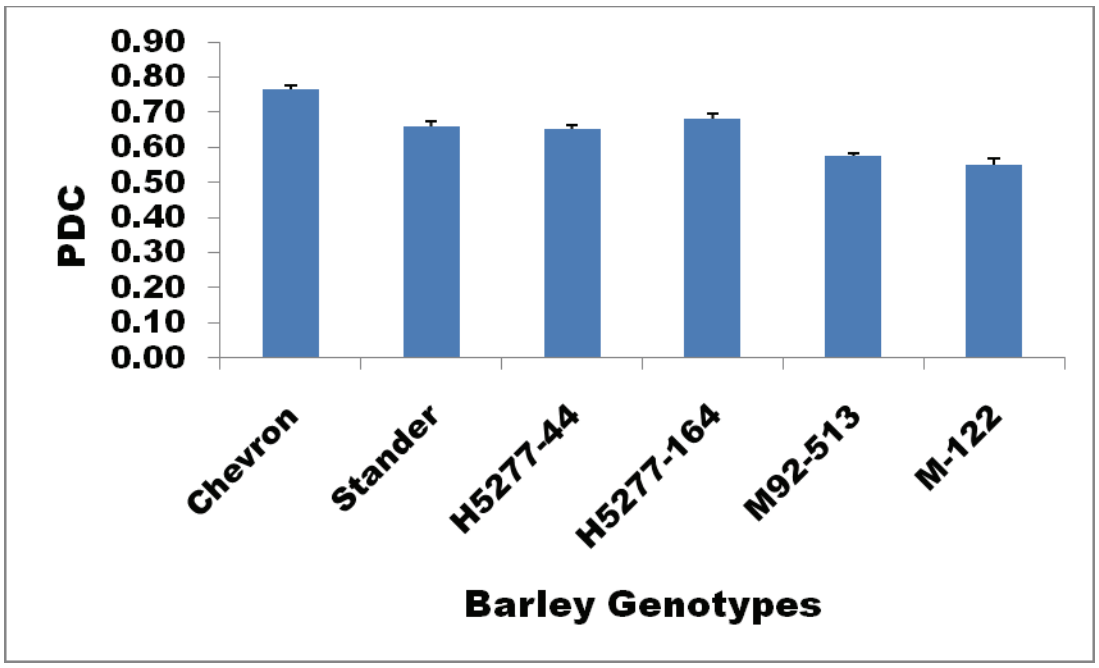

Fig. 5.2b

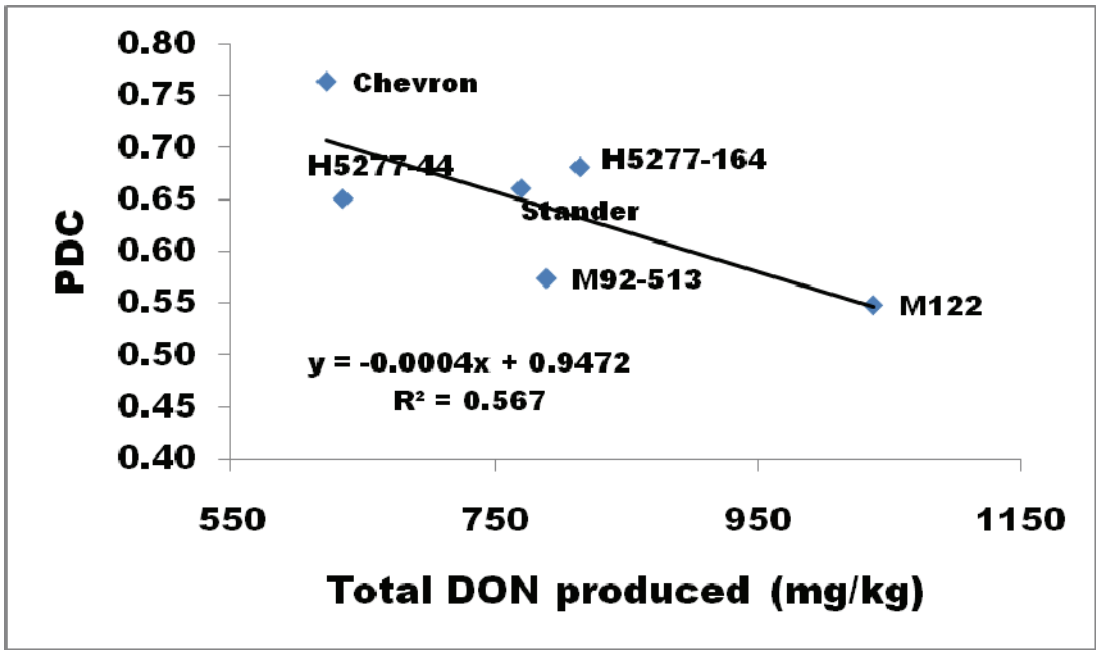

Fig. 5.2c

CHAPTER 6

SUMMARY, CONCLUSIONS, AND SUGGESTIONS FOR FUTURE RESEARCH

6.1. General summary and conclusions

Fusarium head blight, caused by *F. graminearum* is the major disease of barley and wheat in Canada and worldwide. The damaging effects of the disease include yield losses along with mycotoxin contamination of grains. These toxins are detrimental to human and animal health (Sutton 1982; Parry et al. 1995; Bai and Shaner 2004). Complete resistance to FHB in barley was not detected in more than 25,000 barley accessions screened. Genotypes showing only moderate resistance to FHB have been released for commercial cultivation (Choo 2006). Though different methods exist to control the disease, breeding for host resistance remains the best option (Bai and Shaner 1994).

Two-row barley types are more resistant to FHB than six-row barley because of closed florets. The molecular mechanisms of barley resistance to FHB are not well understood. Although sources of FHB resistance have been found in the wild genotypes, transferring of the FHB resistance genes from these genotypes to elite cultivars is quite difficult. Quantitative trait loci for FHB resistance have been identified on all seven chromosomes of barley, but no major QTL was found to be directly responsible for FHB resistance. Often, these QTL were associated with apparent resistance, such as heading date, plant height, etc. (de la Pena et al. 1999; Zhu et al. 1999; Ma et al. 2000; Dahleen et al. 2003). Based on more than 100 QTL identified in both wheat and barley it is evident that several mechanisms of resistance exist (Buerstmayr et al. 2009; Choo et al. 2006). Screening of barley genotypes for FHB resistance based only on disease severity is inadequate. Also, screening breeding lines under multiple environments, locations and years, which is often inconsistent over years, is expensive and time consuming.

Among more than 15 trichothecenes produced by *F. graminearum*, DON is the major toxin. Apart from downgrading the quality of grains, DON also acts as a virulence factor in FHB disease development (Proctor et al. 1995). The chemotypes of *F. graminearum* producing high quantity of DON were more virulent, their by increasing susceptibility of the host (Atanassov et al. 1994). It has been documented that certain

