Metabolic profiling and multivariate analysis to phenotype cultivars of wheat varying in resistance to fusarium head blight.

By

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To my wife, my children, and my parents

Abstract

Bread wheat (*Triticum aestivum* L. em. Thell.) is the most important cultivated crop in Canada. Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is the principal disease of wheat in North America, causing severe losses in grain yield and quality. Breeding for cultivar resistance is considered the most practical way to manage this disease. High spatiotemporal variance makes screening for disease resistance based on visual assessment of symptoms slow, difficult, and expensive. Development of a cheaper, rapid, accurate, and high throughput tool for screening resistance is the highest priority for wheat breeders.

A novel method based on metabolite profiling technology was applied to discriminate resistance in wheat genotypes against FHB. The present investigation reports on three linked studies. The first study involved the detection and application of metabolites to distinguish susceptible (Roblin) and resistant (Sumai3) wheat cultivars. In the second study, cultivars varying in level of resistance were discriminated (descending order: Wangshubai, AW488, Nobeoka Bozu, BRS177, Frontana, CEP24). Finally, biomarker metabolites related to susceptible and resistant near isogenic lines (NILs) with alternate alleles for FHB resistance on the 2DL chromosome were identified. The metabolites were extracted from spikelets in a mixture of methanol-water/chloroform, and analyzed using GC-ion trap-MS (studies 1-2) or GC-TOF-MS (study 3). Compound identification and quantification was achieved manually and/or using automated software for peak deconvolution (AMDIS), library search (MSRI and NIST libraries), and peak alignment and quantification (MET-IDEA).

Several hundred peaks were detected, but only 55, 79, and 120 metabolites were identified in studies 1, 2, and 3, respectively. The metabolites significantly varied in abundance among cultivars/NILs varying in resistance. A resistance biomarker metabolite was defined based on univariate analysis or with high factor/canonical loading to vectors that discriminated resistant genotypes or groups of genotypes. The resistance biomarker metabolites included metabolites related to the phenylpropanoid pathway such as *p*-coumaric acid, cinnamic acid,

several coumarins, and benzoic acid; important signaling molecules such as myo-inositol; signal-related metabolites including hexadecanoic/octadecanoic acid; and other resistance-related metabolites such as aminobutyric acid. Metabolite profiling technology has enormous potential as a high throughput tool for screening resistance to FHB in wheat genotypes.

Résume

Le blé à pain (*Triticum aestivum* L. em. Thell.) est la culture la plus importante au Canada. Le flétrissement des plantules des céréales causé par *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] est la maladie du blé la plus dévastatrice en Amérique du Nord. Elle cause des pertes tant dans le rendement que dans la qualité du grain. La résistance est le moyen le plus efficace pour gérer cette maladie. Une variabilité spatio-temporelle élevée rend difficile et coûteuse l'évaluation de la résistance basée sur les symptômes. Le développement de moyens abordables, rapides, et à haut débit pour le criblage de la résistance est la priorité des sélectionneurs.

Une nouvelle méthode s'appuyant sur le profilage métabolique a été utilisée pour déterminer la résistance à la fusariose dans des lignées de blé. Trois études ont été accomplies lors de la présente investigation : 1) la détection et l'usage de métabolites afin d'identifier les cultivars de blé étant sensibles (Roblin) et résistants (Sumai3) 2) l'identification de cultivars variants dans leur résistance 3) l'identification de biomarqueurs métaboliques dans des lignées isogéniques ayant un contraste d'allèles au locus quantitatif (LQ) FHB-résistant, sur le chromosome 2DL. Les métabolites ont été extraits dans des épillets dans une mixture de méthanol, d'eau et de chloroforme et analysés par CG-piège à ions-SM (étude 1-2) ou par CG-temps de vol-SM (étude 3). L'identification et la quantification des composés ont été accomplies manuellement et/ou en utilisant un logiciel automatisé pour la déconvolution des pics (AMDIS), pour la recherche dans la librairie (NIST) et pour l'alignement et la quantification des pics (MET-IDEA).

Plusieurs centaines de pics ont été détectés mais seulement 55, 51 et 120 métabolites ont été identifiés dans les études 1, 2, et 3, respectivement. Afin d'évaluer la résistance, nous avons considéré comme composés biomarqueurs les métabolites variants significativement en abondance parmi les cultivars/NILs variant en résistance. Ces composés étaient soit reliés aux voies phénylpropanoïdes, soit d'importantes molécules de signal ou encore d'autres

métabolites reliés à la résistance. Le potentiel du profilage métabolique comme technique permettant le criblage à haut débit pour l'évaluation des cultivars/lignées de blé variant dans leur résistance au FHB est discuté.

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List of Scientific and Statistical Abbreviations

AAFC	Agriculture Agri-Food Canada
AFLP	Amplified fragment length polymorphism
AMDIS	Automated mass spectral deconvolution and identification system
ANNs	Artificial neural networks
ANOVA	Analysis of variance
AtNPR1	*Arabidopsis thaliana* NPR1 gene
AUDPC	Area under disease progress curve
BA	Benzoic acid
BTH	Benzo (l, 2, 3) thiadiazole-7-carbothioic acid s-methyl ester
CAN score	Canonical score
CAS	Chemical abstract service number
CDA	Canonical discriminant analysis
CPSR	Canada prairie spring red wheat
CPSW	Canada prairie spring white wheat
CWAD	Canada western amber durum wheat
CWRS	Canada western red spring wheat
CWRW	Canada western red winter wheat
CWSWS	Canada western soft white spring wheat
d	Day
DA	Discriminant analysis
dai	Day after inoculation
DH	Double haploid
DON	Deoxynivalenol
DSP	Disease severity phenotypes
ESTs	Expressed sequenced tags
FA	Factor analysis
FDK	Fusarium damaged kernels
FHB	Fusarium head blight
GC/EI-TOF	Gas chromatography/electron ionization-time of flight

GC/MS	Gas chromatography mass spectrometry
ha	Hectare
hai	Hour after inoculation
HCA	Hierarchical cluster analysis
HPLC	High-pressure liquid chromatography
IRt marker	Retention time and ion marker
JA	Jasmonic acid
LC/MS	Liquid chromatography mass spectrometry
m/z ratio	Mass charge ratio
MATLAB	Matrix laboratory
MetAlign	Metabolomics alignment software
MET-IDEA	Metabolomics ion-based data extraction algorithm
MPP	Metabolic profile phenotypes
MSFACTs	Metabolomics spectral formatting, alignment, and conversion tools
NIL	Near isogenic lines
NIST	National Institute of Standards and Technology of the USA
NMR	Nuclear magnetic resonance
NPK	Nitrogen, phosphorus and potassium
PCA	Principal component analysis
PR gene	Pathogenesis related gene
PR Protein	Pathogenesis related protein
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphism
RP	Roblin pathogen-inoculated
RRC	Resistance-related constitutive metabolite
RRI	Resistance-related induced metabolite
RT	Retention time
RW	Roblin water-inoculated
SA	Salicylic acid
SAR	Systemic acquired resistance

SP	Sumai3 pathogen-inoculated
SSR	Simple sequence repeat
SVM	Support vector machines
SW	Sumai3 water-inoculated
TMS	Trimethylsilyl
TOF-MS	Time of flight mass spectrometer
VCG	Vegetative compatibility group

Contribution of authors

This thesis comprises three studies, leading to three manuscripts (Chapters 3, 4, and 5). The first manuscript (Chapter 3) was coauthored by Dr. A.C. Kushalappa, Y. Dion, S. Rioux, A. Comeau, V. Yaylayan, W.D. Marshall, and D.E. Mather. I designed the first study, defined the approach, completed the experiment, fulfilled the data processing and analysis, and wrote the manuscript. Dr. Kushalappa supervised the research by providing helpful suggestions throughout various steps of the study, from the beginning to the end. Dr. Marshal supplied the GC/MS equipment; Dr. Yaylayan provided GC/MS expertise, while Y. Dion, S. Rioux, and A. Comeau provided the wheat cultivars, and Dr. Mather gave me valuable comments and suggestions.

The second manuscript (Chapter 4) was coauthored by A. C. Kushalappa, Y. Dion, S. Rioux, A. Comeau, V. Yaylayan, and W. D. Marshall. The contributions of the authors were the same as described for Chapter 3.

The third manuscript (Chapter 5) was coauthored by V. Paranidharan, A. C. Kushalappa, O. Mamer, and D. Somers. The contribution of Dr. Kushalappa was as described before in Chapter 3. Dr. Paranidharan assisted me in modifying the analytical platform, Dr. Mamer supplied the GC/MS equipment, and Dr. Somers provided the seeds of near isogenic lines with alternate alleles in 2D QTL for the third study.

Chapter 1

Introduction

Common wheat (*Triticum aestivum* L. em. Thell.) is one of the most important cultivated plants. It is a cool season crop grown in various climatic regimes. The range of annual precipitation required for wheat cultivation is from 250 to 1750 mm. However, in most wheat-growing areas the annual precipitation ranges from 375 to 875 mm (Hyena, 1987).

The principal cultivated food crop in Canada is wheat. Canadian wheat is well known for its high protein content. Canada's wheat production in 2006 was 27.276 million metric tons (Statistics Canada, 2006). On a global scale, Canada ranks third among major wheat producers and second among wheat exporters, following the USA. Canadian farmland area is only 7 percent of the total land area of 2,272 million acres available.

One of the most important diseases of wheat in Canada is fusarium head blight (FHB) caused mainly by *Fusarium graminearum* Schwabe (Schroeder and Christensen, 1963; Sutton, 1982; Clear and Abramson, 1986; Wong et al., 1992; Bai and Shaner, 1994; Parry et al., 1995). Hard red spring and durum wheat are the common hosts, but it can infect corn, barley, and oats. The main symptom of the disease is whitening of infected spikelets leading to eventual death. Under humid conditions, salmon-pink to orange masses of spores (spordochia) develop on the glumes. Besides large yield losses in years with severe epidemics, *F. graminearum* produces several mycotoxins in wheat kernels, including deoxynivalenol (DON) or vomitoxin and zearalenone, both of which are hazardous to human and livestock health. DON is produced in the wheat grains before they are harvested. Frequent outbreaks of FHB in eastern Canada and in Manitoba have threatened Canadian wheat production, and the pathogen has continued its spread towards the west (Fernando, 1999). As a result, FHB reduces both the quantity and quality of the wheat yield and has some indirect effects on the dairy production of animals fed with toxin-contaminated wheat (Bai and Shaner, 1994). Reduced seed germination and seedling blight of infected seeds are other effects of the disease. Human and animals consuming toxin-

contaminated wheat may suffer serious health threats. It is believed that some *Fusarium* mycotoxins may be associated with human alimentary toxic aleukia. Vaginal prolapse and vulva vaginitis of female pigs due to consumption of cereals contaminated with *F. graminearum* or *F. culmorum* have also been documented. The symptoms on poultry that are fed such grains are stunting and poor feathering (Bai and Shaner, 1994; Parry et al., 1995).

The FHB pathogen reproduces both asexually and sexually in nature. It can survive as mycelium, ascostroma, and chlamydospores. Ascospores, the propagules of the sexual stage, develop in the perithecia on the host debris. The macroconidia, mycelia, and chlamydospores survive in the soil and microconidia are not produced. During the saprophytic phase, the pathogen can develop on plant debris and initiate infection. The pathogen first initiates a biotrophic relationship with the host. Within a short time after infection, the host tissues are killed and the spike turns white because of the necrotrophic growth of the pathogen (Bushnell et al., 2003). Several weed species, including species of *Agropyron*, *Agrostis*, *Avena*, *Bromus, Cenchrus*, *Lolium, Medicago,* and *Trifolium*, harbor *Fusarium* species. Continuous cropping of wheat or a wheat-maize rotation favors severe epidemics because of improved pathogen survival due to an increased availability of nutrients, as well as greater inoculum production by the fungus. Temperature and moisture are two key environmental factors influencing FHB epidemics. The optimum conditions for infection of heads by *F. graminearum* are 25 ^{o}C and saturation humidity for at least 24 h. Under such conditions, FHB is capable of destroying a wheat field in a few weeks (Sutton, 1982; Clear and Abramson, 1986; Abramson et al., 1987; McMullen et al., 1997a).

Current disease management includes the use of resistant cultivars, fungicides, and cultural practices. These measures are inadequate in years with severe epidemics. Effective fungicides for foliar application are limited. Folicur 432F (tebuconazole) was introduced for controlling FHB in the Prairies in 1999 (McMullen, 1999; Fernando, 1999). Biological control of FHB is in its formative years. The logic behind biological control is to advance the decomposition of infected residue on the soil to decrease the primary inoculum for the next year. Burying infected residue by plowing promotes the

rapid decomposition of the residue. This results in the reduction of pathogen spore load in the spring.

The consensus is that resistance is central in any integrated FHB management program (Gilbert and Tekauz, 1999). Chinese breeders have screened more than 30,000 wheat cultivars, lines, and accessions in order to find resistance to FHB. Researchers have found the well known Sumai3 and Ning derivatives, which are currently widely used in breeding programs (Weizhong, 1999).

There are two main classes of resistance to FHB in wheat (Mesterhazy, 1995). Passive resistance involves passive mechanisms associated with phenotypic traits such as plant height, presence of awns, spikelet density, time of flowering, length of flowering, anther morphology, diversity of florets, and the length of time that flowers remain open (Miedaner, 1997).

Active resistance of wheat to FHB is more complicated and includes five proposed types of mechanisms: type I, resistance to initial infection; type II, resistance to the spread of infection in the spike; type III, resistance to kernel infection; type IV, tolerance (tolerant wheat plants show the same level of disease severity but vary in grain yield); and type V, resistance to mycotoxin accumulation. Resistance types III and IV are generally undefined (Schroeder and Christensen, 1963; Mesterhazy, 1995) and not much is known about them (Stack, 1997). The first two types of resistance are the most widely used by breeders and type II resistance is the most studied. In general, manipulation of resistance types III, IV, and V is very difficult and their use as a screening tool in breeding is expensive. Wheat resistance to FHB is quantitatively inherited and controlled by 2-5 major genes (Miedaner, 1997; Fernando, 1999; Gilbert et al., 2000).

The following factors make the breeding for FHB resistance in wheat very challenging:

1. There is a lack of precise knowledge about the genetic mechanisms of resistance, including the number of genes and their different chromosomal locations (Parry et al., 1995; Miedaner, 1997; Gilbert et al., 2000)
2. The fungus can infect wheat during a very short temporal window, between anthesis and the soft dough stage of kernel development

(10-20 days after anthesis) (Schroeder and Christensen, 1963; Sutton, 1982). The earlier the spike is infected, the more severe the disease. When scoring spikes for disease symptoms as a measure of resistance, lack of flowering uniformity of the spikelets at the time of inoculation can cause errors in disease assessment.

3. There is no wheat cultivar immune to FHB and most existing wheat cultivars are susceptible. A few wheat cultivars are relatively resistant. All durum (a tetraploid wheat lacking the D genome) wheat cultivars are more susceptible to FHB than the common wheat cultivars (Parry et al., 1995).
4. There is no conclusive evidence about race-specific (vertical) resistance to FHB in wheat (Parry et al., 1995).
5. Resistance to FHB is a quantitative trait and controlled by several QTL (Bai and Shaner, 1994; Feredric et al., 1999; Waldron et al., 1999; Anderson et al., 2001; Kolb et al., 2001; Bai et al., 2003a; Somers et al., 2003; Yang et al., 2005)
6. Although trichothecenes are phytotoxins, their production is not required for the appearance of disease symptoms. DON is a virulence factor and it is possible to reduce FHB severity by introducing genes for resistance against trichothecenes. However, attempts to correlate DON content and disease resistance scores were not conclusive (Leonard and Bushnell, 2003; Lemmens et al., 2005).

Conventional methods currently being used to evaluate quantitative resistance to FHB in wheat cultivars are time consuming and expensive. These methods are principally based on the visual assessment of disease severity (Parry et al., 1995). Double haploid and marker assisted selection using molecular markers such as RFLP, AFLP, and RAPD have recently been used to study FHB resistance (Feredric et al., 1999; Waldron et al., 1999; Anderson et al., 2001; Kolb et al., 2001; Shen et al., 2003). DNA molecular techniques are rapid and require only a small amount of plant tissue at any growth stage. With all of these techniques, existing polymorphisms among different cultivars, if any, are revealed. The limited knowledge of the mechanism of resistance, the number of genes involved, and their functions prevent molecular techniques alone from resolving the dilemma of FHB

resistance. The number of resistance genes has been estimated based on a number of studies to vary between one and six (Bai et al., 1989; Snijder, 1990). In different varieties, resistance genes have been reported to be on different chromosomes (Waldron et al., 1999; Weizhong, 1999; Ban and Watanabe, 2001; Kolb et al., 2001). In Sumai3, resistance genes were suggested to be on chromosomes 1B, 2A, 5A, 6D, 7D, 2B, 3B, 6B, and 7A (Weizhong,1999; Kolb et al., 2001). A recent study has shown the involvement of seven QTLs for type I resistance on chromosome arms 2DS, 3AS, 3BS, 3BC (centromeric), 4DL, 5AS, and 6BS, and four QTLs for type II resistance on chromosomes 2DS, 3BS, 6BS, and 7BL, suggesting a pleotrophic effect for QTL on the 2DS chromosome. The most successful and widely used source of FHB resistance is the 3BS QTL of Sumai3 and its derivatives that explain 15-60 percent of phenotypic variation of type II resistance in different studies (Waldron et al., 1999; Shen et al., 2003; Somers et al., 2003; Zhou et al., 2003; Yang et al., 2005). The importance of the D genome of Sumai3 in conferring resistance to FHB was emphasized in one study (Ban and Watanabe, 2001), but rejected in other studies (Gilbert et al., 2000; Kolb et al., 2001). In addition to 3BS, 5A and 6BS are also likely locations for FHB resistance genes from Sumai3. More recently a study on 174 DH lines from a cross between DH181 (resistant) and AC Foremost (susceptible), showed the involvement of the D genome (QTL on 2DS chromosome) in both types I and II resistance against FHB (Yang et al., 2005).

The impacts of the environment on disease symptoms are magnified when challenged with such a resistance controlled by multiple QTLs, with conceivable mixed small to large effects expressing in various ways that demonstrate as different resistance types.

Our knowledge of the mechanisms of resistance to FHB is limited. Although modern approaches, such as molecular assisted selection, combined with conventional methods, have enhanced the throughput of screening methods, they remain slow and have contributed very little to our knowledge about the mechanisms of resistance. Genes are upstream of a continuous flow of encrypted information from genotype to deciphered visible phenotype of the FHB-wheat pathosystem. There is less inconsistency in DNA-marker-based disease resistance selections. Unlike DNA markers, the

phenotype is influenced and masked by the external microclimate so DNA markers are preferable for breeding purposes. The key to the FHB resistance puzzle lies in the parallel studies of the FHB resistance genome, transcriptome, proteome, and metabolome.

In the post-genomic era, there is an urgent need to assign functions to genes in order to pyramid genes controlling suitable traits for crop improvement. Metabolomics, an evolving field of systems biology, aims to decipher gene function at the metabolite level (Fihen et al., 2000; Dixon, 2001; Fiehn, 2002; Sumner et al., 2003; Goodacre et al., 2004). Metabolomics generates large data matrices with astronomical dimensions. However, often only a few metabolites relate to the function of a gene or a trait (Goodacre et al., 2004). The identification of a set of metabolites that can explain the functions associated with a gene is a big challenge in systems biology.

There has been enormous progress in recent years in the development of new tools to study genetic diversity in plants based on their secondary metabolites. Metabolite profiling enables us to discriminate genetic variation within plants (Roessner et al., 2001), and combined with mRNA and protein analyses, provides deeper insight into complicated regulatory processes and mechanisms in resistance and can be used accurately for resistance evaluation (Fiehn et al., 2000a). According to the gene expression concept, FHB resistance genes are transcribed into mRNA (transcriptome) and subsequently translated into enzymes. These enzymes may play a direct role in defense (PR proteins) or contribute in setting metabolic pathways through which the dynamics of metabolic networks are modified in a manner to prepare for an enhanced defensive state. A sufficient knowledge about the pathways contributing to FHB resistance will help reveal the unknown aspects of the underlying mechanisms of the resistance and improve the efficiency of screening germplasm. It may be possible to quantify resistance by measuring the amount of these hypothetical biomarker metabolites in wheat cultivars varying in resistance to FHB as an indicator of the functional level of resistance gene(s).

The general assumption is that the quality and quantity of these resistance biomarker metabolites vary among cultivars of wheat with different types and levels of resistance to FHB. Based on this assumption, it may be

possible to develop and use metabolic criteria to distinguish disease resistance by means of metabolite profiling. To date, no one has used this novel approach to discriminate FHB resistance in different wheat genotypes.