chemotypes produce varying amounts of DON (Logrieco et al. 1990) and also under field conditions (Carter et al. 2002). Though DON is not needed for the initial invasion of host (Jansen et al. 2005), it plays a major role in the spread of infection within spike (Bai et al. 2001a; Lemmens et al. 2005). The enzymatic conversion of DON to less toxic D3G has been reported in *Arabidopsis* (Poppenberger et al. 2003) and in wheat (Lemmens et al. 2005, 2008). Another mechanism of resistance is due to inhibition of the synthesis of type B trichothecenes by ferulic acid, which is produced in high amounts in grains (Boutigny et al. 2008, 2009). The natural extract of phenolic compounds from wheat bran showed complete inhibition of type B trichothecene biosynthesis *in vitro* (Boutigny et al. 2010).

Understanding the FHB resistance lies in functional genomics approaches like transcriptomics, proteomics and metabolomics (Fiehn et al. 2000). Several PR proteins have been identified in wheat and barley against FHB (Zhou et al. 2005; Geddes et al. 2008; Shin et al. 2008). Metabolomics approach has been used and several PR and RR metabolites have been identified in wheat (Hamzehzarghani et al. 2005, 2008a, b; Paranidharan et al. 2008).

Metabolic profiling has been used to discriminate genetically modified traits in tobacco, *Arabidopsis* (Mungur et al. 2005; Roessner et al. 2001), wheat cultivars varying in resistance against FHB using GC-MS technology (Hamzehzarghani, et al. 2005, 2008a; Paranidharan et al. 2008), and salt-stressed from non-stressed tomatoes (Johnson et al. 2003) using GC-MS. However, despite its high sensitivity and availability of huge mass spectral data resources, it lacks the ability to detect non-volatile metabolites (Johnson et al. 2003). Tomato fruit metabolites have been identified using LC-Q-TOF and the mass spectra are published in MoTo database (Moco et al. 2006). In *Arabidopsis* recombinant inbred lines have been used to identify hundreds of metabolic QTL and each or a few of these were related to genomic positions. Several co-located QTL controlled certain metabolic pathways (Keurentjes et al. 2006). Though metabolomics approach to screen for resistance in barley against FHB cannot detect enzymes that detoxify trichothecenes, the detoxified products of enzymes can be detected using metabolomics approach (Mirocha et al. 1994; Lemmens et al. 2005). Thus, metabolomics as a tool can be used to identify several mechanisms of resistance in barley against FHB, such as PR and RR metabolites, including antimicrobial, signaling, and cell walls enforcement

compounds, trichothecene toxins and their detoxified products, and pathogen enzyme degredation products. Thus, metabolic profiling can be an attractive alternative tool for breeders for high throughput phenotyping of resistance.

In this study, mass spectrometry based metabolomics approach has been explored to phenotype resistance in barley against *F. graminearum*. It was hypothesised that six-row barley genotypes varying in resistance to FHB also vary in their metabolic profiles. The objectives of this study were: 1) to discriminate resistant (Chevron) and susceptible (Stander) six-row barley cultivars varying in resistance to FHB, based on metabolic profiles and to identify antifungal activity of selected RR metabolites *in vitro*; 2) to study the effect of antifungal RR metabolites on type B trichothecene inhibition; 3) to identify the biomarker metabolites in barley for screening barley breeding lines.

Chapter 3 describes the application of mass spectrometry based metabolomics approach to discriminate two six-row barley cultivars varying in resistance aginst FHB. In this study, 'Chevron' and 'Stander' were mock-inoculated or pathogen-inoculated disease severity was assessed, and metabolites were profiled using LC-hybrid-MS. The disease severity, based on PSD and AUDPC, separated 'Chevron' to have high level of type-I disease resistance from 'Stander'. The LC/MS data output was aligned and peaks were deconvoluted using XCMS, and the CAMERA algorithm based on R platform was used to detect adducts, isotopes and neutral losses. Out of a total of 1826 peaks detected 496 were treatment significant metabolites. These were further classified as RRC (194) and PR (26) metabolites, but none was RRI metabolite (Table 3.1). Out of 194 RRC and 26 PR metabolites, 47 and 6 were putatively identified, respectively. And they belonged to different metabolic pathways such as the phenylpropanoid, flavonoid, terpenoid, and fatty acid (Fig 3.5).

The RR metabolites identified belonged to three major metabolic pathways: the phenylpropanoid, flavonoid, and fatty acid pathways. These metabolites have several plant defense properties. *p*-coumaric and sinapic acid, identified as RRC metabolites, are antifungal metabolites. Inhibition of type B trichothecene biosynthesis by a phenolic compound, ferulic acid, *in vitro* through its antioxidant property has been studied (Boutigny et al. 2009). The natural extracts of phenolic acids from wheat bran showed complete inhibition of biosynthesis of type B trichothecenes *in vitro* (Boutigny et al.

2010). *p*-coumaric acid and sinapic acids also play a role in cell walls lignification process and their by decreasing further spread of pathogen (Humphreys and Chapple, 2002). In our study, *p*-coumaric and sinapic acids showed a significant inhibition of *F. graminearum* mycelial biomass *in vitro*. The LD_{50} values varied among metabolites, with greater inhibition by capric acid (Table 3.2). In present study 16 different flavonoids, flavonols, and kaempferol glucosylated forms were identified and linked to flavonol glucoside metabolic pathway (Fig. 3.6). Flavonoids isolatd from carnation showed antifungal activities against *F. oxysporum* (Galeotti et al. 2008). Since glucosides of kaempferol, as identified here, were not commercially available only kaempferol was evaluated for biomass inhibition. However, it was poorly soluble in aqueous methanol, and thus, resulted in low inhibition of mycelial biomass. Capric and lauric acids were identified as major fatty acid metabolites. *In vitro* studies both showed a significant inhibition of *F. graminearum* mycelial biomass. Hence, capric acid had the lowest LD_{50} value (0.1mM) and highest RE among all the tested RR metabolites for antifungal activities (Table 3.2).

This is the first report of the detection of DON detoxification product, DON3-*O*-glucoside in barley (Fig. 3.3). This metabolite was detected in low abundance and also inconsistent among replicates. This was mainly due to the use of a semipolar Jupiter column. Earlier it was reported form wheat as DON detoxified product and was linked to 3BS QTL in wheat FHB resistant cultivar Sumai 3 (Lemmes et al. 2005; Poppenberger et al. 2003).