1.1. General hypothesis

It is hypothesized that wheat cultivars varying in resistance to FHB have different metabolite profiles. The null hypothesis states that, “There is no difference in the metabolite profiles of different wheat cultivars known to vary in their resistance to FHB”.

1.2. Objectives

1. To identify metabolites produced in the wheat-FHB pathosystem.
2. To classify selected cultivars varying in the level of FHB resistance based on their metabolic profiles.
3. To establish metabolite phenotypes for selected susceptible and resistant near isogenic lines with alternate alleles for FHB resistance QTL in order to identify resistance-related biomarker metabolites.

Chapter 2

General literature review

2.1. Wheat

Bread wheat (*Triticum aestivum* L. em. Thell.) is the staple crop for many people around the world. It is one of the best examples of an allopolyploid agricultural plant. An allopolyploid is an organism that has two or more complete sets of chromosomes derived from different species, with chromosome sets that are not fully homologous. Tetraploid wheat (4n=28, AABB) evolved from a cross between two different diploids (2n=14, AA and BB). Wild emmer (*T. dicoccoides*) is the ancestor of tetraploid wheat (as well as emmer and durum wheat). Hybridization between two diploid wild grasses (*T. urartu*) and a wild goat grass such as *Aegilops searsii* or *A. Speltoides* resulted in wild emmer. This hybridization occurred in the wild, long before domestication of wheat. Some 8000 years ago hexaploid wheat (6n=42, AABBDD) evolved from diploid and tetraploid ancestors (Griffiths et al., 1999). Bread wheat has several hundred varieties that can be grouped in one of eight classes according to kernel hardness, color, size, shape, milling and baking quality, and food application (Hyena, 1987).

Bread wheat has the capacity to grow in relatively different climates ranging from the Arctic Circle to the equator. However, most of its production is between latitudes 30-60^{o}N and 27-40^{o}S. Canada has long cold winters and short hot summers that support the culture of spring wheat. Limited rainfall affects the amount of grain yield. However, Canadian wheat grain is well known worldwide for its high protein content. Another attribute for grouping wheat cultivars into classes is the time of planting and harvesting (Hyena, 1987). Wheat may grow in winter and/or spring seasons. The planting and harvesting time of winter wheat are the fall and the spring/summer respectively, while those of spring wheat are the spring and the summer/fall.

Canadian wheat is also categorized into six classes as follows (Ontario Wheat Board, 2002):

1) Canada western red spring (CWRS) wheat is a hard wheat with superior milling and baking quality.

2) Canada western amber durum (CWAD) wheat has a high yield and an excellent pasta-making quality. Durum or Macaroni wheat is the only tetraploid species of wheat widely cultivated today. High protein content and gluten strength of durum wheat make it good for pasta.

3) Canada prairie spring red (CPSR) wheat has medium hard kernels and medium dough strength.

4) Canada western red winter (CWRW) wheat is a hard wheat with excellent milling quality.

5) Canada prairie spring white (CPSW) wheat is suitable for making various types of flat breads.

6) Canada western soft white spring (CWSWS) wheat with low protein content is suitable for making cake and cookies (Ontario Wheat Board, 2002).

Wheat is the most important cultivated crop in Canada and the country is third among major wheat producers and second among wheat exporters following the USA (Statistics Canada, 2002a; Wainio and Zahniser, 2002). During the last decade, total Canadian wheat production has decreased 24 percent. About 40 percent of Canada's total crop area is devoted to wheat production. Almost 80 percent of Canadian wheat is produced in the Prairie Provinces of Alberta, Saskatchewan and Manitoba (Statistics Canada, 2003).

The land area occupied by wheat fields in Canada in the last two decades has varied between 10 to more than 14 million ha, most of which have been seeded to spring wheat. Between 32,000 and 40,000 hectares of the total land cultivated with wheat are in Quebec, which is less than 4 percent of the total area that wheat is grown on in Canada (Statistics Canada, 2002b).

A short growing season forces growers to plant high quality spring and durum wheat. Red spring wheat is the most common class of wheat planted in the Prairie Provinces. Soft white winter wheat is mainly produced in Ontario and across other eastern provinces in small quantities. The durum wheat share is only 5 percent of the total wheat grown in the Prairie Provinces. However, it is an important component of Canadian wheat exports.

2.1.1. Growth stages of wheat

Some systems have been created to describe the growth and developmental stages of wheat among which the Zadok's scale (Zadoks et al., 1974) is the most widely used. This scale has a two-digit code for each stage. The major growth stages of the wheat plant are seed germination, seedling, tillering, stem elongation or jointing, booting, heading, flowering or anthesis, milk, dough, and ripening. There are ten major stages from 0-9 with each main stage subdivided into ten (0-9) minor stages (Zadoks et al., 1974).

At the apex of each tiller, one inflorescence appears. The inflorescence of wheat is a spike with a main axis called a rachis. The rachis bears spikelets at the internodes. Each spikelet contains two to five florets borne on a short axis (rachilla) (Fig. 2.1).Two sterile bracts (glumes) surround each spikelet and each floret has two bract-like structures - a lemma and a palea. Three stamens with large anthers and a pistil with two styles each having feathery stigma branches are present inside each of the florets (Fig. 2.1).

Two important factors influencing the number of initiated spikelets are temperature and photoperiod. The longer the photoperiod the earlier the spikelet is initiated. Any increase in day or night temperatures raises the spikelet initiation rate. The differentiation of florets inside the spikelets begins in the lower-central portion of the spike and develops in both up and down directions. Inside each spikelet, up to 12 florets may be produced but five or fewer will result in kernel production. At the base of the ovary in the wheat floret are two lodicules that swell during anthesis and cause the flower to open and the anthers to protrude. Within 20 minutes after lodicules swell, anthesis will be completed by the closing of the lemma and palea.

2.2. Wheat fusarium head blight

One of the most important diseases of wheat in Canada is fusarium head blight (FHB), which is also commonly known as wheat scab or tombstone disease. The principal causal agent of the disease is *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch). Hard red spring wheat and durum wheat are the most frequently infected, but the disease is not just confined to wheat. Other small grain crops, including corn, barley and oats, are also susceptible to infection. Spikelets in diseased heads

are killed, whitened and if enough humidity is provided, salmon-pink to orange spore (macroconidia) masses develop on the edges of the glumes. The disease is sometimes called tombstone disease because of the development of white to pink shriveled, scabby kernels. Severe yield losses may be observed in years with an epidemic of FHB. Besides yield losses, *F. graminearum* also often produces several mycotoxins in wheat seeds, which are harmful to human and livestock. Deoxynivalenol (DON) is one of the major toxins, which has raised much concern because it is produced in wheat seeds before harvest.

The importance of FHB, as a major plant disease, is now increasing throughout the world, especially in North America. Recent epidemics of FHB have drawn the interest of researchers (McMullen et al., 1997a). The disease has been frequently reported in most wheat growing areas of the world and at least 17 *Fusarium* species have been associated with this disease. Although *F. graminearum* is the major pathogen of the disease in many countries (Schroeder and Christensen, 1963; Sutton, 1982; Clear and Abramson, 1986; Wong et al., 1992; Bai and Shaner, 1994; Parry et al., 1995), other species such as *F. culmorum*, *F. avenaceum* (*Gibberella avenaceum*), *F. poae,* and *Microdochium nivale* have also been cited as the causal agents of FHB. However, *F. graminearum* and *F. culmorum* have been reported to be the main causal agents for FHB (Parry et al., 1995).

2.2.1. Situation of the disease worldwide and in Canada

FHB has become a prevalent and major threat to wheat production worldwide (Snijders, 1990; Sutton, 1982; Bai and Shaner, 1994; Gilbert et al., 2000). FHB infections have been reported from most places where cereals are grown (McMullen et al., 1997a) including Canada (McMullen et al., 1997a), the USA (Bai and Shaner ,1994), Australia (Burgess et al., 1987), Bulgaria, Czechoslovakia, France, Germany, Hungary, Italy, the Netherlands, Romania, Switzerland, Yugoslavia, England, China, India, and Japan (McMullen et al., 1997a). *F. graminearum* causes epidemics over large areas but shows local variability in severity. This variability is due to differences in cultivars planted, crop rotations, date of anthesis, and microclimate. In severe epidemics, all heads in the field may show symptoms (Bai and Shaner, 1994).

The first record of heavy infection of wheat by this fungus was from southern Manitoba (Red River Valley) in 1984 (Clear and Abramson, 1986). In 1993, the highest level of the disease was observed in southeastern Manitoba where Roblin, a highly susceptible cultivar, was most widely grown. *F. graminearum* has also been reported from southeastern Saskatchewan in durum wheat. Severe epidemics of FHB have occurred in spring wheat fields in Manitoba and some Northern states of the USA (Bai and Shaner, 1994). It is believed that increasing interest in FHB research in Canada has its roots in the 1993 epidemic (Gilbert et al., 2000). In the western Prairies, a high level of *F. graminearum* was observed for the first time in west central Alberta in Canada Prairie Spring wheat samples. Since then, low levels of *Fusarium* diseased kernels (FDK) have been observed mainly due to drought. The disease was well established in Saskatchewan by 1998. Wheat scab epidemics during 1927-1980 have occurred approximately once every 9 years in wheat fields in Ontario. It has been observed in Ontario, Quebec, the Maritime Provinces, Manitoba, and the Peace River region of Alberta (Sutton, 1982). FHB has spread toward the west from southeastern Manitoba to Saskatchewan and Alberta, replacing less pathogenic species of *Fusarium* (Clear and Patrick, 2000).

2.2.2. Potential damage of FHB

FHB reduces both the quantity and quality of the grain. Direct damage is due to weight reduction of the grains while indirect damage results from production of two important mycotoxins called DON and zearalenone (Bai and Shaner, 1994). There is a reduction of seed germination and seedling blight of infected grains. Scabby head incidence in China varies from 50 to 100 percent, with 20-40 percent grain loss in severe epidemic years (Bai and Shaner, 1994). FHB is the second among leading wheat diseases in China after stripe rust (*Puccinia striiformis*), infecting roughly one quarter of the country's total wheat production area, making China the largest wheat scab-infected region of the world. Favorable climatic conditions for FHB epidemics in the soft red winter wheat areas of the USA caused the development of scab damage averaging of 25 percent or 2.72 million tons of grain yield reduction on 6.1 million ha (McMullen et al., 1997a). Total estimated loss from wheat

scab in Minnesota, North and South Dakota and Manitoba was reported to be about US$1 billion in the 1993 epidemic. This is one of the highest annual economic losses ever, due to a plant disease in North America. The amount of wheat lost in Manitoba in 1992 was 176 million bushels (worth $704 million), 20 million bushels more than the sum of all losses in Minnesota, North and South Dakota.

2.2.3. The pathogen

Fusarium graminearum Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) is considered the principal causal agent of FHB around the world. The fungus produces ascospores, macroconidia, and chlamydospores in its life cycle. Ascospores are mainly three-septate, hyaline, 17-25.5 µm long, and 3-5 µm wide, and formed in purplish-black perithecia. Perithecia are developed on stromata on the host tissues and host debris. Microconidia are not produced, but macroconidia, chlamydospores, and mycelia are formed in soil. The macroconidia are less homogeneous in septation than the ascospores and are 3-7-septate with a foot cell varying from 35-62 µm long by 2.5-5 µm wide (Leonard and Bushnell, 2003).

Continuous wheat cropping, in wheat-maize and wheat-rice rotations, enhances the food resources for better survival and higher inoculum production of the pathogen, and eventual development of severe epidemics. Perithecia are initiated on crop residues on the soil surface during the saprophytic phase. Inoculum is spread mainly by wind and rain splattering, but arthropods, such as mites, and systemic growth may also play roles. Several weed species, including species of *Agropyron*, *Agrostis*, *Avena*, *Bromus, Cenchrus*, *Lolium, Medicago,* and *Trifolium* harbor isolates of the *Fusarium* species. Therefore, the fungus can survive between wheat crops in living or dead host tissues. The ascospores are produced after maturation of asci in the perithecia and are disseminated by the wind. The ascospores, macroconidia, chlamydospores, and hyphal fragments can all serve as inocula, although the main initial sources of inocula are considered ascospores and macroconidia. Humidity in the form of rain or heavy dew is a factor required for initial ascospore release.

Following deposition of inocula on spikelets, the pathogen starts its pathogenic phase. The germination occurs mainly on anthers during anthesis. The fungus can invade the floret through crevices between the palea and lemma, directly from epidermal cells of interior floral surfaces, wounds, and stomata. Some fungal growth stimulators in glumes like choline chloride and betaine hydrochloride may favor the fungus to colonize the host. These two important metabolites can also be found in other parts of the plant such as the palea, the lemma, the glume, the rachis, and the grain, although compared to the anthers there are lower concentrations of these metabolites in these parts (Bushnell et al., 2003).

Several studies have shown that severe FHB epidemics occur consistently in wet weather conditions (Sutton, 1982; Clear and Abramson, 1986; Abramson et al., 1987; Burgess et al., 1987; McMullen, 1997a). FHB develops within 3-4 days in warm and humid conditions. The disease progression rate of FHB will increase following any rise in humidity (at 25 ^{o}C). The optimal infection of cereal ears to FHB by *F. graminearum* occurs after at least 24 h of warm (25 ^{o}C) and humid (HR=100%) conditions. Within a short time after infection, the spikelets start to turn white (Leonard and Bushnell, 2003).

2.2.3.1. Variability of *F. graminearum*

There is a high genetic diversity among isolates of *F. graminearum* (Mesterhazy, 2003). A number of studies have been carried out on the genotypic diversity of *G. zeae* using different techniques such as the vegetative compatibility group (VCG) and RAPD markers. The results have shown high levels of diversity in the genotypes of the pathogen from single wheat heads or from different fields (Bowden and Leslie, 1992; Ouellet and Seifert, 1993; Dusabenyagasani et al., 1999). VCG data must be generated via pair wise comparisons among all strains, which tends to lead to an exponential increase in the number of tests as the sample size increases. Miedaner (1997) believes that the quantitative inheritance of wheat resistance lowers the risk of adaptation of *G. zeae* to host resistance. Until now, there is no evidence for race specificity or breakdown of resistance to FHB (Young, 1996; Gilbert et a., 2000).

Correlation between resistance of wheat to *F. graminearum* and *F. culmorum* (Fernando, 1999) suggests that the same FHB resistance gene(s) may be effective against both species of the pathogen (Mesterhazy, 1995, 1997). The practical advantage of a general *Fusarium* resistance in both winter and spring types of wheat is that selection against a good pathogenic isolate of an important species may lead to FHB resistance to all important isolates and species (Mesterhazy, 2003).

2.2.4. Fusarium head blight control options

Cultural practices, biological and chemical control options, as well as use of resistance cultivars are different ways of controlling FHB (Gilbert et al., 2000). The cultural measures include avoidance of such rotations as wheat-corn or wheat-rice as well as burying plant residues. The biological control of FHB relies on adding some species of *Trichoderma* for residue management and promoting residue decomposition. Considering incomplete resistance of wheat to FHB and the lack of highly effective fungicides, a key element in FHB management is reducing inoculum of *F. graminearum* in the host debris and other reservoirs. Chemical fungicides that farmers could use for this purpose in North America are few in number. Folicur (tebuconazole) has been recently registered for heading application (McMullen et al., 1997b; McMullen, 1999). Despite its immediate action, chemical control is not a long-term option. One of the most promising methods for FHB control is genetic resistance, but complete resistance in commercially available cultivars is not at hand, especially in certain cultivars of winter and durum wheat.

2.3. Wheat breeding for resistance against FHB

Scientists, who work on FHB, unanimously believe that breeding for resistance is at the core of any integrated FHB management program (Gilbert and Tekauz, 1999). The disease has been challenging breeders for a long time and many breeding programs are now underway to develop new scab-resistant varieties by screening cultivars based on scab severity and DON production. Resistance or susceptibility of wheat to FHB is determined in a relatively short temporal window (10 to 20 days) which is the time between the beginning of anthesis and the soft dough stage of kernel development when

wheat spikes are susceptible to infection by *F. graminearum* (Schroeder and Christensen, 1963). The earlier the spike is infected in this time span the greater is the severity of disease.

The first efforts to find wheat resistant to FHB commenced in 1941. Progress and Haynes Bluestem cultivars were reported to be the most resistant wheat cultivars over half a century ago (Hanson et al., 1950). Chinese breeders have attempted massive screening of more than 30,000 wheat genotypes to find resistance to FHB and have achieved great progress. One significant problem in screening for FHB resistance and testing its components lies in the poor reproducibility of FHB resistance tests, due to the significant effect of environmental variability on the expression of symptoms, implying a high genotype × environment interaction variance (Dill-Macky, 2003).

Many researchers believe that the measurement of the spread of FHB in the head presents one of the most reliable estimates of a cultivar's resistance. Such estimates are generally conducted via visual assessment of disease symptoms. In natural infections and field trials, the disease incidence is measured as the percentage of infected heads (spikes). Disease severity, however is a better indicator of cultivar resistance, and is measured as the percentage of infected spikelets in a spike (Wilcoxon et al., 1992). Disease index, the product of disease incidence and disease severity, may also be used as a measure of cultivar resistance against FHB. These three means of evaluation are used by researchers in North America (Groth et al., 1999). Post harvest examination of kernels for yield, test weight (kg/hl; weight of grain per half liter) and visually scabby kernels are also used for the evaluation of FHB. Differences in FHB-resistance between wheat cultivars may also be assessed based on DON detoxification in wheat grains. Miller et al. reported that within 72 h, the relatively resistant cultivar Frontana could degrade 18 percent of DON compared with 5 percent in Casavant (a susceptible cultivar) (Miller et al., 1986).

RFLP, AFLP, and RAPD have been used to study FHB resistance (Feredric et al., 1999; Waldron et al., 1999; Anderson et al., 2001; Kolb et al., 2001). Detection of resistance genes in large populations requires a powerful and rapid technology and therefore improving the throughput of screening

tools remains an ongoing need. There are two main kinds of mechanisms involved in resistance. The passive and active mechanisms are defined as those that act dependently or independently of the physiological status of the plant, respectively (Mesterhazy, 1995).

2.3.1. Passive resistance

Passive resistance involves mechanisms associated with phenotypic traits such as plant height, the presence of awns, spikelet density, time of flowering, length of flowering, anther morphology, the position and diversity of florets, and the length of time that flowers remain open (Miedaner, 1997). Tall wheat varieties without awns and varieties that do not protrude their anthers have shown reduced susceptibility to the disease. The effect of increasing plant height on reducing infection is independent from active resistance components (Mesterhazy, 1995). The longer the upper internodes, the less likely the heads will be exposed to the inocula of the pathogen carried by splashing rainwater (Parry et al., 1995). The presence of awns increases the probability of capturing airborne or water splashed spores, thereby increasing the chances of exposure to head blight infection (Mesterhazy, 1995). Less exposure of the anthers to spores helps the plant to escape infection. The betaine- and choline-type metabolites found in the pollen are thought to enhance the possibility of the fungal infection (Leonard and Bushnell, 2003). The role of passive resistance against heavy epidemics is not significant and the real solution is believed to be an increased active resistance (Mesterhazy, 2003).

2.3.2. Active resistance

Resistance to FHB has five components (type I through V) inherited independently. These components often show linkage in many genotypes (Masterhazy, 2003):

Type I is resistance to initial infection (Schroder and Christensen, 1963) and is measured as incidence, which is the percentage of heads showing symptoms regardless of the number of spikelets in the head. Preformed morphological and/or biochemical resistance factors or induced active defense responses, are listed as possible operating factors in type I resistance.

Type II resistance is resistance to the spread of the disease in infected tissue (Schroeder and Christensen, 1963) which is derived from Sumai3 and related wheat lines and inherited in a polygenic way. Type II resistance is measured by inoculating an individual floret in a spikelet near the middle of the spike and observing the spread of symptoms to adjacent spikelets. Symptoms are typically visible in the inoculated spikelets within 3-7 d and spread to other spikelets by 8 to 12 d. The spikelet-to-spikelet spread occurs through the rachis and type II resistance reduces this spread. In a study by Schroeder and Christensen (1963) on two susceptible and resistant cultivars, the spread rates of the fungus in the rachis were different. There is evidence that the fungus induces appositions of highly heterogeneous materials at and around the site of entry and intense lignin accumulation in the cell wall of the resistant cultivars (Kang and Buchenaur, 2000). While the expression of the type II resistance in the floret and rachis does not stop fungal development, it retards the growth of the fungus sufficiently to allow the rachis to uphold function in support of grain filling in portions of heads above infected spikelets.