In Chapter 4, ten RR metabolites, which were previously evaluated for *F. graminearum* biomass inhibition (Chapter 3; Bollina et al. 2010; Kumaraswamy et al. 2011), were further evaluated for their ability to inhibit trichothecene biosynthesis. It was hypothesized that plant endogenous compounds could inhibit type B trichothecene production by *F. graminearum*. The LD_{50} mM concentrations of these RR metabolites in basal liquid media were evaluated for trichothecene biosynthesis inhibition of *F. graminearum in vitro* (Table 4.1). Five day old cultures were centrifuged, separated from liquid media, and lyophilized to obtain fungal dry biomass. Biomass was around 50% of control in all the treatments (Table 4.2). From the filtrate the type B trichothecenes were extracted and analyzed using LC-hybrid-MS. The trichothecenes were completely

inhibited in lauric, *p*-coumaric, sinapic, ferulic, naringenin, quercetin, and methyl jasmonate at LD_{50} mM concentrations for biomass inhibition. Whereas, in capric acid there was, complete inhibition of 3ADON but not DON. Both DON and 3ADON were detected in quinic acid and caffeic acid but the inhibition relative to control was very high. Phenolic acids were reported to accumulate at the site of infection (Matern and Kneusel 1988) and most predominant phenolic acid in wheat and barley was ferulic acid (Kim et al. 2006). Biosynthesis of type B trichothecene inhibition was 99% at 1mM concentration and at 2.5 and 5 mM there were complete inhibition (Boutigny et al. 2009). Also, phenolic acid mixtures extracted from wheat bran showed a complete inhibition of type B trichothecene biosynthesis (Boutigny et al. 2010). The concentration of ferulic acid observed in wheat bran was 1007 $\mu g\ g^{-1}$ and at 0.5 mM of ferulic acid concentration complete inhibition *in vitro* was observed (Boutigny et al. 2010). The mechanism is considered to be due to their antioxidant property. Similarly, it is possible that other phenolic, flavonoid, and fatty acids with antioxidant and pro-oxidant properties might also inhibit the synthesis of trichothecenes. Different phenolic acids, flavonoids, carotenoid, and linolenic acids are known to have antioxidant and pro-oxidant properties and these could modulate biosynthesis of mycotoxins (Burow et al. 1997; Huang et al. 1997; Ponts et al. 2006). Constitutive occurrence and induction of these RR metabolites upon pathogen infection could inhibit the toxin biosynthesis by *F. graminearum*.

Chapter 5 describes the LC-hybrid-MS based metabolomics application to metabolic phenotyping of barley resistance to FHB and to identify potential biomarker metabolites. Six-row barley genotypes 'Chevron', H5277-44, H5277-164, M92-513, 'M122', and 'Stander' were selected for the identification of biomarker metabolites. Several changes to methodology, compared to study 1 (Chapter 3), were made to improve detection of RR metabolites. Chevron and Standercultivars used in the first study (Chapter 3) were repeated here to see the consistency of occurrence of RR metabolites, in spite of minor changes in methodology. The use of relatively more polar chromatography column, Kinetex, instead of Jupiter in study 1, has detected DON and its detoxified product D3G. Metabolite extraction protocol was also changed slightly; aqueous methanol content was decreased to 50% from 65% from first study to extract highly polar trichothecenes (Berthiller et al. 2005). Analysis of raw data files using a newer version of

XCMS with slight changes in some of the parameters like band width (bw=10), change in *m/z* difference (*mzdiff*=0.01), and *mz* width (*mzwid*=0.005) to maximize the probability of correct alignment has increased the number of metabolites detected. Increase in incubation time from 48 hpi to 72 hpi may have increased the trichothecene levels. Inspite of these modifications, 18 RR metabolites were common in 'Chevron', between the study 1 and 3. Development of a comprehensive and untargeted universal method for plant metabolite extraction, identification and quantification remains a challenge for plant metabolomics (Hegeman 2010).