Type III is resistance against kernel infection (Mesterhazy, 1995, 1997). In some cultivars, the kernel colonization rate by the fungus is less than what is expected based on visual disease severity assessments. Based on this, Mesterhazy (1995) concluded that kernel resistance to infection is different from other types of resistance. Some studies propose that in some wheat lines resistance factors come into play that reduce kernel infection when other parts of the floret are colonized enough to display visible symptoms (Mesterhazy et al., 1999). The stage of kernel development, the presence of phenolic metabolites, and the resistance of specific layers of the seed coat, as well as rate of DON transfer from chaff to kernel (if DON promotes fungal spread in the head) are several factors that limit kernel infection.

Type IV resistance against FHB, so called tolerance, is resistance to yield loss in the presence of disease (Mesterhazy, 1995). FHB causes shriveled kernels and reduces kernel weight. Weight loss has a positive correlation with disease severity. Type IV resistance was suggested for those wheat genotypes showing less kernel weight loss when infected with the fungus (Mesterhazy et al., 1999).

Type V is resistance against mycotoxins. This resistance is due to toxin degradation, or insensitivity of the plant to toxin and/or limitation of the amount of toxin build-up in head tissues. The fungus initially launches a biotrophic relationship with the host tissue but later moves to a necrotrophic phase. The switch from biotrophy to necrotrophy is mediated by DON and other toxins produced by the FHB pathogen. These toxins inhibit protein synthesis by interfering with peptidyl transferase. Several lines of evidence show that DON or possibly other related trichothecenes are in part responsible for chlorosis and browning of heads in FHB (Bushnell et al., 2003). There are two modes of action postulated regarding the role of DON in FHB. DON may promote spread of the fungus by limiting activation of defense responses or by direct toxic effect on the cells of the rachis. Regardless of any possible role DON may have in pathogenesis, resistance leading to lowered toxin accumulation in the kernel is important due to end-product quality considerations.

The rates of spread (type II resistance) and penetration (type I resistance) are both accelerated and reduced simultaneously and closely related in several synthetic wheat lines carrying the D genome from *Aegilops squarrosa* (Mesterhazy, 2003). However, resistance is better measured by evaluating the rate of spread than that of penetration. Tolerance (type IV) is also independent from other traits. Tolerant wheat cultivars maintain an adequate yield despite the presence of disease. Resistance components, despite independent inheritance, have linkage but the genetic basis for the close correlation is unknown. One explanation may be that resistance genes have pleiotropic effect for all the traits or perhaps different genes for the respective traits are linked. Another possibility is that separate genes may have modifying effects (Mesterhazy, 2003).

Type II resistance is the most studied, and much less is known on resistance types III and IV. In general, manipulation of resistance types III, IV, and V is very difficult and their screening is expensive (Mesterhazy, 2003). Therefore, they are not used in current breeding programs. The number of genes, the types of resistance, and their different chromosomal locations clearly indicate the challenge of breeding wheat varieties for FHB resistance.

2.3.3. Genetic complications of resistance to FHB

Wheat plants can escape the infection of FHB by early or late heading. When most of the susceptible wheat plants are diseased in an FHB epidemic, some plants may remain healthy and free from any infection or symptom. The genotypes of these plants are susceptible, but their phenotypes are similar to resistant ones. This is a passive resistance referred to as apparent resistance. This may occur when an aggressive pathogen, susceptible plant, and favorable environmental conditions do not coincide (Kolb et al., 2002). Tolerance is another form of resistance where the susceptible wheat plant can produce a good yield despite an epidemic of FHB. This ability is genetically controlled but the mechanisms are not well understood. A pathogen can infect a wheat plant but the plant has the ability to resist the damage. As opposed to apparent resistance, true resistance is genetically controlled by resistance genes. The host-pathogen interaction may be a non-compatible (resistant) or a compatible (susceptible) one. The reason(s) for susceptibility may be the lack of recognition that is due to a lack of plant receptors (products of resistance genes) or pathogen elicitors (products of avirulence genes of the pathogen) (Flor, 1971). If both elicitor (*i.e.*, pathogen signal) and receptor exist, their binding brings about recognition and thereby triggers intra- and inter-cellular signal transduction pathways. Consequently, a non-compatible interaction between host and pathogen will result in recognition of the presence of the attacking pathogen by the plant. This will lead to preparations for defense, which will eventually lead to resistance (Wink, 1999). Signal transduction is the process by which environmental, developmental, and hormonal signals regulate cellular responses. In cases where either the pathogen lacks elicitor(s) and/or the plant lacks a corresponding receptor(s), the recognition of the pathogen by the host plant fails, as does the triggering of the cellular defense mechanisms leading to a compatible (susceptible) reaction and infection of the plant.

True resistance can be either vertical or horizontal. A plant genotype may be R (resistant) or r (susceptible). Pathogens may also be avirulent (A) or virulent (a) (Agrios, 2005). In this simple probability space, there are four pathosystem events: AR, Ar, aR and ar. Among these, only the AR gene combination is incompatible. The probability of occurrence of each

pathosystem in a natural population is related to the frequency of alleles involved in that stochastic event.

Vertical resistance is monogenic with the disadvantage of it being unstable (Agrios, 2005). Horizontal resistance is controlled by many resistance genes with small effects and is therefore durable. In horizontal resistance, all plant varieties have the same level of resistance (Agrios, 2005) and function equally well against most strains of the pathogen. However, working with horizontal resistance in field experiments is very difficult since the effect of each minor gene is usually very small and easily masked by the experimental error.

Quantitative resistance has the advantage of durability. However, analyzing this kind of resistance is too time and labor consuming. Unlike HR, quantitative resistance is not race specific. The most important key step in QTL (quantitative trait loci) analysis is discrimination of resistance. If QTLs are on different chromosomes, there is no linkage and their inheritance will be independent of each other. Otherwise, they may have a loose or tight linkage. Sometimes there is a mixture of independent and loosely or tightly linked QTLs in the same plant, which increases the complexity and makes the analysis of mixed QTLs more difficult (Fig. 2.2) (Xu, 1997). QTLs are highly influenced by environment and therefore spatiotemporal variance of the traits controlled by them is very high. QTL analysis requires using a series of both field and laboratory experiments followed by extensive statistical approaches. Relying on polymorphic molecular markers and linkage maps, QTL mapping comprises growing and evaluating large populations of plants and applying the appropriate statistical tools. Molecular markers such as RFLP and RAPD segregate as single genes and unlike QTLs, they seem unaffected by the environment. Furthermore, they are highly polymorphic, which implies that many QTLs can be mapped in a single cross. They enable breeders to develop high quality linkage maps (Kao et al., 1999).

The inheritance of FHB resistance in wheat is an example of quantitative resistance of mixed QTLs (Feredric et al., 1999; Waldron et al., 1999; Anderson et al., 2001; Kolb et al., 2001; Bai et al., 2003b; Somers et al., 2003). It contains different forms of passive resistance, apparent resistance (tolerance and escape), and true resistance which is expressed in five

different types. This makes resistance of wheat to FHB one of the most complex and unique types of plant resistance to a disease.

2.3.4. Sources of resistance

Chinese plant breeders made a significant contribution in identifying highly resistant wheat germplasm, such as Sumai3 and Ning7840, which have shown stable resistance to fungal spread in the spike across different environments (Bai et al., 2003a). In Canada, the Semiarid Prairie Agricultural Center (SPARC) labeled wheat cultivars as “very poor”, “poor”, “fair”, “good”, and “very good” for resistance to FHB. Most wheat cultivars that fall in the range of “poor” to “very poor” resistance to FHB mature in approximately 100 days. Some Canadian cultivars such as AC Barrie, AC Cora, Kapetawa, AC Majestic, and McKenzie have better tolerance to FHB (AAFC, 2004).

Despite the fact that increasing numbers of commercial cultivars now have some degree of resistance, most available cultivars of wheat with a high yield potential are still susceptible. Combining desirable agronomic traits and resistance to FHB is a challenge for breeders.

Spring wheat genotypes such as Sumai3, Nobeoka Bozu, and Frontana are some important universal sources of resistance against FHB (Mesterhazy, 2003). The breeding of winter wheat is more difficult than spring wheat and requires use of spring wheat sources or construction of more resistant germplasm from moderately resistant parents by gene pyramiding (Mesterhazy, 2003). It was hypothesized that there are one, two and three major resistance gene(s) in moderately susceptible to moderately resistant, resistant, and resistant to highly resistant wheat cultivars, respectively (Bai and Shaner, 1994). Resistance against FHB in Nobeoka Bozu and Wangshuibai has been reported to have 5-6 genes with additive dominant functions (Bai et al., 2003a).

There are hybrids of *T. aestivum* and other Triticaceae such as *Rogerun* and *Leymus* that have proved to be more resistant to FHB than Sumai3. Within some Triticaceae genera including *Hysterix, Agropyron* (*Elymus*), *Haymaldia* (*H. villosa*)*, Thinapyrum* (*T. intermedium*) and *Hordeum* (*H. californicum*) are species with FHB resistance (Mesterhazy, 2003).

Several QTLs have been identified for resistance to FHB (Feredric et al., 1999; Somers et al., 2003). In different varieties, the resistance QTLs have been observed on different chromosomes. Sumai3 resistance QTLs were reported on chromosomes 2A, 5A, 7A, 1B, 2B, 3B, 6B, and 7D (Weizhong, 1999). The importance of the D genome in conferring resistance in Sumai3 was negated by Gilbert et al. (2000) and Kolb et al. (2001) but emphasized by Ban and Watanabe (2001). In another study, the occurrence of three QTLs for FHB resistance located on 3BS, 2D, and 6B as together accounting for approximately 52 percent of the phenotypic variation was reported by Shen et al. (2003). A resistance QTL was detected on the chromosome 2DL of a F1-derived DH (double haploid) line from the cross *T. aestivum* 'Wuhan-1' × *T. aestivum* 'Maringa' (Somers et al., 2003). In this study, a QTL associated with DON level was also detected on the chromosome 2DS. A recent study has shown the involvement of 7 QTLs on chromosomes 2DS, 3AS, 3BS, 3BC, 4DL, 5AS, and 6BS in type I resistance and 4 QTL on chromosomes 2DS, 3BS, 6BS, and 7BL in type II resistance (Yang et al., 2005). The most widely used source of resistance is a QTL on the 3BS chromosome of Sumai3, which explains 15-60 percent of phenotypic variation in type II resistance (Somers et al., 2003; Zhou et al., 2003; Yang et al., 2005). In addition to 3BS, the Sumai3 also carries FHB resistance QTLs on the 5A and 6BS chromosomes. A summary of some important aspects of FHB resistance is listed as follows:

1) There is no strong evidence about race-specific resistance to FHB
2) DON contents produced in susceptible cultivars are greater than in resistant ones
3) Resistance in wheat varieties to FHB used in Canadian breeding programs can be grouped into three categories (Gilbert et al., 2000; Fedak et al., 2002)
    i) Spring wheat from Asia (Chinese cultivars such as Sumai3 and Ning derivatives along with the Japanese cultivar Nobeoka Bozu), European cultivars such as Ringo Star, Novokrumka, and Frontana, which have been used as a source of FHB resistance for winter wheat in Canada
    ii) CIMMYT wheat, such as CIMMYT-1, CIMMYT-11

iii) North American Breeding Lines

a - Older breeding lines with poor agronomic traits, such as FHB #21, and FHB #37

b - Newer breeding lines, such as AC Voyageur, Alsen, BW278, HY644, and Mcvey

4) To improve the selection of resistant wheat cultivars, a large genetic variance of the host population and a small genotype × environment (GE) interaction variance are required (Miedaner, 1997)

2.4. Metabolite profiling as a new approach to study resistance

2.4.1. Functional genomics

In the post-genomic era, there is an urgent need to assign functions to orphan genes in order to pyramid genes controlling suitable traits for crop improvement, such as resistance to plant diseases. Metabolite profiling is an approach through which a selected number of metabolites are identified and quantified (Fiehn, 2002). The task of functional genomics is analysis at the levels of gene expression (transcriptomics), protein translation (proteomics) including post-translational modifications, and the metabolic network (metabolomics), with the aim of defining the phenotype and bridging the genotype-to-phenotype gap (Goodacre et al., 2004). Metabolomics, the missing ring of the omic's chain and an evolving field of systems biology with its unique advantages, aims to decipher gene function at the metabolite level (Fiehn et al., 2000b; Dixon, 2001; Dixon et al., 2002; Sumner et al., 2003). Comprehensive identification and quantification of all the metabolites of an organism is a functional genomics methodology that can contribute to our understanding of the complex molecular interactions in biological systems (Bino et al., 2004). Metabolite profiling has, for instance, enabled the classification of two potato tuber systems grown either *in vitro* or in the soil (Roessner et al., 2001), yeast mutants (Allen et al., 2003), genetically modified organisms (Choi et al., 2003), salt-stressed tomatoes (Johnson et al., 2003), and resistance in plants against disease stress (Hamzehzarghani et al., 2005). Metabolite profiling when conducted along with mRNA and protein analysis can help to reveal the resistance mechanisms (Fiehn et al., 2000a). Therefore,

multi-parallel studies on genome, transcriptome, proteome, and metabolome are central to today's functional genomics (Weckwerth et al., 2004). The future is metabolic engineering of natural product pathways as a practical strategy for the enhancement of plant disease resistance (Kristensen et al., 2005).

Plants activate a network of metabolic pathways, rather than a linearly linked pathway following pathogen invasion (Barabasi and Oltvasi, 2004). The most important metabolites with antimicrobial and/or signal transduction properties that play a role in plant defense are derived from the phenylpropanoid, isoprenoid, alkaloid or fatty acid/polyketide pathways. Antimicrobial secondary resistance-related metabolites are classified into two main groups. Phytoalexins are the metabolites that are synthesized *de novo* and phytoanticipins are those that are formed before infection (Dixon, 2001).

Pathogen-inoculated wheat spikelets have been shown to induce disease response genes, mRNAs, proteins, and metabolites within a few hours after pathogen inoculation (Pritsch et al., 2000; Muthukrishnan et al., 2001). The expressed sequence tags (ESTs) from a cDNA library of spikes of Sumai3, a cultivar with resistance type II, when inoculated with *F. graminearum* has been reported to induce a variety of stress-related genes. These include genes encoding (pathogenesis-related) PR-1, PR-2 (β-1, 3-glucanase), PR-3 (chitinase), PR-5 (thaumatinlike-proteins), phenylpropanoid pathway enzymes, and oxygen metabolism enzymes. Genes encoding proteins implicated in pathogen recognition, signal transduction and induction of defense-related gene transcription such as MAP and protein kinases, transcription factors, and resistance gene analogues have been detected (Kruger et al., 2002).

A systemic acquired resistance (SAR)-like mechanism has been recognized in wheat. The application of salicylic acid (SA)-like metabolites such as benzo (l, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) has also been shown to induce SAR in wheat against powdery mildew (*Erysiphe graminis*) (Gorlach et al., 1996). SAR is associated with the accumulation of SA and expression of the PR genes (Ryals et al., 1996). The *Arabidopsis* NPR1 (AtNPR1) gene, a key regulator of SAR, was shown to increase the susceptibility or resistance to a variety of pathogens after undergoing loss-of-function or overexpression mutations, respectively (Durrant and Dong, 2004).

The enhanced disease resistance by the overexpression of AtNPR1 was related to the faster response of the plant to SA and BTH. The translocation of NPR1 into the nucleus and its interaction with the TGA proteins is vital for the expression of the PR1 gene and is central in governing disease resistance (Durrant and Dong, 2004). Makandar et al. (2006) demonstrated that the expression of the AtNPR1 transcript, which regulates the activation of SAR in wheat, conferred a heritable, type II resistance to FHB without any change in grain yield (Makandar et al., 2006). FHB resistance-related transcripts, proteins, and metabolites in wheat, resulting in the detection of disease response genes and PR proteins that are induced following pathogen inoculation, have been documented (Li et al., 1999; Pritsch et al., 2000; Siranidu et al., 2002). The specific activity of guaiacol-peroxidase and polyphenol oxidase was reported to be significantly greater in resistant wheat cultivars during the milk stage as compared to non-inoculated control plants (Mohammadi and Kazemi, 2002).

In non-stressed wheat plants, there is no thickening of the cell wall or any phenolic substance production to limit the pathogen during the first 10 days of pollination (Bushnell et al., 2003). Flavonoid metabolites protect the testa against fungal invasion. Soluble phenolic acids like ferulic acid and *p*-coumaric acid accumulate in spikes during testa differentiation. Within 10 days after anthesis, the amount of *p*-coumaric acid accumulates to about 10 mg/100 kernels, a level that is enough to inhibit the fungal growth *in vitro*. The phenolic substances may contribute to FHB resistance in the testa by binding to the cell wall and improving its physical strength. The majority of phenolic metabolites are synthesized in the aleuron layer, which is formed 12-25 days after anthesis and added to the amounts accumulated during testa development (Bushnell et al., 2003).

Several metabolites have been related to resistance of wheat cultivars against disease stresses. The accumulation of phenylpropanoid metabolites and phenolic acids also occurs after the infection of wheat by other pathogens (Southerton and Deverall, 1990; Kofalvi and Nassuth, 1995; Okazaki et al., 2004). The total phenolic acids in wheat have a positive correlation with wheat resistance to diseases including rust (unidentified), Karnal bunt (*Neovossia indica*) and Take-all (*Gaeumannomyces graminis* var. *tritici*) (Gogoi et al.,

2001). Higher concentrations of free phenolic metabolites have been found in the resistant wheat cultivar Frontana as compared to the susceptible cultivar Argent when inoculated with *F. graminearum* (Siranidou et al., 2002). Mycelial growth inhibition of *F. graminearum* by phenolics such as *p*-coumaric and ferulic acids has been reported in a number of studies (Smart and Flores, 1997; Russel et al., 1999; McKeen et al., 1999). A significant increase occurred in the levels of these phenolics in the glumes of a resistant wheat cultivar after inoculation with *F. graminearum* (Siranidou et al., 2002). The common phenolic acids in wheat are cell-bound and in the form of O-[5–O-(*trans*-feruloyl)-α-L-arabinofuranosryl]-(1►3) –O– β – B – xylopyranosyl-(1►4) -D-xylo pyanose (FAXX). The Russian aphid-wheat caused a significant increase in SA level in wheat 96 hours after infestation (Mohase and van der Westhuizen, 2002). Benzoic acid, another PAL pathway metabolite and the immediate precursor to SA and its derivatives, also possesses antifungal activity (Bénigne-Ernest et al., 2002).

2.4.1.1. Technological platform

The success of any breeding program depends on the screening methodologies. In almost all wheat breeding programs up to now, variables such as disease severity, DON content, and FDK have been used for evaluation of resistance to FHB. Due to the complexity of the genetics of *Fusarium* resistance, a higher screening capacity is required in order to accelerate a breeding program (see pages 5-6).

The advent of new technologies is an opportunity for breeders to adopt and integrate new tools into their conventional methods to enhance the efficiency of breeding. Enormous advances over the last few years in developing new tools to study genetic diversity in plants show that metabolite profiling is a way that will enable breeders to develop a comprehensive phenotyping of genetically different plants (Roessner et al., 2001). The goal of a metabolomic profiling experiment is to quantify all the metabolites in one snapshot of a cellular system that represents the cell or tissue in a given state at a given point in time (Goodacre et al., 2004).

Analytical technologies for metabolite profiling vary according to their selectivity, speed, and sensitivity. The ability of a technique to separate a

desired analyte/signal in a mixture without interference from other analytes is referred to as the selectivity. The sensitivity is defined as the ability of a tool to detect a low signal level. Some tools have rapidity but lack sensitivity and selectivity, such as NMR. Some other tools such as mass spectrometry methods, including LC/MS and GC/MS, are good in both selectivity and sensitivity but need a relatively longer time for analysis (Sumner et al., 2003). Besides a high efficiency in separating complex biological matrices, GC/MS is a relatively inexpensive method. However, the problem is that it can only analyze volatile metabolites. It is possible to analyze semi to non-volatile metabolites, but they must be volatilized through chemical derivatization.

In GC/MS the components of a mixture are separated while traveling in an inert carrier gas (such as helium) through a capillary column in a heated oven with a ramping temperature based on their boiling points and affinities to a stationary phase that covers the inner surface of the column. The next step is the bombardment of the gaseous form of the metabolites in a hot evacuated chamber by a beam of electrons possessing energy sufficient to break chemical bonds and produce ionized fragments with different mass/charge ratios. In other words, the energy of electrons (70eV) is high enough to cause both fragmentation and ionization. After mass ions are produced, they are separated by means of a device called a mass analyzer or mass spectrometer (MS) according to their mass/charge ratios. A quadrupole, an ion trap, and a time of flight (TOF) mass spectrometer are well known mass analyzers (Mellon et al., 2000). A Quadrupole MS is two pairs of parallel metallic rods, each opposite pair with the same polarity while adjacent pairs with the opposite polarity. These polarities are created by connecting them to a DC voltage source. Another RF voltage is also used at the same time to scan for selected ions. Varying RF voltage will create various RF magnetic fields in time that will allow only selected mass ions (with certain m/z ratio) to pass through. Other mass ions, whose m/z ratios are more or less than the m/z ratio of these mass ions, do not have a stable oscillatory trajectory in the Quadrupole and so are rejected. As a result, the mass ions with the same mass/charge ratio leave the analyzer at the same time, which are eventually counted by a detector. In the ion trap MS both ionization and mass analysis occur in the same place. The electrons emitted by a filament ionize the

molecules of the sample and create the mass ions, which are then scanned over a range between 20 and 650 Daltons. The disadvantage of the ion trap MS is that the dynamic range has a low “upper limit” of 650 Daltons and that it cannot accept large samples.