In this study, thousands of peaks were initially detected, and different sieving techniques were used to identify biomarkers. The multiple peaks of a compound were sieved based on CAMARA algorithms, inconsistent peaks among replicates were excluded using EXCEL. Pair wise comparison based on *t*-test sieved metabolites with highly variable abundances, which generally lead to erroneous conclusions based on multivariate analyses. In the third study, 1430 RR metabolites were selected. These RR metabolites were further grouped into RRC (1138) and RRI (291) metabolites (Table 5.2). Out of 1138 RRC metabolites 90 were assigned putative names and in the case of RRI metabolites 40 were putatively identified (Table 5.3). We also identified DON and D3G as resistance indicator metabolites. The greater PDC was observed in the most resistant cultivar Chevron (76%) and least in 'M122' and M92-513 genotype, which converted only 55 and 57% of DON, respectively (Fig. 5.2b). A total of 16 biomarker metabolites were selected based on high significance, high fold change in abundance relative to susceptible, and with known plant defense properties (Table 5.4). The major potential biomarker metabolites proposed here were: *fatty acid pathway*: jasmonic acid, methyl jasmonate, and linolenic acid; *phenylpropanoid pathway*: *p*-coumaric acid, sinapic acid; *flavonoid pathway*: naringenin and naringenin glucoside and several catachin and kaempferol glucosides.

The general protocol established here can also be used to identify mechanisms of resistance in wheat and barley controlled by more than 100 QTL. Alternatively, the recombinant inbred linescan be profiled to identify metabolic QTL. These metabolites can be related to proteins and eventually to genes, with the advent of genome sequencing of not only wheat and barley but also other model plants.

6.2. Suggestions for future research

1. In this study several RR metabolites were putatively identified based on accurate mass, fragmentation pattern, and isotope ratio. For some compounds pure standards were purchased and the fragmentation pattern was matched. For other RR metabolites, to confirm the identity, either authentic standards need to be purchased or they have to be purified and fragmentation patterns should be matched for correct metabolite identification.
2. The mechanisms involved in the type B trichothecenes biosyntheses inhibition by tested RR metabolites are considered to be due to antioxidant property of metabolites. Further determination of antioxidant levels of these metabolites are needed to determine this mechanism. Inhibition of synthesis at transcriptional level should be determined based on gene expression studies (Boutigney et al. 2010). Also their real concentration in a genotype and inhibition at that concentration should be established.
3. We have identified biomarker metabolites based on metabolomics of six genotypes. Inhibition of these metabolite production using chemicals or through mutants lacking production of these RR metabolites can be used to study the effects of these RR metabolites. Since the effects are small it may, however, be difficult to prove these effects.
4. Only few selected RR metabolites were evaluated in our studies for *F. graminearum* biomass inhibition and for trichothecene production inhibition. Other RR metabolites reported here should be studied for their effect on both biomass and trichothecene biosynthesis inhibition, and also their concentration in resistant plants should be quantified.
5. Only DON and 3ADON were identified as trichothecenes produced by *F. graminearum*. Other mycotoxins like NIV, ZEN and Fusarion should be identified and their detoxification products should be identified.
6. We have identified significant amounts of naringenin and related synthesis product kaempferol and their conjugated forms. These and other biomarker

metabolites, identified here could be used in metabolic pathway engineering to overexpress, in order to increase resistance in barley genotypes.

7. We have used metabolic profiling tool to detect and identify RR metabolites. Generally, this takes about an hour to run the samples. High throughput tools, based on direct infusion, can be developed to screen barley genotypes for resistance against FHB. Alternatively, HPLC tools also can be developed to identify the resistance biomarkers identified here and used in barley resistance improvement.

CHAPTER 7

CONTRIBUTIONS TO KNOWLEDGE

The following scientific contributions to knowledge can be considered as original in this thesis:

- This is the first study to apply liquid chromatography, and mass spectrometry-based metabolomics tools to phenotype 6 six-row barley genotypes for FHB resistance, based on metabolic profiles.
- Ten RR metabolites were evaluated for *F. graminearum* biomass reduction, and their LD_{50} mM concentrations of metabolites have been reported.
- Resistance equivalence (RE) was developed for selected RR metabolites, higher the RE value, higher is the potential of resistance.
- Complete inhibition of type B trichothecene biosyntheses by lauric, *p*-coumaric, sinapic, ferulic, naringenin, quercetin and methyl jasmonate were reported.
- We are the first to report a DON detoxification product DON-3-*O*-glucoside in six-row barley genotypes.
- Resistance indicator metabolites such as TDP and PDC were identified; their application to discriminate mechanisms related to DON synthesis by the pathogen and DON detoxification by the plants has been demonstrated.