In the TOF-MS, the individually charged particles subjected to a potential difference V attain the same energy (eV) and therefore the lighter particles have a shorter TOF over a given distance. The accelerated particles are passed into a field-free region where they are separated in time by their m/z values and counted by a detector. Faster electronics are required for adequate resolution because the arrival time of successive ions can be very short (less than 10^{-7} second) (Mellon et al., 2000). At the end, a detector will count the number of each mass ion at any given time and record it to a computer. A detector is a conductive surface where, after collision of mass ions with this surface, a certain electric current is generated. The ion current is saved on a computer. The computer is previously calibrated and therefore it knows which mass ion generates which current and so determines which mass ion exists in that certain moment of time.

LC/MS can analyze the nonvolatile metabolites directly after extraction and there is no need for derivatization (Kiston et al., 1996; Mellon et al., 2000; Sumner et al., 2003). However, it lacks a mass spectral library and thus its application in non-target analysis is limited.

2.4.1.2. Software platform

Unlike the significant progress in data acquisition, metabolomics has devoted less effort to advance methods of data extraction, visualization, and interpretation. Mass spectrometry relies on ion extraction and the use of retention times and mass spectra of peaks to find the best targets that fits a metabolite, a process called library matching for metabolite identification (Duran et al., 2003). If the sample is clean and there are pure standards, this will work well, but in most cases, many biological samples are far from pure. Before tentative identification (library matching) and quantification of individual peaks, there are some problems to be resolved. The migration of the metabolites through a column with similar speeds raises the problem of "overlapping" peaks or "co-eluting" metabolites. Perhaps one of the biggest

challenges in dealing with complex samples is to how quickly and accurately separate target signals from background noise. Both identification and quantification of metabolites are slowed down by two kinds of noises. The first is random noise originating from instruments and the second is matrix-specific noise coming from the sample (Dromey et al., 1976; Stein, 1999). The subtraction of known patterns of chemical noise (stored in digital files) from the sample signal (matrix subtraction) commonly referred to as baseline correction can improve the separation of target signals from background noise.

Commercially available software packages to complete automated, rapid, and flexible reduction of large complex chromatographic/spectrometric data sets are just becoming available. They will assist researchers studying metabolomics to generate well-organized, two-dimensional data matrices and analyze the GC outputs using statistical software such as SAS and MATLAB (Stein, 1999; Tolstikov et al., 2003; Duran et al., 2003; Draper et al., 2004; Broeckling et al., 2006). MSFACTs was one of the first software packages developed to assist the automated import, reformatting, alignment, and export of large chromatographic data sets. This enables a more rapid visualization and interpretation of metabolomic data. It aligns integrated chromatographic peak lists and extracts information from raw chromatographic ASCII formatted data files (Duran et al., 2003).

MetAlign is a tool designed for non-targeted data analysis by mass spectral fragment detection and alignment across treatments and replicates. It corrects baseline noise and aligns retention time of mass spectral fragments through multiple iterations of calculations/corrections of shifts in GC/MS profiles. Elimination of background noise makes deconvolution and identification of peaks/components an easier task.

The Automated Mass Spectral Deconvolution and Identification System (AMDIS) (Stein, 1999) is one of the best software packages. It was developed by the National Institute of Standards and Technology (NIST) (Tolstikov et al., 2003). The software can read most data file formats and perform mass spectral “clean up” through mass spectral deconvolution prior to library searching. In addition, user library creation is simple and spectra can be searched against the NIST or any other user-defined database. AMDIS

performs noise analysis, component perception, and spectral deconvolution, extracting pure component spectra and related information stored in different output files such as “elu” and “fin” file formats (Stein, 1999).

Although most of the software packages perform qualitative data processing including alignment and deconvolution of overlapping peaks, they do not provide quantitative data. MET-IDEA (Metabolomics Ion-based Data Extraction Algorithm) is a new tool of choice that has revolutionized the application of simple basic algorithms to extract ion abundance data related to separate or co-eluting metabolite peaks in complex GC/MS data sets. The MET-IDEA software imports raw file in “net.cdf" format and requires an input list of a series of ion/retention times (IRt). An IRt list can be manually-generated and edited within MET-IDEA. It can also be imported in a tab-delimited text format from metabolite databases or extracted from AMDIS output (“elu” and “fin”) files. MET-IDEA software processes one representative TIC from a data folder and extracts the list of retention times and ion markers (IRt) for each component. The generated ion list is saved as an “ion” file, and subsequently the software continues to the calibration step. It scans the data folder and recognizes all “net.cdf” files (samples). The MET-IDEA software applies directed extraction of ion intensity values based on a list of IRt values and writes the results to a single tab-delimited text file with metabolite identifiers as column headings and sample names as rows with a user-defined filename (Broeckling et al., 2006).

2.4.1.3. Data mining

Data analysis techniques may be classified into two main groups (supervised and unsupervised) that can be predictive and/or descriptive. The majority of metabolomics data analyses are based on “unsupervised” techniques with the goal of pattern-recognition. Unsupervised methods perform the job of clustering while supervised ones classify the data sets. The fundamental difference between clustering and classification is that in the clustering the data points are unlabelled, assuming no prior knowledge of the previous grouping of samples. In classification, the data points have “tags” or there are pre-defined groups. For example, hierarchical clustering arranges unlabelled data points (samples) from an experiment in a way such that the

more similar samples cluster tightly together. In contrast, in a classification method, such as support vector machines (SVM) or artificial neural networks (Caudill and Butler, 1992), a training set (a portion of data or a different data set) is used to discover the pattern. The algorithm will learn to classify the labeled data into preset categories (Goodacre et al., 2004).

2.4.1.3.1. Supervised methods

Discriminant analysis (DA) and partial least square regression analysis are examples of supervised modeling techniques that can be both exploratory and explanatory (Goodacre et al., 2004). DA is a multivariate statistical technique used to construct a predictive/descriptive model to classify each observation into one of the groups based on observed predictor variables. In DA, multiple quantitative entries (measured variables such as metabolites) are used to discriminate single classification variables (such as a cultivar). DA is used to study group differences through identification of significant discriminating variables to differentiate groups and classify new observations into pre-existing groups (Johnson, 1998; Johnson and Wichern, 2002; Goodacre et al., 2004).

2.4.1.3.2. Unsupervised methods

The unsupervised methods such as hierarchical cluster analysis (HCA), principal component analysis (PCA), factor analysis (FA), and canonical discriminant analysis (CDA) are far more useful than supervised ones (Goodacre et al., 2004). Cluster analysis is a completely unsupervised method that measures similarity between two clusters or observations and then assigns the observation to the cluster to which it has more similarity (Johnson, 1998). A data matrix, such as a set of metabolites, is used to classify observations based on their metabolic profiles by doing HCA to investigate differences and/or similarities in the metabolite profiles between the genotypes and the treatments. The Euclidean distance between group centers is used to construct a similarity measure and a dendrogram is developed using the Cluster procedure of SAS.

PCA and FA reduce a set of correlated variables (metabolic profiles) into a small number of hidden orthogonal (uncorrelated) factors. The principal

assumption of factor/principal component analyses is that there exist a number of latent variables (factors/PCs) accounting for the correlations among observed variables. Variation in all correlated measured response variables (metabolites), accounted for by the same latent factor/PC, is summarized in the factor in as much as the initial data can be represented using a few new uncorrelated variables (factors/PCs) (Johnson, 1998; Johnson and Wichern, 2002).

Canonical discriminant analysis is a dimension-reduction technique similar to PCA. CDA develops linear combinations of the measured variables (metabolites) or so called CAN-vectors summarizing between-class variation. It increases the resolution of the clustering pattern by minimizing within-cluster variance and maximizing the between cluster variance (Johnson, 1998). The CANDISC procedure of SAS can be used to compute squared Mahalanobis distances (distances in variance scale) among samples (cultivars for example) means. The values of canonical variables are used to plot all samples in a canonical 3D-space of three CAN-vectors, to aid in the visual interpretation of group differences/similarities. The loading of a measured variable (metabolite) to each CAN-vector is used to explain its influence on grouping criteria (such as hidden functions of host-pathogen interaction).

Figure 2.1. Wheat inflorescence: 1-2=spike, 3=spikelet, 4=glume, 5=lemma, 6=palea, 7=lodicules, 8-10=appearance of pistil, 11=pollen on stigma branch, 12=cross-section of a spikelet (modified from Hyene, 1987).

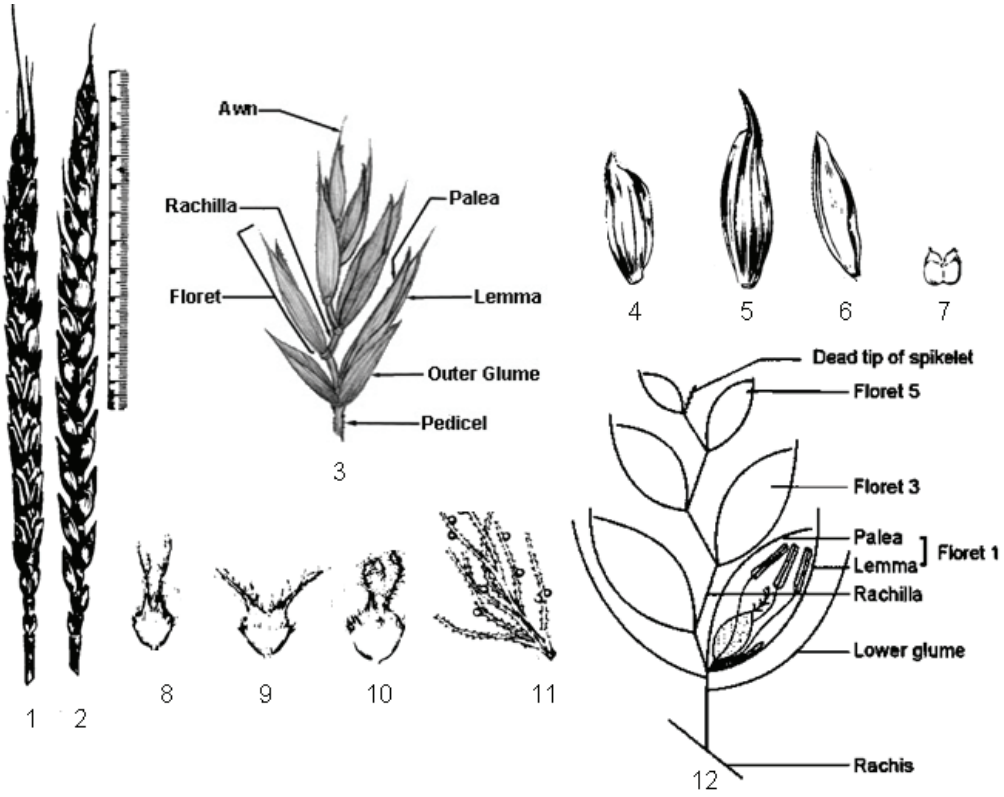
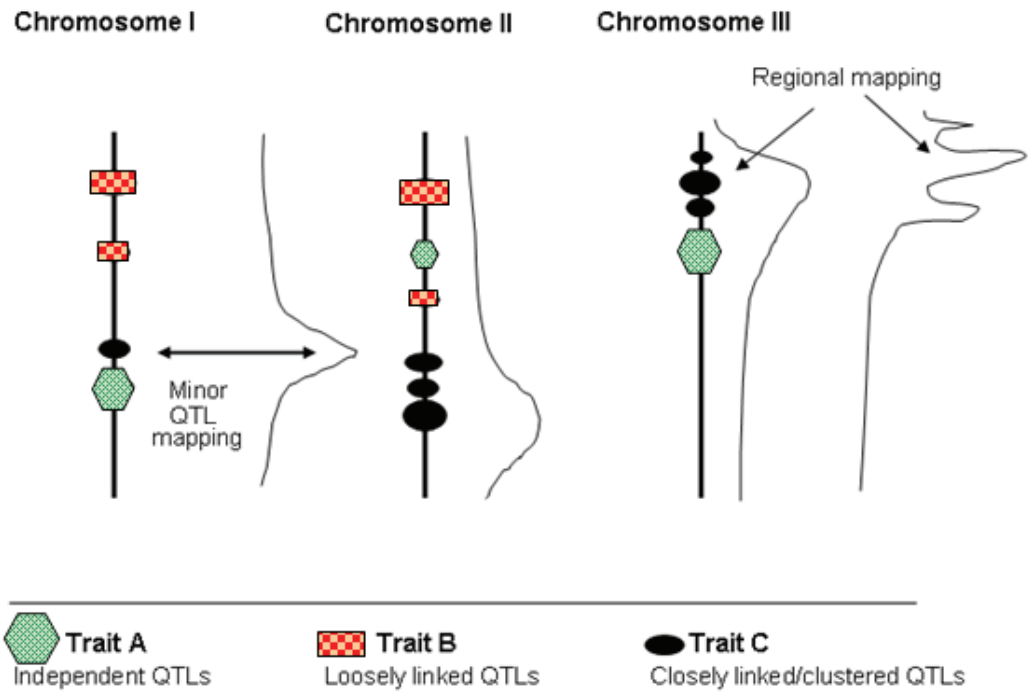

Awn
Rachilla
Palea
Floret
Lemma
Outer Glume
Pedicel
3
4
5
6
7
1
2
8
9
10
11
Dead tip of spikelet
Floret 5
Floret 3
Palea
Lemma
Floret 1
Rachilla
Lower glume
Rachis
12

Figure 2.2. Different kinds of quantitative resistance loci (trait A, independent QTL, trait B, loosely linked QTL, trait C, tightly linked or clustered QTL) (modified from Xu, 1997).


Chromosome I
Chromosome II
Chromosome III
Regional mapping
Minor
QTL
mapping
Trait A
Independent QTLs
Trait B
Loosely linked QTLs
Trait C
Closely linked/clustered QTLs

Chapter 3

Metabolite profiling and factor analysis to discriminate quantitative resistance in wheat cultivars against fusarium head blight

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Preface to Chapter 3

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The results of this work, in parts, authored by Hamzehzarghani, H., Kushalappa A.C., Dion Y., Rioux S., Comeau A., Yaylayan V., Marshall W.D., and Mather D.E. were also presented as posters with abstracts at the 3rd Canadian Workshop on Fusarium Head Blight (2003) and 2nd International Symposium on Fusarium Head Blight Incorporating the 8th European Fusarium Seminar (2004).

Hypothetically, any genetic change at the plant cell level can lead to a detectable change in the cell metabolite profile. There are many examples of using metabolite profiling for discriminating plant systems. Metabolite profiling has been used to identify blackcurrant (*Ribes nigrum* L.) genotypes resistant to gall mite (*Cecidophyopsis ribis* Westw.) (Brennan et al., 1992). Transgenic and non-transgenic alfalfa cultivars have been differentiated using principle component analysis of their HPLC chromatograms (Chen et al., 2003). Roessner and co-workers used GC/MS to identify differences between potato tubers grown *in vitro* or in soil (Roessner et al., 2000). The same technique was used to distinguish four distinct genetically modified potato genotypes (Roessner et al., 2001). Similarly, control and salt-treated tomatoes of two varieties were differentiated on the basis of their metabolic fingerprints (Johnson et al., 2003). Metabolic profiles of potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* have been studied by means of GC-electron ionization-time of flight mass spectrometry (GC/EI-TOF) (Wagner et al., 2003). Diseases of potato tubers were discriminated using volatile fingerprinting and modeling with the aid of a neural network (Kushalappa et al., 2002). Fiehn et al. (2000b) were able to identify some new

uncommon plant metabolites in *Arabidopsis* using gas chromatography low-resolution mass spectrometry (MS).

The reproducibility of retention time and the ability to identify metabolites in a complex mixture have led Fiehn's analytical methods (Fiehn et al., 2000a) to be used in a number of other studies.

In wheat, the role of phenolic metabolites in the plant's resistance to FHB has been revealed through comparative HPLC studies of different susceptible and resistant cultivars' cell wall-bound phenolic metabolites isolated from glumes, lemmas and paleas (Siranidou et al., 2002). It is plausible to hypothesize that each wheat cultivar has a different pre- and post-inoculation metabolite profile that could be distinguished using GC/MS technology. This technique could be used in discriminating between FHB-susceptible and resistant wheat cultivars. Consequently, the objective of this study was to detect metabolites produced in the wheat-*F.graminearum* pathosystem and to distinguish susceptible and resistant wheat cultivars based on their respective metabolite profiles.

3.1. Abstract

Metabolic profiles of spikelets of the wheat cultivars, Roblin and Sumai3, the former being susceptible and the latter being resistant to fusarium head blight (FHB), were analyzed using GC/MS to develop a technology to discriminate FHB resistance. Over 700 peaks were detected and 55 metabolites were tentatively identified. The abundance of 49 of these metabolites increased following pathogen inoculation, including 23 in Roblin and 26 in Sumai3. However, only five metabolites were significantly different between both cultivars and inoculations. The metabolite *m*-hydroxycinnamic acid was detected in all four treatments. It was six-fold more abundant in Sumai3 following pathogen inoculation, with no corresponding change in Roblin. The level of myo-inositol in Sumai3 was greater than in Roblin, and increased in both following pathogen inoculation. The metabolites common to all treatments were subjected to factor analysis to identify, based on significant factor loadings, groups of metabolites associated with susceptibility or resistance to FHB. Pathogen inoculation of the resistant cultivar Sumai3 was associated with the highest scores for the first and second factors, which therefore can be used to screen for resistance to FHB. The first factor was associated with a higher abundance of several fatty acids and aromatic metabolites, while the second factor was associated with metabolites such as *p*- and *m*-coumaric acids, myo-inositol and other sugars, and malonic acid. Pathogen inoculation treatments outscored water-inoculated controls for the third factor, with the susceptible cultivar Roblin receiving the highest scores. Consequently, the third factor may be useful in explaining susceptibility/ pathogenesis. The third factor had positive correlation with metabolites from different groups, mostly amino acids, fatty acids, and aromatics. The possible roles of the various metabolites detected in plant defense against pathogen-stress, their metabolic pathways of synthesis, and their potential application in screening wheat cultivars for resistance to FHB are discussed

Keywords: Factor analysis, functional genomics, *Fusarium graminearum*, *Gibberella zeae*, horizontal resistance metabolomics, *Triticum aestivum*, Wheat scab

3.2. Introduction

Fusarium head blight (FHB; scab), caused by *Fusarium graminearum* is ranked as the most important disease of wheat in North America (Comeau, 1999). Under warm and humid conditions, it can cause severe losses in yield and it can significantly reduce grain quality by producing a wide range of mycotoxins (Bai and Shaner, 1994; Parry et al., 1995). Control of the disease by chemical, cultural or biological methods is very difficult (Liu and Wang, 1991; McMullen et al., 1997a; Fernando, 1999) and host resistance is considered the most promising method (Gilbert et al., 2000). The nature of FHB resistance in wheat is considered either passive (associated with phenotypic traits) or active (associated with reduction of pathogen development or quantitative resistance) (Mesterhazy, 1995). The mechanisms of wheat's resistance to FHB are not well understood, but quantitative trait loci associated with resistance have been identified (Bai and Shaner, 1994; Kolb et al., 2001). Resistance to FHB is classified into five different types (Hammond-Kosack and Jones, 1996): type I = resistance to initial infection; type II=resistance to spread of infection in the spike; type III=resistance to kernel infection; type IV=tolerance and type V=resistance to mycotoxin accumulation. The first two types have received the most research attention (Schroeder and Christensen, 1963; Mesterhazy, 1995).

Wheat cultivars with improved resistance to FHB have been developed based on selection for low disease severity, often using simple disease ratings and without prior knowledge of mechanisms involved in resistance (Fedak et al., 2002). Thorough assessment of quantitative resistance parameters for FHB (e.g. infection efficiency, latent period, sporulation, disease progress over time, etc.) in large breeding populations is useful in further improving resistance and in advancing the understanding of resistance mechanisms, but will be prohibitively costly to conduct in greenhouse or in field environments across seasons and regions. Furthermore, breeders are looking for mechanisms to pyramid disease resistance genes into an elite cultivar. Resistance screening methods that provide an understanding of the disease resistance mechanisms are desirable to incorporate quantitative disease resistance genes into cultivars.

A few studies have been conducted to detect FHB resistance-related transcripts, proteins and metabolites in wheat. Several disease response genes and pathogenesis-related (PR) proteins induced because of pathogen inoculation have been identified (Pritsch et al., 2000; Muthukrishnan et al., 2001). A greater abundance of free phenolic metabolites (especially *p*-coumaric acid) has been detected in the glumes, lemmas and paleas of the resistant wheat cultivar Frontana as compared to the susceptible cultivar Argent, when inoculated with *F. graminearum*, (Siranidou et al., 2002). Under *in vitro* conditions, *p*-coumaric and ferulic acids were observed to have synergistic effects in inhibiting mycelial growth of the two isolates of *F. graminearum* (Siranidou et al., 2002).

A comprehensive metabolite profiling study targeted at the discrimination of disease resistance has yet to be reported for wheat, or any other plant species. Here, we hypothesize that wheat cultivars that differ in resistance to FHB will also vary in their metabolic profiles inherently and/or in their early response (24 h) to inoculation with *F. graminearum*. The objectives of this study were to develop a technology to profile metabolites of wheat spikelets, with and without pathogen stress, and to identify metabolic criteria that might be applied to differentiate FHB resistance in wheat.

3.3. Materials and methods

3.3.1. Plant and pathogen production

The seeds of spring wheat cultivars Roblin and Sumai3, the former susceptible (Fedak et al., 2002) and the latter exhibiting a high level of type II resistance to FHB (Liu and Wang, 1991; Shen et al., 2003), were obtained from the Centre de recherché sur les grains (CEROM) and Agriculture and Agri-Food Canada (AAFC). Wheat was grown from seed in 15 cm pots and maintained in a greenhouse at 22 ± 3^{o}C. At tillering (GS 25) and booting (GS 40) plants were fertilized with 100 ml of a 0.2 percent solution of 20-20-20 NPK (Zadoks et al., 1974).

Seven day old cultures of *Fusarium graminearum* (isolate 99-15-35) (obtained from Dr. S. Rioux, CEROM, QC) were inundated with water and filtered through two layers of cheesecloth. Spore suspensions were made in

aqueous solutions of 0.02 percent Tween 80. The spore concentration was adjusted to 10^5 macroconidia ml^{-1}.

3.3.2. Inoculation and incubation

At anthesis (GS=60-69) (Zadoks et al., 1974) the spikes were area-source inoculated by placing 10 µl of the macroconidial suspension into the middle floret of each of four spikelets located roughly at mid-spike. To evaluate disease severity, a single spikelet located roughly at mid-spike was inoculated (Gary et al., 2000). Each sample (*i.e.*, experimental unit) consisted of 16 spikelets, made up of four replicate sets of four spikelets, each set being represented by a single plant's spike. Spikelets inoculated with 10µl of distilled water containing 0.02 percent Tween 80 served as controls. After inoculation, the plants were covered with plastic bags and sprayed inside with water to provide a saturated atmosphere. The plants were kept in a greenhouse maintained at 20 ± 3 ^{o}C. The plastic bags were removed after 24 h.

3.3.3. Disease severity assessment

Spikelets with FHB symptoms were monitored after inoculation, at 4 d intervals, until 20 days after inoculation (dai). A spikelet showing discoloration, necrosis or visible mycelia was considered diseased. The FHB severity (*y*) was assessed as the number of diseased spikelets in a spike. Several FHB severity (y) values over time were used to construct a disease progress curve. The disease severity over time was subsequently reduced to a single value by calculating the area under the disease progress curve (AUDPC) (Franc and Nether, 1997; Shaner and Finney, 1977):

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

where

i is the number of the individual observation (*i* = 1 – *n*)

n is the total number of observations.

t is time after inoculation (days),

y is the FHB severity,

3.3.4. Metabolite extraction and GC/MS analysis

Sets of 16 inoculated spikelets, 4 inoculated spikelets from each of the four spikes, were harvested at 24 hai, and served as sampling units for metabolite extraction and analysis. Immediately after harvesting, samples were crushed in liquid nitrogen to suppress hydrolytic activity. Metabolites were extracted following the methods developed by Fiehn et al. (Fiehn et al., 2000a, b), with some minor modifications. The metabolites from the ground spikelet samples (300 mg) were first extracted in a mixture of methanol and distilled water (28:1, v/v), centrifuged at 12000 g for 5 min, the supernatant decanted and 1.4 ml of distilled water added. Chloroform was added to each pellet, and this was vigorously resuspended and mixed. The resultant mixture was centrifuged at 12000 g for 5 min, and the supernatant added to the water-methanol fraction. The two fractions (water-methanol and chloroform) were separated using centrifugal fractionation at 3500 g for 15 min. From the top portion, 1ml of the methanol-water fraction was removed, concentrated five-fold using a SpeedVac concentrator, and then freeze-dried. To 1 ml of the chloroform fraction, 1 ml of 3 percent v/v H_2SO_4 in methanol was added to transmethylate fatty acids and lipids. The chloroform fraction was washed 3 times with distilled water, dried by adding anhydrous Na_2SO_4, and concentrated by means of a SpeedVac concentrator. Since most metabolites were non-volatile, both fractions were separately derivatized by adding N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) to render them volatile at GC oven temperatures. Methoxyamine hydrochloride in pyridine was added to the dried residue (methoximation) because direct derivatization of sugars such as fructose causes confusing peaks related to hexoses (Roessner et al., 2000).

A 1 µl aliquots of the methanol-water and chloroform fractions of the spikelet extract were independently injected into the port of a GC/MS (GC 3400xc with Voyager® ion trap mass analyzer; Varian®, QC, Canada) equipped with an autosampler. The injection port temperature was maintained at 230 ^{o}C. A capillary DB-5MS column (0.25 µm film thickness, 0.25 mm inner diameter and 30 m long, Supelco, Canada) was used with helium as a carrier gas (flow rate of 1 ml min^{-1}). The oven temperature was programmed to remain at 70^{o}C for 5 min, followed by a 5 ^{o}C min^{-1} rise in temperature up to

280 ^{o}C, and a further 3 min at 280 ^{o}C. The gas was ionized and the abundance of mass ions ranging from 45 to 600 *m/z* was determined using an ion trap mass analyzer. The mass ions were scanned at the rate of one spectrum s^{-1}. For each scan, the GC/MS output consisted of scans and abundance for ions 46-600 *m/z*.

3.3.5. Metabolite identification and quantification

The GC/MS outputs on scans and mass abundance were processed using Saturn Lab software. The metabolites were identified using a NIST library mass spectrum search program (version 1.6). For each peak, the consistency of major fragments of the spectrum across the four blocks (replicates) was manually investigated, using the Pivot Table feature of Microsoft Excel, sorting the data based on the retention time. The observed spectra of each peak for different blocks were compared with the ten topmost choices in NIST to construct the identity of a metabolite using the retention time as a reference. Only the metabolites that had detectable peaks in all the four blocks, of at least one treatment, were considered for further analysis. The peaks, especially those with low abundance, were inconsistent across blocks, as they were close to the noise level. In the table of metabolites automatically generated by the Saturn software, when peaks were not detected in all blocks of a treatment, the corresponding retention time regions of the peaks in the chromatograms were inspected for all blocks. When peaks, similar in spectra, were detected across all the four blocks, the identities and abundance of metabolites were determined using the automated component table builder of the Saturn lab software. When no suitable match in the NIST hits was found for a certain spectrum of a peak, the peak was designated as unidentified, and its first five most abundant mass ions were recorded in a descending order of abundance. Compounds that occurred only in one (unique), 2 or 3, but not in all four treatments SW, SP, RW and RP (Sumai3/Roblin-Water/Pathogen-inoculated, respectively) were considered as discriminatory.

3.3.6. Experimental design and statistical analysis

The experiment was designed as a factorial design randomized in complete blocks, with four treatments: two cultivars, Roblin and Sumai3, combined with two inoculations, - with water (control) or with the pathogen. The evaluations were conducted on four different time occasions - therefore four time blocks. The experimental unit for metabolite profiling consisted of a pooled sample of 16 inoculated-spikelets harvested 24 h after inoculation. In addition, four central spikelets of each of the four spikes from four plants of each cultivar were inoculated with the pathogen and were used for disease severity assessment. The FHB symptoms on these spikelets were assessed non-destructively over a period of 20 d after inoculation to construct disease progress curves.

Disease severity and AUDPC were subjected to ANOVA, using SAS (Khattree and Naik, 2000). The data on metabolic profiles, which consisted of several metabolites (55 that were consistent among replicates) and their relative abundance (the ion-trap mass analyzer output from the GC/MS analyses) were subjected to a univariate analysis of variance using SAS (Khattree and Naik, 2000), to identify metabolites with significant ($P \leq 0.05$) differences between cultivars and between inoculations. The metabolites that were significantly induced in their abundance following pathogen inoculation, and metabolites deemed novel based on univariate ANOVA, were designated as pathogenesis-related (PR) metabolites (SP>SW and RP>RW). The level of metabolites that occurred in all the treatments (48 metabolites, 31 from methanol-water and 17 from chloroform extracts of the spikelets) were subjected to a factor analysis following the FACTOR procedure, using the principal components method, implemented in SAS (Johnson, 1998; Johnson and Wichern, 2002; Khattree and Naik, 2000). The contribution of each metabolite to a treatment and the relationships among treatments were investigated using the factor loadings of the metabolites and factor scores of the treatments, respectively. The factor scores explained the spatial location of treatments, indicating the relationships between treatments where a positive factor score was associated with a positive factor loading for metabolites. The values of the factor scores increased with an increase in the abundance of the metabolites that loaded to a given factor.

3.4. Results

3.4.1. Disease progress

Fusarium head blight symptoms developed in all the pathogen-inoculated spikes, but not in all the pathogen-inoculated spikelets. Within a spike, the infection spread from the pathogen-inoculated spikelets to non-inoculated spikelets in the susceptible cultivar Roblin but not in the resistant cultivar Sumai3. This indicated that the latter cultivar expressed type II resistance. The number diseased spikelets (y) at 4, 8, 12, 16 and 20 dai were 0, 0.25, 0.50, 0.75 and 0.75 in Sumai3 and 1.25, 2.00, 3.00, 7.00 and 8.00 in Roblin, respectively. A value of y>1.0 means the infection had spread to spikelets beyond the inoculated central spikelet. The area under the disease progress curve (AUDPC) was 7.50 in Sumai3 and 39.00 in Roblin, implying that Sumai3 was more resistant to FHB than Roblin.

3.4.2. Metabolic profiles

3.4.2.1. Metabolites of wheat-FHB system

More than 700 peaks were detected in the wheat-FHB pathosystem, out of which 55 metabolites were tentatively identified, including 38 and 17 metabolites from the methanol-water (hydrophilic) and chloroform (lipophilic) fractions of plant extracts, respectively (Table 3.1). Of 55 metabolites detected, 48 metabolites were common to all treatments. Among the seven treatment-discriminatory metabolites only arabinoic acid-gamma lactone; ribofuranose; and D-ribose were unique to Sumai3, while the remaining four were common to ≤3 treatments. Only 11 and 9 metabolites were significantly ($P \leq 0.05$) different between cultivars and between inoculations, respectively, including five metabolites that were significantly ($P \leq 0.05$) different both between cultivars and between inoculants (Table 3.1). The abundance of *m*-cinnamic acid was highly significant ($P \leq 0.01$) both between cultivars and between inoculations. The metabolites detected belonged to diverse functional groups such as fatty acids, sugars, aromatics, amino acids, and phenolics (Table 3.2).

3.4.2.2. Factor analysis of metabolites

The 48 metabolites common to all treatments, including both the methanol-water and the chloroform fractions (Table 3.1), were subjected to factor analysis. The first three factors accounted for virtually all (F1=39%; F2=36%; F3=25%) the variance in the selection of metabolites with significant factor loadings (Table 3.1). Factor scores, showing their association among treatments, are presented in Fig. 3.1, while the factor loadings of metabolites contributing to factor scores are shown in Table 3.1. The metabolites with significant factor loadings to each of the first three factors are listed in Fig. 3.1 (see caption). A three-dimensional scatter plot (F1 × F2 × F3) of treatments' factor scores indicated a clustering of treatments.

3.4.2.3. Wheat cultivar-specific metabolites

Among the 55 metabolites identified, 54 and 49 were detected in water-inoculated Sumai3 (SW) and Roblin (RW) cultivars, respectively (Table 3.1). This included 11 metabolites, which varied significantly between cultivars, irrespective of inoculations. Of the 24 metabolites identified in water-inoculated spikes, only two, L-proline and butanedioic acid (= Malic acid), were at significantly higher levels in Roblin than in Sumai3. Conversely, only 8 metabolites were significantly higher in water-inoculated Sumai3 than in Roblin: butandioic acid; *m*-cinnamic acid; myo-inositol; D-fructose; 1,2,3-propanetricarboxylic acid; D-fructose RT2; glucose RT2 and alpha-D-glucopyranoside. Five metabolites were detected only in Sumai3 but not in Roblin: propanoic acid, D-ribose, arabinoic acid-gamma lactone, D-glucuronic acid and benzoic acid.

3.4.2.3.1. Metabolite loading and cultivar discrimination

The first and second factors differentiated cultivars and treatments, respectively. Pathogen-inoculated Sumai3 (SP), compared to SW, had positive F1 scores (SP>SW), while inoculated Roblin, (RP) had negative F1 scores (RW>RP). Irrespective of inoculation, Roblin had a negative F2 score, while Sumai3 had a positive F2 score (Fig. 3.1). The metabolites with significant factor loadings to these factors can be used to discriminate the cultivars (Table 3.1). Roblin, which had a significant positive F1 score, had

significant factor loadings for fatty acids. Sumai3, with negative F2 score, had significant factor loadings for *p*-cinnamic acid, galactose and an unidentified peak (retention time 23.39-23.43, mass spectra 217, 73, 218, 147, 219, 45, and 305). However, there were metabolites with significant negative factor loadings for both F1 and F2. Alpha-linolenic acid; an unidentified peak (retention time 23.39-23.43, mass spectra 95, 67, 123, 81, 82, 69, and 55); octadecanoic acid; L-valine; benzene, 1, 3-bis (1, 1-dimethyethyl)-N, N-bis [2-TMS] ethaneamine; 9, 12-octadecadienoic acid (Z, Z)-, and butanoic acid had significant negative loadings for F1, indicating a higher abundance of these metabolites in Sumai3. Metabolites with significantly more abundance in Roblin, such as L-proline; 8, 11-octadecadienoic acid; glycine; phosphoric acid; and an unidentified peak (retention time 25.14-25.18, mass spectra 345, 73, 255, 147, 346, 347, and 45) had negative factor loadings for F2.

3.4.2.3.2. Wheat-FHB-related metabolites

Induced metabolites, novel metabolites or those with elevated abundance following pathogen inoculation were classified into three groups. R- and S-metabolites were unique to the susceptible cultivar Roblin (RP>RW) and the resistant cultivar, Sumai3 (SP>SW), respectively. C-metabolites were metabolites in greater abundance in pathogen-inoculated samples of both cultivar (RP>RW and SP>SW). The common metabolites were further grouped into R>S-C-metabolites (RP>SP), and S>R-C-metabolites (SP>RP) (Table 3.1). Among the 49 metabolites, 12, 14, and 23 were R, S and C-metabolites, respectively. Among common metabolites, 11 and 12 were R>S- and S>R-C-metabolites, respectively. The FHB resistance response can as a result be differentiated into susceptible and resistant, based on 23 Roblin (R/R>S-C) and 26 Sumai3 (S/S>R-C) metabolites induced following pathogen inoculation.

The metabolites also varied in their abundance following pathogen inoculation. Some metabolites whose levels increased more in Sumai3 than in Roblin were α-D-glucopyranoside, hexadecanoic acid, octadecanoic acid, pentadecanoic acid, *m*-cinnamic acid, heptadecanoic acid, hexadecanoic acid, myristic acid and tetradecanoic acid. The metabolite ribofuranose was the only metabolite specific to SP. Benzoic acid, propanoic acid and D-glucuronic acid

were detected in RP, SP and SW but not in RW. The abundance of propanoic acid was at least seven times greater in the pathogen-inoculated treatments than in the water-inoculated ones.

Among the 55 metabolites identified, only 15 were significantly different among treatments, including 14 that were induced after pathogen inoculation (defense-related or DR metabolites). Among DR metabolites, nine were significantly induced. However, only five of these varied significantly among cultivars (Fig.3.2). Myo-inositol, *m*-cinnamic acid, α-D-glucopyranoside and D-fructose were significant both between cultivars and between inoculants (Fig.3.2). The difference in abundance of *m*-cinnamic acid was highly significant ($P<0.01$) between inoculations (SP>SW) and cultivars (S>R). Myo-inositol levels were significantly different between SP and RP (SP>RP) and between SW and RW (SW>RW). The abundance of α-D-glucopyranoside was significantly different between SP and SW, but not between RP and RW. The same trend was observed for D-fructose (Fig.3.2). The metabolites L-alanine and hexadecanoic acid were significantly different between cultivars (Sumai3>Roblin). The metabolites butanedioic acid (= malic acid); 1,2-ethanediamine; 1,2,3-propanetricarboxylic acid; and glucose RT2 were detected in significantly higher quantities in pathogen-inoculated spikelets compared to water-inoculated ones, irrespective of cultivar.

The levels of several metabolites (15 and 10 metabolites in Sumai3 and Roblin respectively) were reduced following pathogen inoculation. However, none was significantly different between inoculants, although two were significantly different between cultivars.

3.4.2.4. Factor loadings and resistance discrimination

The first and second factors had positive factor scores for resistance while the third factor had a positive factor score for susceptibility. The clustering pattern of treatments, according to the three factor scores, was used to explain several possible hidden functions. These included a) pathogenesis or susceptibility function and b) defense or resistance function. This was done by relating the significant positive factor loadings of metabolites to susceptible or resistant cultivars, and to water- and pathogen-inoculated treatments, and then clustering of the treatments based on factor scores.

3.4.2.5. Pathogenesis/susceptibility function

Factor scores for F3 discriminated the levels of pathogenesis. A higher positive factor score of F3 was associated with i) a pathogen-inoculated (RP, SP) *vs.* a water-inoculated (SW, RW) cluster of treatments, irrespective of cultivars; ii) the susceptible cultivar, inoculated with pathogen (RP) *vs.* the resistant one (SP); iii) the resistant cultivar *vs.* the susceptible one, inoculated with water (SW *vs.* RW). This indicated an association of metabolites with significant factor loadings to F3 with pathogenesis or susceptibility of cultivars (section 3.1). The metabolites with significant F3 loadings (causing higher positive F3 scores) were fatty acids such as 8, 11-octadecadienoic acid and phenolic metabolites such as phenol, 2,4-bis (1,1-dimethylethyl-) and benzene-related metabolites such as 2,4,6-Tri-t-butylbenzenethiol and amino acids such as glutamine.

3.4.2.6. Defense/resistance function:

Factor scores for F1 and F2 differentiated the resistant and susceptible cultivars (section 3.1). A greater positive F2 score was associated with: i) the resistant cultivar cluster of two treatments (SP, SW) as opposed to that of the susceptible cultivar (RP, RW), irrespective of inoculation agents; ii) the resistant pathogen-inoculated cultivar (SP) as opposed to the susceptible pathogen-inoculated cultivar (RP); iii) the resistant water-inoculated cultivar (SW) as opposed to the susceptible water-inoculated cultivar (RW). The metabolites with high positive F2 loadings that partly (also confounded with cultivar-specific metabolites) explained defense functions were *m*-cinnamic acid, (also *p*-cinnamic acid); myo-inositol; fructose; galactose; glucose; 1,2,3-propanetricarboxylic acid RT2; α-D-glucopyranoside; malonic acid and an unidentified metabolite (retention time: 23.39-23.43 min, mass spectrum: 217, 73, 218, 147, 219, 45, and 305).

The highest positive F1 score was associated with: i) a pathogen-inoculated resistant cultivar (SP) as opposed to others (RW, RP, and SW). As a result, a higher positive F1 score means a higher level of resistance (note: moderately high positive F1 scores for RP and RW and a negative F1 score for SW). The metabolites with high positive F1 loadings that partly (also confounded with cultivar-specific metabolites) explained defense functions

were: benzene, (1-Butylopentyl)-; tetradecanoic acid; pentadecanoic acid; heptadecanoic acid; hexadecanoic acid; octadecanoic acid; myristic acid; hexadecanoic acid; octadecanoic acid and 2-monostearin.