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APPENDICES

Chapter 3

Appendix 3.1: Supplementary Table 3.1 Experimental median accurate masses (*m/z*), retention times (RT) and respective CAN1 loadings of resistance-related (RR) and pathogenesis-related (PR) metabolites detected in mock- or *Gibberella zeae*-inoculated barley genotypes in a negative mode of ionization based on LC-ESI-LTQ-Orbitrap (total of 217 metabolites).

Expt. Med mass (m/z)	Expt. Med (RT-min)	PR & RR Metabolite	CAN1 loadings	CAN2 loadings	Expt. Med mass (m/z)	Expt. Med (RT-min)	PR & RR Metabolite	CAN1 loadings	CAN2
86.70735	41.65	RRC	**0.93**	-0.37	536.1887	16.95	RRC	**0.93**	-0.36
88.01635	2.41	RRC	0.89	-0.28	538.1733	2.3	RRC	0.84	0.53
97.96765	43.87	RRC	**0.92**	-0.30	543.9722	40.05	RRC	**0.91**	0.37
97.97715	2.32	RRC	**0.93**	0.31	548.1527	2.44	RRC	0.87	0.49
104.0112	2.53	RRC	**0.96**	0.10	553.1913	22.54	RRC	0.67	-0.21
113.0197	2.44	RRC	**0.94**	-0.35	557.1705	2.34	RRC	0.81	0.44
129.0427	2.42	RRC	**0.94**	0.22	561.2219	2.54	RRC	**0.95**	0.26
130.0268	2.44	RRC	**0.97**	0.15	563.321	42.67	PRs	-0.46	0.86
133.0377	2.31	RRC	0.86	0.04	564.3244	42.67	PRs	-0.50	0.83
155.9264	43.87	RRC	0.88	-0.24	576.3126	39.01	RRC	0.83	-0.14

156.0061	2.44	RRC	**0.95**	-0.09	582.2088	28.93	RRC	0.97	-0.19
158.5949	41.66	RRC	0.67	0.60	583.212	28.93	RRC	**0.97**	-0.17
161.0687	2.42	RRC	0.83	0.48	584.2095	18.46	RRC	0.68	-0.56
164.0474	34.43	RRC	0.86	0.02	584.2243	28.83	RRC	**0.97**	0.05
166.0498	2.35	RRC	**0.94**	0.20	584.2245	27.83	RRC	**0.98**	-0.03
172.1458	40.63	RRC	0.88	-0.18	585.2276	27.83	RRC	**0.97**	0.05
174.1117	2.28	RRC	0.87	-0.20	585.228	28.83	RRC	**0.97**	0.02
182.1656	2.48	PRr	-0.76	0.18	588.1655	2.3	PRs	0.66	0.74
186.1617	41.94	RRC	0.80	0.05	590.3296	33.82	RRC	0.74	-0.56
192.0272	2.45	RRC	**0.95**	-0.31	594.1576	23.86	RRC	**0.98**	0.18
192.0634	2.34	PRs	-0.45	0.85	594.2792	41.58	PRs	-0.48	0.45
193.3971	2.45	RRC	**0.95**	0.22	595.2827	41.58	PRs	-0.43	0.42
196.0583	2.33	RRC	0.81	0.51	598.2252	18.45	RRC	0.61	-0.61
198.3752	2.43	RRC	**0.92**	-0.20	606.1943	23.62	RRC	0.74	-0.64
200.1776	43.22	RRC, PRs	0.52	0.30	608.1726	25.79	RRC	**0.99**	-0.08
202.1568	33.61	RRC	0.76	-0.63	609.1759	25.79	RRC	**0.99**	-0.08
203.0193	2.89	RRC	**0.96**	0.04	614.0248	41.72	PRr	0.10	0.63

216.1724	36.05	RRC	0.62	-0.17	614.114	2.37	PRr	0.31	0.43
224.0687	34.53	RRC	-0.58	0.27	614.1294	19.15	RRC	0.84	-0.53
234.5175	2.46	RRC	0.81	0.25	614.1388	25.64	RRC	**0.96**	-0.07
250.1567	37.83	PRs	**0.96**	0.27	615.2812	33.07	RRC	**0.98**	-0.07
266.1555	40.88	PRs	-0.47	0.12	615.7788	2.51	RRC	0.38	-0.22
268.1511	40.88	PRs	-0.05	0.73	616.2848	33.07	RRC	**0.98**	-0.04
271.9943	2.42	RRC	0.83	0.47	618.1636	22.56	RRC	0.76	-0.15
282.1408	34.55	RRC	**0.99**	-0.16	622.2722	36.9	RRC	0.87	-0.44
284.1985	40.91	RRC	0.72	-0.56	625.1709	25.65	RRC	**0.99**	-0.05
288.2298	39.29	RRC	**0.99**	0.05	630.2293	23.52	RRC	**0.99**	-0.11
290.0063	2.46	RRC	0.88	0.40	632.119	2.5	RRC	**0.95**	-0.32
294.1437	35.72	RRC	**0.98**	-0.10	637.2355	36.91	RRC	**0.93**	-0.32
303.1077	2.34	RRC	0.89	0.41	637.2638	33.08	RRC	**0.97**	-0.12
306.2231	42.98	PRs	0.50	0.86	638.1833	25.64	RRC	**1.00**	-0.02
307.6407	33.08	RRC	**0.96**	-0.11	639.1865	25.65	RRC	**1.00**	-0.02
308.1985	39.33	RRC	**0.95**	-0.24	640.1888	25.63	RRC	**1.00**	-0.03
312.1163	2.28	RRC	0.88	0.17	643.2507	36.91	RRC	0.78	-0.61

316.0792	2.49	RRC	**1.00**	0.04	646.1494	25.65	RRC	**0.90**	-0.20
317.0855	2.53	RRC	**0.94**	0.24	651.2213	2.42	RRC	**0.99**	0.02
318.221	34.25	PRr	0.01	0.83	654.1778	25.77	RRC	**1.00**	-0.01
327.9869	2.6	RRC	0.80	0.44	676.1622	28.46	RRC	**0.91**	-0.31
328.1308	26.02	RRC	0.87	-0.34	677.1656	28.46	RRC	**0.91**	-0.30
328.2605	41.52	PRs	0.55	0.51	678.1684	28.46	RRC	0.88	-0.36
329.2642	41.52	PRs	0.74	0.42	690.1776	28.43	RRC	**0.91**	-0.26
332.074	2.39	RRC	**0.95**	-0.30	692.3886	42.64	RRC	**0.95**	0.11
334.2174	39.15	RRC	**0.96**	0.21	698.1885	2.53	RRC	**0.94**	0.31
336.1962	38.23	RRC	**0.97**	-0.16	704.2087	33.07	RRC	**0.99**	-0.14
338.0945	2.32	RRC	**0.93**	0.21	705.212	33.07	RRC	**1.00**	-0.10
339.2094	39.79	RRC	**0.91**	-0.41	706.1704	25.65	RRC	**1.00**	-0.05
340.1307	26.02	RRC	0.89	-0.32	710.2044	22.81	RRC	**0.97**	-0.09
346.2907	42.77	RRC	0.89	0.45	710.3533	38.33	RRC	**0.98**	0.12
354.11	24.69	RRC	0.58	-0.74	711.2077	22.8	RRC	**0.94**	-0.19
357.0774	2.53	RRC	**0.94**	-0.14	722.2197	31.08	RRC	**0.99**	0.05
358.1065	2.28	RRC	**0.98**	-0.18	723.2229	31.09	RRC	**1.00**	0.01

358.141	26.02	RRC	**0.95**	-0.12	732.2617	20.88	RRC	0.72	-0.62
360.1907	39.48	RRC	0.74	-0.67	736.3496	36.05	RRC	0.89	-0.37
361.1942	39.48	RRC	0.76	-0.65	740.2144	21.41	RRC	**0.93**	-0.32
374.1935	21.87	RRC	0.79	-0.55	740.2147	24.94	RRC	**0.98**	0.01
374.1936	22.86	RRC	**0.91**	-0.39	741.2181	24.93	RRC	**0.94**	-0.27
379.8136	41.52	RRC, PRs	0.20	**0.93**	741.2181	21.4	RRC	**0.97**	-0.03
388.1928	39.48	RRC	0.89	-0.06	746.2765	28.45	RRC	0.72	-0.56
393.1104	21.42	RRC	**0.97**	-0.05	746.2771	26.62	RRC	**0.90**	-0.35
399.173	2.58	RRC	**0.95**	0.06	747.2798	28.45	RRC	0.89	-0.36
410.3025	43.1	RRC	0.24	0.56	748.3863	38.25	RRC	0.85	-0.13
414.152	20.3	RRC	**0.96**	-0.22	756.2097	19.57	RRC	**0.98**	-0.09
415.1555	20.29	RRC	**0.96**	-0.25	756.2097	22.81	RRC	**0.95**	-0.14
417.6882	34.42	RRC	0.83	-0.35	762.4667	42.02	RRC	**0.98**	-0.10
418.089	27.09	RRC	**0.96**	-0.19	768.4279	42.8	RRC	0.84	0.15
418.1315	2.4	RRC, PRs	0.84	0.54	769.4318	42.81	RRC	**0.95**	0.07
418.1831	21.87	RRC	0.84	-0.47	770.2249	21.73	RRC	**1.00**	-0.05
419.0923	27.1	RRC	**0.94**	-0.12	774.4665	42.43	RRC	0.89	-0.44