There was a negative association between the factor score and the level of the metabolite(s) with negative factor loading(s), which means a negative correlation of the F-score (as a measure of resistance) with the level of the metabolite. The smaller factor scores were associated with a reduced level of defense/resistance and an increased amount of susceptible/PR metabolites. The metabolites 9,12-octadecadienoic acid (Z, Z)-,; α-linolenic acid; benzene,1,3-bis (1,1-dimethylethyl)-; N,N-bis [2-trimethylsiloxylethyl] ethaneamine; L-valine and an unidentified peak at retention time 24.89-24.93 (mass spectrum 95, 67, 123, 81, 82, 69, and 55) had negative loadings to F1. SP and SW had the highest and lowest F1 scores, respectively. Benzoic acid had the highest abundance in SW. Glycine, L-proline and 8,11-octadecadienoic acid had significant negative loadings to F3. Pathogen-inoculated spikelets of both cultivars (SP and RP) with the highest F3 scores compared to the water-inoculated ones (SW and RW), had the lowest quantities of these metabolites. These contributed to the greater F3 score, the susceptible/pathogenesis factor.

3.4. Discussion

With the progress in initiatives to profile genome and gene expression of plant-pathogen interactions, metabolite profiling is increasingly in demand in attempts to attain a fuller understanding of plant defense mechanisms against various environmental stresses, including pathogen stress. Wheat breeders on the other hand are looking for rapid, easy and precise tools to screen for resistance to FHB. In addition, a better understanding of the functions of FHB resistance genes would help them pyramid suitable resistance genes into elite cultivars. In the present study, GC/MS metabolite profiling of wheat spikelets at 24 hai allowed the identification of several plant-pathogen interaction-specific metabolites, as well as a putative relation of induced metabolites to wheat cultivars' resistance or susceptibility to FHB. Many of these are known to play significant roles in the metabolism of plants, leading to the production of defense-related metabolites

(Fiehn et al., 2000b; Roessner et al., 2000 and 2001; Dixon et al., 2002). The technique developed here could be further used to study genetic and/or environmental variations in resistance, providing a knowledge base that could be used to improve FHB resistance in wheat cultivars.

In this study, we discriminated wheat FHB resistance on the basis of several metabolite profiling criteria: a) potential DR/PR metabolites unique/specific to a cultivar resistant or susceptible to FHB; b) potential DR/PR metabolites common to both the susceptible and resistant cultivars but in higher abundance in one of these cultivars; c) metabolites with significant factor loadings to factor scores or treatments. This approach allowed us to suggest possible explanations for between-cultivar differences in resistance against FHB.

Both susceptible and resistant cultivars either produced some novel metabolites or increased the levels of pre-existing metabolites following pathogen inoculation. These metabolites were designated here as potential PR metabolites, and where the levels of these increased significantly ($P \leq 0.05$), as PR metabolites, in a manner similar to PR proteins and PR genes (Pritsch et al., 2000; Muthukrishnan et al., 2001). In this study, 49 potential DR/PR metabolites were identified, including 12 metabolites that were unique to Roblin, 13 unique to Sumai3 and 23 common to both cultivars. Of 23 common metabolites, 11 and 12, respectively, were in higher abundance in R and S. There were in total 23 and 26 potential PR metabolites from Roblin (R/R>S) and Sumai3 (S/S>R), respectively. These metabolites could be used to discriminate FHB-responses between cultivars varying in their resistance against FHB. However, of 55 metabolites detected here, only fifteen were significantly different between treatments, including nine that were PR-metabolites (Table 3.1, Fig. 3.2), such as myo-inositol, *m*-cinnamic acid, α-D-glucopyranoside, and D-fructose. Even though *m*-cinnamic acid was detected in all the treatments, its level in SP was about six times greater than that in SW. Its levels were low in RW, and only slightly changed following pathogen inoculation. A higher abundance of sugars such as myo-inositol, an important signal molecule, and glucose, a precursor of shikimic acid and monomer of cellulose and hemicelluloses, both at significantly higher levels in Sumai3, than Roblin, can also account for the resistance of Sumai3 to FHB (Buchanan

et al., 2000). Benzoic acid (BA) was induced in the resistant cultivar Sumai3 when inoculated with the pathogen. BA was not detected in RW, but was detected in low abundance in RP. BA can easily be converted to cinnamic acid, a key metabolite in the phenylpropanoid pathway. Decarboxylation of *trans*-cinnamic acid to BA and further 2-hydroxylation of BA to salicylic acid (SA) has also been reported (Leon et al., 1995). Some aromatic metabolites such as BA and salycilic acid (SA), besides their role in signal transduction, are directly antimicrobial (Hammond-Kosack and Jones, 1996). A higher level of glutamine helps the plant cell recycle ammonia ions liberated from phenylalanine. A higher level of glutamine is therefore considered as resistance discriminatory and further evidence for a more active phenyl ammonia lyase (PAL) pathway in Sumai3. Glutamine was detected at increased levels in pathogen-inoculated plants of both cultivars, but the increase was greater in SP.

Though the levels of several metabolites detected in this study were lower in pathogen- than water-inoculated spikes, they may still be involved in plant defense (Dixon et al., 2002; Muthukrishnan et al., 2001). Proline and glycine had negative factor loadings to F3 indicating lower abundance of these amino acids in pathogen-inoculated spikelets of both cultivars (with high F3 scores). One class of defense response genes in wheat is a group of resistance (R)-genes encoding for proline/glycine rich proteins (Li et al., 1999). A lower level of proline and glycine in a resistant cultivar, particularly in a defense/induced state, may be due to a higher cellular demand for these amino acids, which allows for synthesis of proline/glycine rich R proteins. Fatty acids, such as linolenic acid and 9,12-octadecadienoic acid (Z,Z)-, had negative F1 loadings, suggesting greater resistance at lower levels of these fatty acids in SP. The metabolite α-linolenic acid is the first precursor for the production of jasmonic acid (JA) which is a key signal molecule. JA induces PAL and several PR proteins (Buchanan et al., 2000; Digiacomo et al., 2002). The F1 score was maximum for SP and minimum for SW, which may indicate that the high abundance of α-linolenic acid, with negative factor loading in SW, was reduced in Sumai3 following pathogen attack, as it was used for the synthesis of JA.

A factor analysis of the metabolites, common to all treatments was used to explain the hidden (pathogenesis and defense) functions, underlying FHB resistance in wheat. The large difference in F1 scores between SP and SW indicated an increased level of DR/PR metabolites following pathogen inoculation. Such a difference was not as significant in Roblin (Fig. 3.1). Also, the highest F2 scores for pathogen-inoculated Sumai3 can partly explain the higher level of resistance due to increase in the abundance of metabolites with positive and significant factor loading to F2 (Table 3.1), though the effect could in part be due to cultivar differences. Therefore, the metabolites with a positive and significant factor loading to F1 and F2 can be used to discriminate FHB resistance in Sumai3. In contrast, the F3 mainly explained pathogenesis, as the pathogen-inoculated spikelets of both cultivars had high positive scores with the highest positive factor score for RP.

Following pathogen attack, plants switch their metabolic pathways from primary metabolite production to an enhanced defense state in order to produce more defense-related metabolites (Dixon et al., 2002; Fiehn et al., 2000b). It appears that PAL is more active in the resistant cultivar Sumai3 than in the susceptible cultivar Roblin. Following pathogen inoculation, the abundance of *m*-hydroxycinnamic acid (and *p*-hydroxycinnamic acid) in Sumai3 increased to a greater degree than in Roblin. PAL converts phenylalanine to *trans*-cinnamic acid, which is also a precursor for salicylic acid. The enzyme cinnamate 4-hydroxylase hydrolyses *trans*-cinnamic acid into 4-coumaric acid, which finally produces coumaric acid, a key metabolite that serves as precursor of other phenolic metabolites and monomers of the cell wall (Blechert et al., 1995; Blount et al., 2000; Buchanan et al., 2000; Dixon et al., 2002). The metabolite 4-hydroxycinnamic acid is a precursor for the production of a group of phytoalexins in oat (*Avena sativa* L.) leaves infected with the rust disease (*Puccinia coronata* f.sp. *avenae*) (Muthukrishnan et al., 2001; Okazaki et al., 2004). The metabolite 4-hydroxy-3-methoxycinnamic acid (ferulic acid) has a role in polymer cross-linking within plant cell walls (Russell et al., 1999).

A higher abundance of 4-hydroxycinnamic acid (*p*-coumaric acid) as well as *m*-coumaric acid was observed in the resistant cultivar (Sumai3). This may be attributed to several hypothetical causes. There is evidence that the

PAL gene is on chromosome 3B which carries the quantitative treat loci (QTL) for resistance to FHB (Li et al., 1999). Therefore, the higher level of *m*-hydroxycinnamic acid in Sumai3 may be due to the PAL gene. This gene (factor) may be less functional or suppressed in the susceptible cultivar, Roblin. A synergistic effect of *p*-coumaric and ferulic acid in inhibiting mycelial growth of two isolates of *F. graminearum* has been observed under *in vitro* conditions (Smart and Flores, 1997). The accumulation of phenylpropanoid metabolites after infection by plant pathogens has been reported (Southerton and Deverall, 1990; Smart and Flores, 1997). Higher concentrations of free phenolic metabolites were found in the resistant wheat cultivar Frontana as compared to the susceptible cultivar Argent inoculated with *F. graminearum*. This is especially true for *p*-coumaric acid in the glumes, lemmas and paleas (Siranidou et al., 2002).

In both cultivars, malonic acid levels were higher in pathogen-inoculated spikes than water-inoculated spikes. This suggests that following pathogen inoculation, both resistant and susceptible cultivars activate the malonate pathway, in addition to the PAL pathway. The former is known to produce phenolics such as isoflavonoids, though it is not as efficient as the PAL pathway for the production of phenolics. Isoflavonoids are important both as fungicidal substances and as signal molecules in plant-microbe communication (Buchanan et al., 2000; Muthukrishnan et al., 2001; Dixon et al., 2002).

Higher levels of sugars, such as myo-inositol and glucose, may also account for the resistance of Sumai3 to FHB. Following pathogen inoculation, the level of myo-inositol in Sumai3 increased to a greater degree than in Roblin. A higher factor loading of myo-inositol to F2, which had a high factor score for the SP treatment, indicates an important contribution of this metabolite in Sumai3's resistance to the pathogen. Myo-inositol is involved in cell signaling in animals and plants (Exton, 1996; Nelson et al., 1998; Poovaiah and Reddy, 1993). Plants with higher levels of resistance to diseases have shown overexpression of inositol (Berridge and Irvine, 1989; Smart and Flores, 1997; Pritsch et al., 2000). A richer pool of inositol-derived metabolites in the resistant cultivar (Sumai3) can supply a higher signal transduction capacity and a more rapid response to the attacking pathogen.

Galactose and glucose had higher factor loadings for F2. A hidden factor may cause the production of some enzymes involved in the synthesis or the enzymatic hydrolysis of these sugars from their parent glycosides. Sugars such as glucose, Galactose, and xylose are all used in the synthesis of hemicelluloses with xylose molecules as side chains (Buchanan et al., 2000).

The highest F1 scores for SP and lowest scores for SW suggest a more active PAL pathway in the Sumai3-FHB system, as opposed to Roblin, which appears to suppress PAL, as the factor scores for RP were lower than for RW.

Fatty acid production by the two cultivars appears to be controlled by functions hidden mainly in F1 for Sumai3 and in F3 for Roblin. Following pathogen inoculation, certain fatty acids appear to increase in both cultivars. There is a greater increase in Roblin, as indicated by higher F3 scores for RP (Table 3.1). The greater increase, after pathogen inoculation, in F3 scores for Roblin *vs.* Sumai3, implies that Roblin may take greater advantage of the JA signal transduction system than Sumai3. The octadecanoic acid pathway produces signal molecules with vital roles in regulating secondary pathways (Blechert et al., 1995). Despite the response in the production of the JA-pathway fatty acids, the disease still progressed in the cultivar Roblin.

Some increase in F3 scores for both SP and RP can be assigned to glutamine, which plays an important role in recycling ammonia ions and guarantees rapid and appropriate functioning of PAL (Buchanan et al., 2000). Greater abundance of glutamine implies greater activity of PAL and phenylpropanoid metabolism in the resistant cultivar Sumai3.

Metabolite profiling associated with factor analysis can be used as a powerful tool in deciphering plant defense responses and for phenotyping cultivar resistance, as we have identified several metabolites that are related to the resistant cultivar, Sumai3, as opposed to the susceptible cultivar, Roblin. There exists a potential to develop this technology for high throughput cultivar screening, once the defense metabolites are characterized. This technology can also be used to recognize the mode of action of the pathotoxin, DON, in pathogenesis, and the five different types of resistance mechanisms (Hammond-Kosack and Jones, 1996). The database on pathways of plant defense could be further exploited through metabolic

engineering. Wheat defense genes against FHB can be identified by relating these PR metabolites to PR gene expressions, including transcriptome and proteome (Muthukrishnan et al., 2001), and this knowledge could be used to pyramid genes into an elite cultivar.

While we have detected only a few metabolites in the wheat-FHB system, plants are known to produce thousands of metabolites (Fiehn et al., 2000b; Sumner et al., 2003), However, we have increased the chance of identifying PR metabolites by extracting and profiling metabolites following pathogen inoculation. In spite of the complexity of resistance in the wheat-FHB system, we were able to recognize groups of metabolites that discriminated resistance and additionally, to explain the possible functions of metabolites in wheat plant defense against *F. graminearum*. However, various steps involved in metabolite profiling such as metabolite extraction, metabolite identification and the use of suitable wheat and pathogen genotypes to prove certain metabolic functions, have to be improved in order to achieve an in depth understanding of wheat-FHB interactions. Following pathogen inoculation, plants use one or more metabolic pathways to synthesize novel metabolites to defend against the attacking pathogen (Kofalvi and Nassuth, 1995; Fiehn et al., 2000b; Dixon et al., 2002). Though we have profiled metabolites only at 24 h following pathogen inoculation, the metabolite synthesis and plant defense are dynamic processes. Further studies involving temporal assessment of metabolites, different wheat and *F. graminearum* genotypes, and environmental variables are required to recognize metabolite function in plant defense.

In this study, metabolites were identified based on a NIST library match and manual comparison of spectra. Since there was no prior knowledge of metabolite identity, GC/MS technology was best suited to this work. However, the identity of the metabolites reported here are tentative and further studies involving spiking with pure metabolites or use of other instruments to identify the metabolite structure are required to authenticate the identities (Sumner et al., 2003).The GC/MS system detects only relatively low molecular weight metabolites (Shen et al., 2003). Application of other hyphenated equipments such as LC/MS/MS, LC/NMR, etc. must be explored (Sumner et al., 2003; Roepenack-Lahaye et al., 2004) for more complex molecules. These

analytical platforms are relatively cost and time effective methods. Likewise, the solvents and extraction methods used in this study were selected to achieve the goal of extracting as many metabolites as possible. More than one extraction method and analytical instrument is required to detect an adequate number of metabolites to explain the nature of resistance in plants against diseases. Metabolite profiling, therefore, can help better understand the functions of metabolites, assist in selecting and pyramiding of suitable/required genes, leading to accelerated wheat-FHB breeding program, especially when this knowledge base is coupled with studies on proteins, mRNAs and genes.

Table 3.1. List of metabolites (abundance = ×10^6; rounded-off) detected in Roblin and Sumai3 wheat cultivars, at 24 h after inoculation with *Fusarium graminearum* or water (control). The eigenvectors and eigenvalues for each of the first three factors calculated by factor analysis of normalized abundance of 49 metabolites (32 from methanol-water fraction and 17 from chloroform fraction) common to all the four treatments (Sumai3 and Roblin inoculated with pathogen or water: SP, SW, RP, and RW). RT=retention time, DR/PR metabolite=defense-related/pathogenesis-related metabolite; F1-F3 are factor vectors; Higher factor loadings show higher contributions of the corresponding metabolites in variability of the factor[1].

RT (mm:ss) Range	Compound Name	M/C[2]	DR-/PR-Metabolite	CG[7]	RP	RW	SP	SW	F1	F2	F3
5.70-.75	1,2-Ethanediamine, (161)[6]	M	S>R (NS;0.09)[4]	AM	0.23	0.19	0.27	0.18	0.64*[3]	0.45	0.62
40.23-.29	2-Monostearin TMS ether, (502)	C	S>R	FA	0.13	0.07	0.13	0.04	0.70*	0.04	0.72*
38.41-.49	α-D-Glucopyranoside,1,3,4,6-tetrakis-O-(TMS)-, (918)	M	S>R (0.04;0.02)	SU	82.00	45.00	123.00	70.00	0.38	0.75*	0.54
25.42-.46	D-Fructose,1,3,4,5,6-pentakis-O-(TMS)-, O-methyloxime, (569)	M	S>R (0.001;0.03)	SU	7.06	6.64	15.08	11.23	0.20	0.98*	0.03
25.63-.67	D-Fructose,1,3,4,5,6-pentakis-O-(TMS)-,O-methyloxime RT2, (569)	M	S>R (0.001;0.03)	SU	4.25	4.14	9.57	7.00	0.21	0.98*	0.01
28.96-.99	Hexadecanoic acid, TMS, (328)	C	S>R	FA	2.87	2.52	3.26	1.67	0.88*	0.02	0.47
29.52-.56	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(TMS), (612)	M	S>R (0.02;0.10)	SU	2.55	2.00	3.72	3.17	0.04	0.97*	0.26
32.47-.50	Octadecanoic acid, TMS, (356)	C	S>R	FA	0.71	0.70	0.87	0.45	0.96*	0.05	0.29

26.73-.80	Pentadecanoic acid, (270)	C	S>R	FA	0.67	0.59	0.73	0.45	0.86*	0.02	0.51
17.80-87	Phenol, 2,4-bis (1,1-dimethyl, (206)	C	S>R (NS;0.07)	PH	0.42	0.32	0.50	0.34	0.52	0.57	0.64*
13.09-.09	Propanoic acid, 2,3-bis [(TMS) oxyl] -,TMS ester, (322)	M	S>R	OA	0.07		0.07	0.01			
28.73-.77	Tris-TMS Malonic acid (320)	M	S>R	OA	0.01	0.01	0.01	0.01	0.27	0.86*	0.43
24.52-.57	1,2,3-Propanetricarboxylic acid, 2-[(TMS)oxyl]-, tris(TMS) RT2, (480)	M	S (0.01;NS)	OA	0.64	0.69	0.74	0.63	0.90*	0.37	-0.22
7.33-.37	Benzene, (1-Butylpentyl)-, (204)	C	S	AR	0.04	0.05	0.05	0.04	0.86*	-0.05	-0.50
12.74-.79	Butandioic acid bis (TMS), (262)	M	S (0.001;NS)	OA	0.02	0.02	0.04	0.04	-0.10	0.98*	-0.18
17.09-.12	Butanedioic acid, [(TMS)oxy]-, bis(TMS) ester (350)	M	S (0.03;NS)	OA	0.33	0.36	0.29	0.28	0.40	*-0.91**	-0.13
26.93-.99	Cinnamic acid, m-(TMS)-, TMS ester, (308)	M	S (0.001;0.001)	PH	0.02	0.02	0.17	0.03	0.65*	0.75*	0.11
25.91-.96	Glucose 2, 3, 4, 5, 6-pentakis-O-(TMS)-, O-methyloxime RT2, (569)	M	S (0.001;NS)	SU	9.03	10.17	23.04	21.27	-0.07	0.99*	-0.16
28.65-.70	Heptadecanoic acid, methyl ester, (284)	C	S	FA	0.03	0.04	0.04	0.02	0.98*	0.14	0.13
37.92-.98	Hexadecanoic acid, 2, 3-bis [(TMS) oxy] propyl ester, (474)	C	S (NS;0.07)	FA	6.83	6.85	7.45	1.95	0.92*	-0.29	0.28
17.88-.91	L-Proline, 5-oxo-1-(trimethysilyl)-,	M	S	AA	0.41	0.50	0.25	0.22	0.33	*-0.93**	-0.13

	TMS ester, (273)										
34.95-.99	Myristic acid, 2, 3-bis (TMS) propyl ester, (446)	C	S	FA	0.20	0.24	0.30	0.12	0.99*	0.10	0.02
23.39-.43	Unidentified: 217, 73, 218, 147, 219, 45, 305	M	S	UN	0.22	0.25	0.54	0.40	0.23	0.97*	-0.09
9.18-.22	Phosphoric acid, bis(TMS)monomethyl ester, (256)	M	S	OA	0.01	0.02	0.01	0.01	0.61*	*-0.69**	-0.39
27.20-.29	Ribofuranose-1,2,3,5-tetraTMS, (438)	M	S	SU			0.41				
25.12-.19	Tetradecanoic acid, TMS ester, (300)	C	S	FA	0.06	0.06	0.07	0.03	0.96*	-0.24	0.16
18.25-.28	2,4,6-Tri-t-butylbenzenethiol, (278)	C	R>S	AR	0.18	0.14	0.15	0.15	-0.07	-0.24	0.97*
29.90-.98	8,11-Octadecadienoic acid, methyl ester, (294)	C	R>S	FA	0.55	0.50	0.41	0.39	0.17	*-0.89**	0.42
27.63-.68	D-Glucose, 2,3,4,5,6-pentakis-O-(TMS)-, (540)	M	R>S	SU	0.81	0.75	0.74	0.42	0.78*	-0.47	0.40
37.14-.20	Docosanoic acid, methyl ester, (354)	C	R>S	FA	0.05	0.03	0.03	0.01	0.42	-0.48	0.78*
20.17-.22	Glutamine tris(TMS)-, (363)	M	R>S	AA	0.03	0.02	0.02	0.01	0.36	-0.48	0.80*
12.39-.41	Glycine, N,N-bis(TMS)-, TMS ester, (291)	M	R>S	AA	0.03	0.02	0.01	0.01	0.08	*-0.75**	0.65