422.2145	30.76	RRC	**0.99**	-0.003	776.4162	40.97	RRC	**0.98**	-0.19
428.1678	23.71	RRC	**0.99**	0.10	776.4828	42.8	RRC	**1.00**	-0.02
432.1049	23.54	RRC	**0.95**	0.29	777.4857	42.8	RRC	**0.97**	-0.20
432.1779	35.41	RRC	0.81	0.50	778.4622	40.98	RRC	0.81	0.45
434.1206	31.02	RRC	**0.91**	0.42	781.4358	33.64	RRC	0.67	-0.38
435.1239	31.01	RRC	**0.93**	0.37	782.4434	42.65	RRC	0.87	-0.12
442.1292	22.32	RRC	**0.96**	-0.27	783.4474	42.64	RRC	0.83	-0.18
446.1163	2.46	RRC	**0.97**	0.15	784.4594	42.54	RRC	**0.93**	0.15
448.1411	37.19	RRC	-0.98	-0.13	786.2202	21.41	RRC	**0.98**	0.03
450.2376	39.3	RRC	**0.92**	-0.04	810.488	40.1	RRC	**0.99**	-0.01
451.241	39.3	RRC	0.78	-0.49	816.2846	2.33	RRC	**0.93**	0.20
456.1076	43.19	RRC	0.80	-0.59	818.465	41.39	RRC	**0.91**	-0.18
457.1745	2.2	RRC	**0.97**	0.23	820.2408	31.66	RRC	0.74	0.67
459.1943	2.25	RRC	**0.91**	0.40	824.4309	40.15	RRC	0.81	-0.32
462.1154	24.05	RRC	**0.99**	-0.01	824.5039	40.94	RRC	**0.97**	-0.13
463.1186	24.04	RRC	**0.99**	-0.01	834.46	40.13	RRC	**0.93**	-0.13
474.2826	40.93	PRs	0.67	0.41	835.3756	34.43	RRC	0.71	-0.64

484.2427	32.43	RRC	**0.98**	-0.09	836.3785	34.43	RRC	0.87	-0.27
490.2771	38.85	PRs	0.01	-0.19	836.4204	40.14	RRC	0.84	-0.16
498.2587	34.78	RRC	**0.99**	-0.10	848.4759	41.36	RRC	**0.97**	0.20
498.7138	33.59	RRC	**0.97**	-0.07	886.5035	41.91	RRC	**0.91**	-0.02
506.2629	41.74	RRC	0.89	0.23	898.5268	42.8	RRC	0.89	-0.15
508.1358	28.85	RRC	-0.59	-0.59	899.5296	42.78	RRC	0.91	-0.20
508.1618	2.32	RRC	**0.96**	0.28	900.5427	42.73	RRC	0.88	0.02
508.2787	42.66	RRC	0.80	0.48	901.546	42.75	RRC	0.86	-0.03
510.2813	38.13	PRr	0.30	0.72	910.4392	37.5	RRC	**0.92**	-0.31
516.1452	2.32	PRs	0.63	0.73	917.3053	40.89	RRC	**0.95**	-0.21
518.1357	2.27	PRs	-0.33	0.85	917.5409	41.68	RRC	0.87	-0.10
520.2625	41.71	PRs	-0.17	0.50	919.1785	2.45	RRC	0.83	0.07
526.2615	31.07	RRC	**0.95**	-0.32	944.4963	42.12	RRC	**0.95**	-0.06
534.1442	2.41	RRC	**0.98**	-0.18	997.4271	33.6	RRC	**0.91**	-0.22
535.1815	2.28	PRs	-0.02	**0.99**					

PR = Pathogenesis related (subscripts: r = in resistant; s = in susceptible genotypes);

RR = Resistance related; RRC = RR constitutive.