7.26-.29	L-Alanine, N-(TMS)-, TMS, (233)	M	R>S (NS;0.001)	AA	0.12	0.02	0.08	0.03	0.25	-0.01	0.97*
12.20-.23	L-Proline, 1-(TMS)-,TMS ester, (259)	M	R>S (0.01;NS)	AA	0.04	0.03	0.02	0.01	0.23	*-0.89**	0.39
25.14-.18	Unidentified: 345, 73, 255, 147, 346, 347, 45	M	R>S	UN	0.28	0.23	0.20	0.12	0.53	*-0.68**	0.51
40.70-.76	Octadecanoic acid, 2,3-bis [(TMS)oxyl]propyl ester, (502)	C	R>S	FA	2.71	2.49	1.94	0.56	0.67*	-0.66	0.34
22.58-.64	Tridecanoic acid, 12-methyl-, methyl ester, (242)	C	R>S	FA	0.30	0.14	0.22	0.13	0.29	-0.13	0.95*
31.88-.91	9,12-Octadecadienoic acid (Z,Z)-, TMS ester, (352)	M	R	FA	1.25	0.22	0.74	1.47	*-0.78**	0.32	0.54
32.01-.04	α-Linolenic acid, TMS ester, (350)	M	R	FA	0.05	0.03	0.03	0.06	*-0.98**	0.03	0.19
11.11-.16	Benzene, 1, 3-bis (1, 1-dimethyl ethyl)-, (190)	C	R	AR	0.22	0.17	0.19	0.33	*-0.94**	0.35	-0.05
42.84-.87	Benzoic acid, 2, 6-bis (TMS)-, (312)	M	R	AR	0.04		0.05	0.06			
30.18-.19	beta-DL-Lyxopyranose, 1, 2, 3, 4-tetrakis-O-(TMS)-, (488)	M	R	SU	0.12	0.03	0.02	0.11	*-0.84**	-0.17	0.52
17.99-18.03	Butanoic acid, 4-[bis (TMS) amino-, TMS, (319)	M	R	OA	0.08	0.05	0.06	0.07	*-0.72**	-0.10	0.69*
34.56-.60	D-Glucuronic acid, 2,3,4,5-tetrakis-O-(TMS)-, TMS, (554)	M	R	OA	0.04		0.05	0.06			

33.95-.99	Eicosanoic acid, (326)	C	R	FA	0.38	0.20	0.29	0.31	-0.38	0.13	0.92*
25.94-26.30	Galactose oxime hexaTMS, (627)	M	R	SU	2.00	1.91	5.86	6.36	-0.27	0.96*	-0.13
10.08-.11	L-Valine, N-(TMS)-, TMS, (261)	M	R	AA	0.03	0.02	0.03	0.04	*-0.90**	0.43	-0.07
11.35-.39	N,N-bis [2-trimethylsiloxylethyl] ethaneamine, (277)	M	R	AM	0.12	0.11	0.11	0.21	*-0.93**	0.34	-0.13
24.89-.93	Unidentified: 95, 67, 123, 81, 82, 69, 55	M	R	UN	0.14	0.12	0.09	0.28	*-0.96**	0.17	-0.21
24.43-.48	1,2,3-Propanetricarboxylic acid, 2-[TMS)oxyl]-, tris(TMS), (480)	M	(0.01;NS)	OA	0.66	0.66	1.03	1.03	-0.18	0.97*	-0.13
20.47-.50	Arabinoic acid, 2,3,5-tris-O-(TMS)-,.gamma lactone, (364)	M		OA				0.37			
23.93-97	Cinnamic acid, p-(TMS)-, (250)	C		PH	0.03	0.03	0.05	0.05	-0.38	0.87*	-0.32
20.38-.40	D-Ribose, 2, 3, 4, 5-tetrakis-O, (438)	M		SU			0.02	0.02			
24.23-.28	Unidentified: 73, 217, 204, 147, 205, 45	M		UN		0.43		0.37			
18.77-.79	Trihydroxybutyric (424)	M		OA	0.01	0.02	0.01	0.02	-0.16	-0.03	*0.99**

R=R-DR=Unique Roblin-DR metabolite s = RP> RW	***12[5]***	Eigenvalues	19.79	18.43	12.78
S=S-DR=Unique Sumai3-DR metabolites =SP>SW	***14***	%Variance explained	0.39	0.36	0.25
R>S; S>R = Common-DR metabolites=common R>S-DR; S>R-DR	***23***	Cumulative % of explained variance	0.39	0.75	1.00
Total R/R>S-DR metabolites = (Unique and Common R-DR) = 12+11	***23***				
Total S/S>R-DR metabolites = (Unique and Common S-DR) = 14+12	***26***				

1 Factor scores contributing to different treatments are shown in Fig. 3.1.

2 M = from methanol-water fraction and C = from chloroform fraction of the extract of spikelets.

3 Relatively high factor loading of metabolites to the respective factor in the column; * =higher levels of factor loading of the corresponding metabolite to the factor in the column; the factor scores for treatments are given in Fig. 3.1.

4 Significance level based on univariate ANOVA (in parentheses the first number is between cultivars and the second number is between inoculations); values $P \leq 0.01$ is highly significant, $P \leq 0.05$ is significant, $P \leq 0.10$ is borderline significant.

5 Number of DR metabolites in different categories mentioned; PR metabolite is pathogenesis-related metabolite = DR metabolites that are significantly induced.

6 Molecular Weight.

7 CG=Chemical Group: AA=amino acid; AM=amine; AR=aromatic; FA=fatty acid; OA=organic acid; SU=sugar; UN=unidentified.

Table 3.2. Total abundance (× 10^6) of different functional groups of metabolites detected in the susceptible (Roblin=R) and resistant (Sumai3=S) cultivars, inoculated with the pathogen (P) or water (W).

Chemical Group	RP	RW	SP	SW
Amine	0.35	0.30	0.38	0.39
Amino acid	0.66	0.61	0.41	0.32
Aromatic	0.48	0.36	0.44	0.58
Fatty acid	16.79	14.70	16.51	7.66
Organic acid	1.87	1.83	2.31	2.53
Sugar	107.80	70.60	181.50	119.60
Unidentified	0.64	1.03	0.83	1.17

Figure 3.1. Scatter plot, based on projections of three factor scores, of factor analysis of abundance of 49 metabolites common to all treatments: Sumai3 pathogen- (SP=♠), Sumai3 water- (SW=♥), Roblin pathogen- (RP=♣) and Roblin water- (RW=♦) inoculated. The factor loadings of metabolites, to different factors, are shown in Table 3.1. The metabolites with significant factor loadings to the first three factors were (see the complete names in Table 3.1): F1=tetradecanoic acid; pentadecanoic acid; heptadecanoic acid; hexadecanoic acid; octadecanoic acid; myristic acid; monostearin; benzene (1-butylopentyl)-; F2=*m*-coumaric acid; *p*-coumaric acid; myo-inositol; fructose; galactose; glucose; propanetricarboxylic acid; α-d-glucopyranoside; malonic acid and butanedioic acid; F3=8,11-octadecadienoic acid; phenol, 2, 4-bis; glutamine; L-alanine and tri-t-butylbenzenethiol

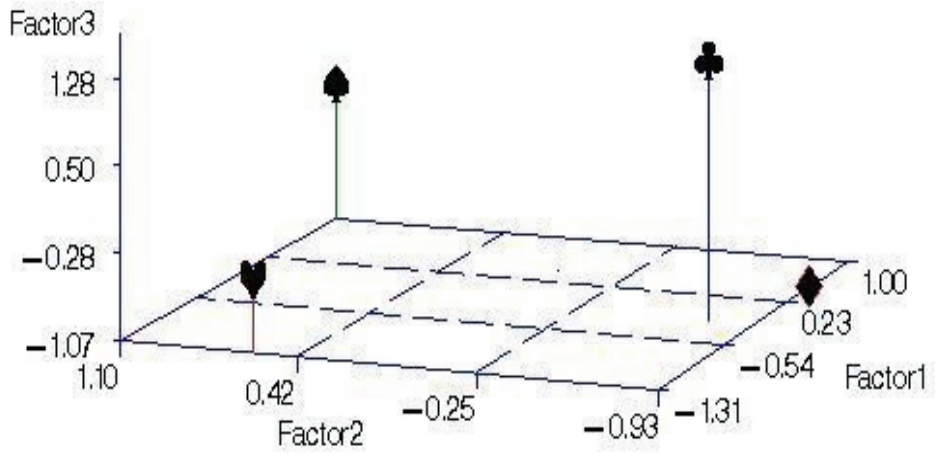
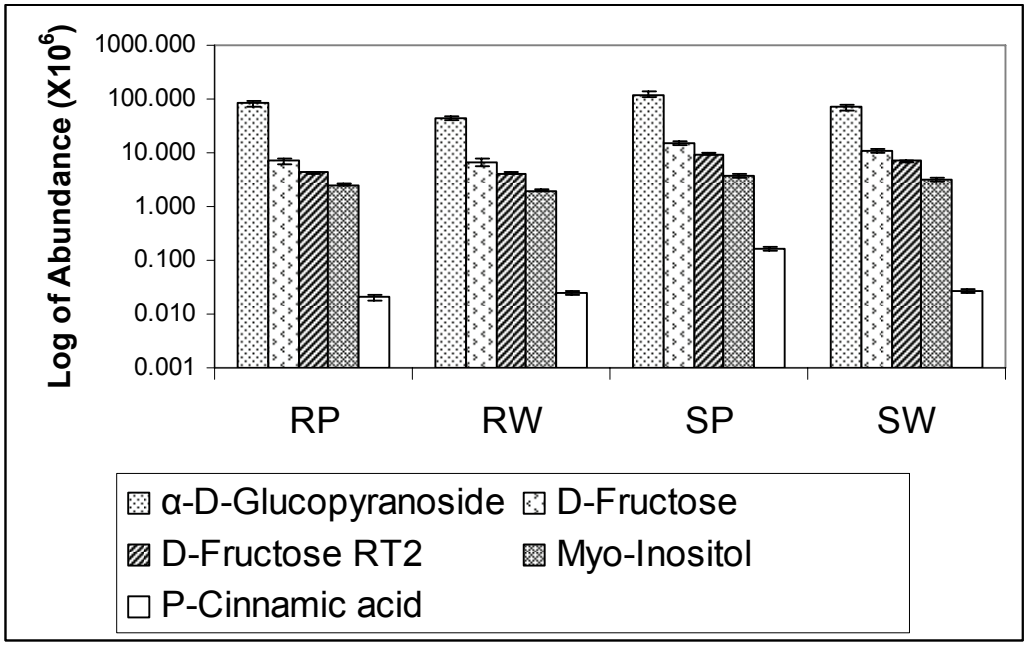

Factor3
1.28
0.50
−0.28
−1.07
1.10
0.42
−0.25
−0.93
Factor2
1.00
0.23
−0.54
−1.31
Factor1

Figure 3.2. Bar graph of the abundance (logarithmic scale) of five PR metabolites, significantly ($P \leq 0.05$) discriminating treatments (RP and RW= Roblin pathogen-/water-inoculated, respectively; likewise SP and SW= Sumai3 pathogen/water-inoculated). Error bars are ±root mean square of error (root MSE). The complete names of metabolites included here are given in Table 3.1. Among nine PR metabolites, only five varied significantly among cultivars are shown here.


Log of Abundance (X10^6)
1000.000
100.000
10.000
1.000
0.100
0.010
0.001
RP
RW
SP
SW
α-D-Glucopyranoside
D-Fructose
D-Fructose RT2
Myo-Inositol
P-Cinnamic acid

Chapter 4

Metabolite profiling techniques coupled with statistical analyses for potential high throughput screening of quantitative resistance to fusarium head blight in wheat cultivars.

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Preface to Chapter 4

Chapter 4 is a manuscript submitted for publication by Hamzehzarghani H., Kushalappa A.C., Dion Y., Rioux S., Comeau A., Yaylayan V., and Marshall W.D. 2007. Metabolite profiling techniques coupled with statistical analyses for potential high throughput screening of quantitative resistance to fusarium head blight in wheat cultivars. Canadian Journal of Plant Pathology, in June 2007.

The results of the present study, in parts, were also presented at the First annual meeting of the metabolomics society held at Kein University, Japan, June 20th to 23rd 2005 and 4th Canadian Workshop on Fusarium Head Blight, Ottawa, Ontario, Nov. 1st to 3rd 2005, both as poster presentations with abstracts. The contribution of the coauthors is described in the "Contribution of Authors" section.

Despite their significantly different pedigrees, the highly resistant (Sumai3) and highly susceptible (Roblin) cultivars we used in a previous study (Chapter 3), were sufficiently different in their resistance to FHB to be easily differentiated, even with the masking effect of background genetic noise. In the current study, differences in the level of FHB resistance of different cultivars were lesser than that of the two cultivars examined in Chapter 3. The cultivars in the present study were from different geographical origins and therefore had a larger genetic variability than those studied in Chapter 3. This experiment presented a good challenge in evaluating the sensitivity, accuracy and throughput of the metabolite profiling technique to screen a large number of cultivars from different sources. In the present study, the following modifications were made to decrease experimental error:

1- GC/MS output files were converted into universal "nef.cdf" format to be amenable to reanalysis with the most updated software platform used in the last study (Chapter 5).The only difference consisted in running the samples in "net.cdf" format with MetAlign to correct for the relatively high background noise. This extra step was essential to obtain a data set to process in the MET-IDEA software.

2- Data processing was improved by deleting metabolites with an abundance of less than 5000 instead of 2000. This decision was made because the abundance of some metabolites coming from the column bleed were higher than 2000.
3- Both polar and non-polar metabolites were analyzed following a more advanced method developed by Fiehn et al. (2000a). The advantage of this method was that it allowed additional refinement of the samples, and therefore improved the consistency of metabolites.
4- In the advanced method, the chemical analysis was further improved by replacing Speed-Vac drying with successive Speed-Vac and freeze-drying. This was done to facilitate the dissolution of the remaining pellet.
5- The metabolite identification protocol was improved using the EXCEL Pivot procedure to compare the mass spectra of the target components with the first fifth NIST hits in order to choose the best match.
6- Injection temperature was increased from 50 ^{o}C to 70 ^{o}C, which would cause solvents to elute faster and reduce the chance of contamination. The maximum temperature was increased from 200 ^{o}C to 280 ^{o}C and was maintained at 280 ^{o}C for 5 minutes. A higher temperature enabled a better detection of more semi- to non-volatile metabolites with higher boiling points. This resulted in a better cleaning of the column, less contamination, and, in turn, improved the consistency of metabolite elution, allowing a more definitive identification.
7- Using a water-bath shaker for the derivatization of metabolites instead of a still water-bath led to a more steady reaction between reactants and metabolites.
8- Instead of moving the test plants to the growth chamber immediately after inoculation, plants were transported there two days before inoculation to provide them with more stable light and temperature conditions and increase their physiological uniformity.

In Chapter 3, we established metabolic criteria to distinguish cultivars varying in their resistance to FHB. In this study, those criteria were further validated and a new set of criteria (based on more cultivars) was defined. We hypothesize that cultivars of wheat varying in their levels of resistance to FHB

should also vary in their metabolic profiles. Metabolic profiles developed for six cultivars were used to discriminate cultivars with different levels of resistance, and assess the procedure's potential future use in high throughput screening of breeding lines.

4.1. Abstract

Fusarium head blight (FHB) causes considerable losses in wheat yield and grain quality. As conventional screening for disease resistance based on five separate types of resistance is inefficient, a metabolomics approach to discriminating resistance was investigated. Spikelets of six wheat cultivars varying in level of resistance were inoculated with *Fusarium graminearum* or water. The spikelet disease severity was quantified and the metabolic profiles were recorded using GC/MS. A total of 214 metabolites were detected, including 79 with significant treatment effects. Univariate analysis of variance identified 41 resistance-related (RR) metabolites and multivariate analysis identified 45 resistance function associated metabolites, including 27 RR metabolites that also explained resistance functions. Highly resistant cultivars Wangshubai and AW488 had 14 and 22 metabolites, respectively, the highest number of constitutive RR and induced RR metabolites. A moderately resistant cultivar BRS177 had 12 induced RR metabolites. The RR metabolites identified here are potential candidate biomarkers for high throughput screening of wheat breeding lines against FHB.

Keywords: Canonical discriminant analysis, disease resistance, functional genomics, *Fusarium graminearum*, metabolomics, phytochemicals, resistance biomarker metabolites, *Triticum aestivum*, wheat scab.

4.2. Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch], causes serious epidemics in wheat leading to severe losses in grain yield and quality. Warm and humid weather conditions, especially at wheat anthesis, are conducive to the development of a FBH epidemic, leading to the production of mycotoxins such as DON in wheat grains (Bai et al., 2001). Genetic resistance is the most desirable method to manage FHB. Expression of the resistance to FHB as five proposed resistance types and the fact that the pathogenicity and progression of the disease are highly influenced by environmental conditions, complicate the evaluation of resistance to FHB (Mesterhazy, 1995). The evaluation of breeding lines based on disease symptoms can be slow and inconsistent from year to year, therefore providing limited information on the mechanism of resistance. Molecular markers that are tightly linked to sets of genes that confer resistance to FHB, such as amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR), have been used as markers in DNA-fingerprinting of wheat genetic materials (Anderson et al., 2001). Yet the functions of the resistance genes remain unknown. The top priority in breeding wheat for resistance to FHB is the development of a high throughput screening method that can also provide information regarding the mechanisms of resistance and resistance gene function.

Metabolomics, an evolving field of systems biology, aims to decipher gene function at the metabolite level (Dixon 2001; Fiehn et al., 2000b; Dixon et al*.,* 2002; Sumner et al., 2003). As with other 'omics' studies, metabolomics generates large data sets of metabolites that always display multicolinearity. Often just a few orthogonal vectors (linear combinations of the metabolites) are available to explain the function of a trait or gene (Goodacre et al*.,* 2004). Identification of a set of metabolites that can explain the functions associated with a trait/gene remains a challenge in systems biology. Multivariate models of metabolite profiles have been used to classify resistance of wheat (Hamzehzarghani et al., 2005) and potato plants (Abu-Nada et al., 2007) to disease stress. Genetically modified organisms (Choi et al*.,* 2003), yeast mutants (Allen et al*.,* 2003), salt-stressed tomatoes (Johnson et al*.,* 2003) and

potato production systems (Roessner et al., 2001) have been similarly classified.

Pathogen-inoculated wheat spikelets have been demonstrated to induce disease response genes, proteins and metabolites within a few hours of the inoculation (Pritsch et al., 2000; Muthukrishnan et al., 2001; Hamzehzarghani et al., 2005). Higher concentrations of free phenolic compounds were observed in the pathogen (*F. graminearum*) inoculated resistant wheat cultivar Frontana than in the susceptible cultivar Argent (Siranidou et al., 2002). Inhibition of *F. graminearum* mycelial growth by phenolics such as *p*-coumaric and ferulic acids has been reported in several studies (Smart and Flores, 1997; Russel et al., 1999). An accumulation of the phenylpropanoid metabolites has been reported after the infection of wheat with other pathogens (Southerton and Deverall, 1990; Kofalvi and Nassuth, 1995; Okazaki et al., 2004).

To our knowledge, there are no studies concerning the use of metabolic profiles and multivariate analysis to discriminate cultivars of plants varying in their levels of resistance to disease stress, or with a view to explaining the underlying resistance functions. A recent study reported on the discrimination of resistant and susceptible wheat cultivars using multivariate analysis (Hamzehzarghani et al., 2005). The objective of this study was to discriminate levels of FHB resistance in wheat cultivars based on metabolic profiles. This study can also provide insights into the possible mechanisms of resistance, for potential application in high throughput screening of wheat breeding lines.

4.3. Materials and methods

4.3.1. Plant and pathogen production

The seeds of spring wheat cultivars, with varying levels of resistance to FHB (cvs. BRS177, Nobeoka Bozu, Wangshubai, Frontana, AW488 and CEP24) were obtained from the Centre de recherché sur les grains (CEROM) and Agriculture and Agri-Food Canada (AAFC). Cultivar CEP24 was considered the least resistant. The seeds were sown in 15 cm pots, and these maintained in the greenhouse at 22±3 ^{o}C. Plants were twice fertilized with a

0.2 percent solution of 20-20-20 NPK, once at tillering and a second time at the boot stage, each time. Seven-day old cultures of *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch; isolate 99–15–35; obtained from Dr. S. Rioux, CEROM, QC], were inundated with water, and then filtered through two layers of cheesecloth. A spore suspension was made up in an aqueous solution of 0.02 percent Tween 80, and adjusted to contain 10^5 macroconidia ml^{-1}.

4.3.2. Inoculation and incubation

Wheat spikes were area-source inoculated by putting 10 µl of macroconidial suspension into the middle florets of each of four mid-spike spikelets (Gary et al*.,* 2000). At anthesis (GS=60-69), one such spikelet was inoculated for disease severity assessment, (Zadoks et al*.,* 1974). Spikelets inoculated with 10 µl of distilled water containing 0.02 percent Tween 80 served as control. After inoculation, plants were kept under saturated atmosphere at 20-25 ^oC for 24 h.

4.3.3. Disease severity assessment

The number of diseased spikelets in a spike was determined at 4 d intervals until 20 d after inoculation (dai). The FHB severity was measured as the proportion of diseased spikelets, including the inoculated spikelets, within a single spike. The area under the disease progress curve (AUDPC) was calculated using disease severity data (Hamzehzarghani et al*.,* 2005).

4.3.4. Metabolite extraction and GC/MS analysis

Sixteen inoculated spikelets (4 mid-spike spikelets from four wheat plants), including the rachis, were pooled as a single sample or experimental unit. These were harvested at 24 hai and crushed immediately in liquid nitrogen to deactivate any hydrolytic activity. The metabolites were extracted according to methods modified from Roessner (Roessner et al., 2000) and Fiehn (Fiehn et al*.,* 2000a, b), with minor modifications. These included changes in the amount of plant tissue and the volume of solvents used, as well as the use of a freeze dryer instead of a SpeedVac to dry the samples. At the outset, 50 µl of 0.2 mg ml^{-1} of ribitol in water or 2 mg ml^{-1} of nonadecanoic

acid in chloroform were added to the polar and non-polar fractions, respectively, to serve as internal standards. One microliter aliquots of these samples were injected into a gas chromatograph (GC) equipped with an ion trap mass analyzer (GC/MS with ion trap analyzer, Varian Inc.) as reported in our earlier study (Hamzehzarghani et al., 2005).

4.3.5. Metabolite profiling

The GC/MS output of total ion chromatograms (Varian Saturn "sms" file format) was converted into a universal "net.cdf" file format using the mass spectral file translator (MassTransit version 3.0.1.16, Palisade Corp., NY) to render the data compatible with several bioinformatic programs used in such studies. The chromatograms were first analyzed using the MetAlign software package to compensate for gradual shifts in baseline signal or background noise (Tikunov et al., 2005). The baseline-corrected chromatograms were analyzed using AMDIS (Automated Mass spectral Deconvolution and Identification System; Version 2.64) for the deconvolution of the peaks, extraction of the baseline corrected mass spectra of the co-eluting components and the identification of the retention times of each component, or mass spectral tags. A mass spectral tag (MST) was defined as a mass spectrum with a consistent fragmentation pattern over scans and across replicates. A MST can be considered a true component of a sample irrespective of its chemical identity (Kopka et al., 2005). The MSTs were tentatively identified by searching MSRI libraries (GOLM metabolome database; Kopka et al., 2005) and the NIST library (version 2.0d, 2005, Palisade Corp., NY) to identify the optimal spectral match. The observed mass spectrum for a peak within the chromatographic profile was compared with each of the ten topmost choices in the NIST and MSRI libraries to assign an identity to a component using its retention time as a reference. If a given spectrum did not provide a suitable fit within the NIST/MSRI libraries across the replicates, the peak was designated as unidentified. All the MSTs detected here, regardless of their identity, were regarded as metabolites in this study.

4.3.6. Data processing

The outputs from AMDIS saved as ”elu” and “fin” files, were processed using the MET-IDEA software (METabolomics Ion-based Data Extraction Algorithm; version 1.2.0) to align the peaks across samples and to calculate their abundance (Broeckling et al., 2006). MET-IDEA processed one representative master total ion chromatogram from the data folder and extracted the list of retention times and ion markers (IRT) for each component across the master profile. The generated ion list was saved as an “ion” file and used for the calculation of the calibration parameters which were stored in a ”cal” file. To apply directed extraction of ion intensity values, MET-IDEA scanned the data folder and recognized all “net.cdf” profiles, based on a list of IRT-values and calibration of the profiles. Finally, the results of the abundance of components were stored in a single tab-delimited text file with compound identifiers as column headings and sample names as rows.

The data were copied into an MS-EXCEL spreadsheet, and using the Pivot Table procedure, the peaks were filtered to remove any peak with a low signal to noise ratio (S/N<20), a low peak purity (<20 percent), peaks from column bleed as well as peaks inconsistent across replicates. A metabolite profile for a sample consisted of the names, the associated retention times and the areas under the peak or the amount of each metabolite relative to the total ion current. Ultimately, the abundance of each peak across the study was adjusted for inter-sample variations by dividing them by the abundance of internal standards as a scaling factor.

4.3.7. Experimental design and statistical analysis

The experiment was designed as a completely randomized block design with four replicates of six cultivars inoculated with pathogen (treatment) or water (control). Each replicate or experimental unit consisted of a pooled sample of 16 spikelets (four spikelets harvested from four spikes of four plants). The data on disease severity (proportion of spikelets diseased out of ten and AUDPC) and on metabolic profiles (abundance of metabolites) was subjected to statistical analyses, using SAS (SAS Institute, 1999).

4.3.7.1. Hierarchical cluster analysis of disease severity

To identify similarities among cultivars and to group them into disease severity phenotypes (DSP), data on disease severity (AUDPC) was subjected to hierarchical cluster analysis, using the CLUSTER procedure in SAS (SAS Institute, 1999).

4.3.7.2. Univariate analysis of variance of metabolite profiles

The metabolite profiles of each of the five resistant cultivars were individually compared to that of the least resistant cultivar CEP24. A metabolite with a significantly ($P \leq 0.05$) or borderline significantly ($P \leq 0.1$) higher abundance in a resistant cultivar compared to the susceptible cultivar, CEP24, was defined as a resistance-related (RR) metabolite.

4.3.7.3. CANDISC and HCA of metabolite profiles

Data on the abundance of metabolite profiles with significant treatment effects for the six cultivars, inoculated with pathogen or water, were subjected to canonical discriminant analysis (CDA). The CANDISC procedure of SAS was used to characterize metabolite profile phenotypes (MPP) and to identify hidden biological functions. The CANDISC procedure of SAS developed linear combinations of the metabolites (CAN-vectors) that summarized between-class variation. This procedure increased the resolution of the clustering pattern by minimizing the within-cluster variance and maximizing the between-cluster variance (Johnson, 1998). The CANDISC procedure computed squared Mahalanobis distances among cultivars using the abundance of their metabolites as response variables. The CAN-vectors are classifiers of the cultivars into metabolite profile phenotypes. The metabolites significantly loaded to each CAN-vector were used to explain the hidden host-pathogen interaction functions, taking into consideration the background relation of MPP to DSP (the disease severity phenotypes).

The canonical score of each treatment from CDA was used for cluster analysis for further classification of treatments. The Euclidean distance between group centers in the canonical space, computed using CAN-scores, was used as a measure of the similarity of groups. Using all the significant CAN-vectors, this procedure allowed the construction of a similarity measure

matrix and a dendrogram, permitting a better visualization of cultivar groupings.

4.4. Results

4.4.1. Disease severity and the DS phenotypes (DSP) of cultivars

All pathogen-inoculated spikelets developed FHB symptoms and the disease severity varied between cultivars. The means of the proportion of spikelets diseased (at 20 dai) and the AUDPCs for the six cultivars, in decreasing order of resistance, were AW488, 0.2 and 0.52; Nobeoka Bozu, 0.233 and 0.60; Wangshubai, 0.267 and 0.62; Frontana, 0.367 and 1.03; BRS177, 0.4 and 1.05; and CEP24, 0.867 and 2.08, respectively. The correlation coefficient between the two disease parameters was 0.96 ($P \leq 0.0001$). A hierarchical cluster analysis of disease severity in six wheat cultivars varying in their level of FHB resistance, based on an AUDPC-based classification, yielded three disease severity phenotypes (DSP). The DSPs and associated AUDPCs, in decreasing order of resistance, were: i) DSP1 (resistant, 0.517-0.617): Wangshubai, AW488, Nobeoka Bozu; ii) DSP2 (moderately resistant, 1.033-1.050): BRS177, Frontana; iii) DSP3 (susceptible or slightly resistant, 2.083): CEP24 (Fig. 4.1). The qualitative descriptors of DSPs (*i.e.,* the resistance categories) used here were arbitrary and chosen to facilitate comparison of DSP to MPP.

4.4.2. Metabolite profiles

Of several hundred peaks detected in the polar and non-polar extracts, 214 MSTs (polar=1-100; non-polar=101-214) consistent across replicates were selected as metabolites occurring in the cultivars tested. These 214 metabolites were tentatively identified using the NIST and MSRI libraries. The quantities of these 214 metabolites from water and pathogen inoculations were separately subjected to univariate ANOVA to filter out the metabolites with less likely significant treatment effects. A total of 79 metabolites had significant treatment effects, of which 54 and 46, respectively, were from pathogen- and water-inoculated cultivars, including 21 common to both treatments. Neither the inoculations nor the cultivars varied in the kind of metabolites detected, but the levels of metabolites differed significantly. The

abundance of 79 metabolites, including 54 from the pathogen-inoculated and 46 from the water-inoculated cultivars were separately subjected to univariate ANOVA to select RR metabolites (Table 4.1).

4.4.2.1. Resistance-related constitutive (RRC) metabolites

RRC metabolites are the homeostatic metabolites from water-inoculated cultivars showing significantly (P<0.05) higher levels in resistant cultivars compared to the susceptible cultivar CEP24 (*i.e.*, RRC=RW>SW). Thirteen RRC metabolites identified in this study were myo-inositol; octadecanoic acid (=stearic acid); 8,11-Octadecadienoic acid; succinic acid; propanoic acid; aspartic acid; galactose; alanine; 2-propenoic acid and four unidentified peaks with MSRI hits (metabolite numbers 36, 45, 48 and 65) (Table 4.1). Twelve potential RRC metabolites were classified as borderline significant RRC metabolites (0.05<P≤0.1). These included 2-butenoic acid; 2, 3, 4-trihydroxybutyric acid; pentonic acid-1, 4-lactone; cyclohexanol; and cadaverine. Twenty-five RRC metabolites were identified. The number of RRC metabolites identified in each cultivar were [(P≤0.05)/(P≤0.1)]: Wangshubai= 12/22 (3, 14, 15, 16, 19, 20, 22, 28, 35, 36, 37, 45, 48, 60, 65, 66, 71, 73, 74, 76, 95, and 135), AW488=0/1 (60), Nobeoka Bozu=1/2 (20 and 60), BRS177=0/0 and Frontana=3/7 (15, 20, 26, 28, 60, 67, and 68).

4.4.2.2. Resistance-related Induced (RRI) metabolites

The RRI metabolites were the metabolites at significantly (P≤0.05) greater levels in pathogen *vs.* water-inoculated spikelets of a given cultivar, along with those of pathogen-inoculated spikelets of the susceptible cultivar CEP24 (RRI = RP>RW>SP). Among the RR metabolites, eighteen were RRI metabolites. These belonged to such chemical groups such as phenolics (61, 84, 126, 120, and 158), organic acids (14, 16, 21, 66, 73, and 118), sugars (55, 60, 82, and 91), ketones (62), and 2 unidentified MSRI hits (59 and 162) (Table 4.1). Some important RRI metabolites were *trans*-*p*-coumaric acid; *p*-cinnamic acid; *trans*-ferulic acid; benzoic acid (phenolics); myo-inositol; glucopyranose; fructose (sugars); fatty acids such as heptadecanoic acid; octadecanoic acid; butanedioic acid; 2-butenoic acid; 2-propenoic acid; propanoic acid and cyclohexanone. There were four borderline significant

(0.05<*P*≤0.10) RRI metabolites that included scopolin, and an important coumarin in the cultivar AW488. Overall, twenty-two RRI metabolites were identified, their number varying from cultivar to cultivar. The cultivar specific RRI metabolites were [(*P*≤0.05)/(*P*≤0.1)]: Wangshubai= 4/6 (66, 73,120, 124, 126, and 162), AW488=10/14 (14, 19, 21, 55, 59, 60, 61, 62, 77, 84, 158, 162, 174, and 118), Nobeoka Bozu=3/3 (60, 126 and 162), BRS177=9/12 (14, 16, 19, 20, 21, 66, 82, 84, 91, 126, 158, and 162) and Frontana=2/6 (14, 66, 124, and 126). Some 41 RR metabolites were identified, including 25 RRC metabolites and 22 RRI metabolites, with six in common (Table 4.1).

4.4.3. Metabolite profile phenotypes and metabolic functions

4.4.3.1. Constitutive metabolite profile phenotypes

The canonical discriminant analysis of the abundance of 46 metabolites measured in the six cultivars following water inoculation summarized 98 percent of their variance in the first three CAN-vectors (Fig. 4.2, a). The CAN1 vector explained 81 percent of the variance in the abundance of metabolites and classified the six wheat cultivars into two main clusters of constitutive metabolic profile phenotypes - CMPP1: AW488, Wangshubai, Nobeoka Bozu with high CAN1 scores, and CMPP2, BRS177, Frontana and CEP24 with low CAN1 scores (Fig. 4.2, a). The cultivars classified in CMPP1 were quite similar to those in the DSP1 and accordingly the CAN1-vector was considered to explain high constitutive resistance. The CAN2-vector explained 14 percent of the variance and separated the cultivars within the CMPP1. Wangshubai, Nobeoka Bozu and AW488 had high, moderate and low CAN2-scores, respectively (Fig. 4.2, a). In contrast, the cultivars in CMPP2 were not separated across the CAN2-vector.

A total of 6 and 16 RRC metabolites had high loadings (>0.4) to the water-inoculated CAN1- and CAN2-vectors, respectively. The RRC metabolites with high loadings to the CAN1- and/or CAN2-vectors were: myo-inositol; 8,11-octadecadienoic acid; propanoic acid; octadecanoic acid; succinic acid; 2-butenoic acid; pentonic acid-1, 4-lactone; cadaverine; octadecanoic acid; propanoic acid and the following five unidentified MSTs (45, 48, 65, 37, and 71)(Table 4.1; *P*≤0.05 shown with stars).

4.4.3.2. Constitutive and induced metabolite profile phenotypes

The first three CAN-vectors explained 98 percent of the variance in the abundance of 56 metabolites measured in the six cultivars after their inoculation with the pathogen. The CAN1-vector explained 62 percent of the variance as it classified the six cultivars into three clusters: CIMPP1, AW488, BRS177 and Frontana; CIMPP2, Wangshubai and Nobeoka Bozu, and CIMPP3, CEP24. The susceptible cultivar CEP24 had the lowest CAN1-score, with higher scores for all the resistant cultivars. Therefore, the CAN1 vector was considered to explain induced resistance. Among the resistant cultivars, BRS177 and Frontana appeared to switch from lower levels of constitutive resistance (Fig. 4.2, a) to higher levels of induced resistance (Fig. 4.2, b).

The CAN2-vector explained 28 percent of the variation and classified Nobeoka Bozu with high scores (Fig. 4.2, b; Fig. 4.3). A total of nine and five RRI metabolites had high loadings (>0.4) for the pathogen-inoculated CAN1- and CAN2-vectors, respectively (Table 4.1). Heptadecanoic acid; phenol, 2,5-bis(1,1-dimethyl ethyl)-; cyclohexanone; *trans-p*-coumaric acid; melezitose (11TMS) alpha-D-Glc-(1,3)-beta-D-Fru-(2,1)-alpha-D-Glc); propanoic acid; benzoic acid; 2-propenoic acid; butanedioic acid; myo-Inositol; scopolin; cadaverine; and one unidentified MSRI component (Table 4.1; $P \leq 0.05$ indicated with stars) are examples of these metabolites.

4.5. Discussion

The present study reports on the application of metabolite profiling techniques, based on GC/MS coupled with univariate and multivariate statistical analyses, to classify quantitative FHB resistance in wheat and to identify resistance-related metabolites. Of 214 metabolites detected in six cultivars, 41 were identified as RR metabolites based on univariate analysis and 45 as resistance function-related metabolites based on multivariate analyses, including 27 that were common to both types. These 27 RR metabolites, of known resistance function, can be used as biomarkers for resistance screening. More stringent screens based on metabolite significance and CAN-loadings, should be used to select metabolites that are more promising. Among the 27 RR metabolites, 19 and 12 were RRC and RRI metabolites, respectively (Table 4.1). The highly resistant cultivar Wangshubai

had the greatest number of RRC metabolites, followed by the moderately resistant cultivar Frontana. The highly resistant cultivar AW488 had the greatest number of RRI metabolites, followed by the moderately resistant cultivar BRS177. The highly resistant cultivar Nobeoka Bozu had few RRC or RRI metabolites. In addition to the number of RR metabolites, their type and quantities may play a significant role in resistance.

As with proteomics and transcriptomics, one of the major problems in metabolomics is data visualization. To select RR metabolites, several filtration steps have been used. Only the metabolites that were significant at $P \leq 0.2$ were retained for further statistical analysis to exclude the metabolites with high biological variability (outliers) from entering into the multivariate data analysis (Sumner et al., 2003). Canonical discriminant analysis was used both as a data reduction and as an exploratory technique to enhance the transparency and improve the data visualization and excavation of the possible mechanisms of FHB resistance in wheat. To classify a metabolite as 'RR,' the criterion of a $P \leq 0.05$ significance level was applied to univariate ANOVA. However, some known antimicrobial metabolites or their precursor molecules were not statistically significant at this level. As a result, metabolites of borderline significance ($0.05 < P \leq 0.10$) were also classified as RR metabolites. To assure these potential metabolic biomarkers, associated with metabolic functions with high CAN-loadings, could serve as effective criteria for screening wheat cultivars for FHB resistance, we searched for their mention in reports regarding phytochemicals with antimicrobial or signaling properties. Approximately one third of the high CAN-loading metabolites, associated with resistance functions, were not classified as RR metabolites due to the elevated variance among replicates, as assessed by univariate ANOVA. This experimental error might be the result of variations in seed population, the use of multiple tissue types within the sample (the lemma, palea, rachis, and reproductive parts), variations in anthesis status at inoculation and the manual extraction of metabolites.

The manual processing of GC/MS output data is slow and labor intensive. Automation is imperative for practical applications of a metabolomic approach to study stresses in plants. The amount of background noise in certain chromatograms was elevated so that baseline correction with MetAlign

(Tikunov et al., 2005) was unavoidable to process the AMDIS data output using MET-IDEA (Broeckling et al., 2006). This enabled the quantification of the abundance of metabolites. These bioinformatic tools have reduced the error in the manual processing of GC/MS output data, as well as increased the speed of processing. The file format limiting the use of these bioinformatic tools was overcome by translating the GC/MS output in Saturn "sms" file format to "net.cdf" using MassTransit. The NIST library (2005, version 2.0d) and the MSRI libraries (Kopka et al., 2005) served as a basis for comparison. The latter collection contains mass spectra generated predominantly from plant sources (Wagner et al., 2003). Even though several of the 214 MSTs were not identified in this study, the MSTs represent analytes that were clearly linked to a chemical structure. These MSTs can be treated in a similar manner as identified metabolites, a name being assigned to them after their first description (Kopka, 2006).

A further limitation of metabolite profiling is the analytical platform. GC/MS was used because the technique has been successfully employed to profile various other biological systems (Roessner et al*.,* 2000 and 2001; Fiehn, 2002). It is very sensitive and has a high resolving power although it lacks the ability to detect non-volatile compounds (Johnson et al*.,* 2003). GC/MS is not applicable to certain known plant defense compounds that are semi-polar, including flavonoids and saponins. Therefore, the use of liquid chromatography and mass spectrometry (LC/MS) might result in a better differentiation of resistance (Vorst et al*.,* 2005). A disadvantage of LC/MS is the lack of a commercial library necessary to the identification of non-target metabolites.

Canonical discriminant analysis identified two metabolic profile phenotypes based on constitutive metabolites, and three based on both constitutive and induced metabolites. The metabolite profile phenotypes were not identical to the disease severity phenotypes. It may be an indication of the existence of more types of resistance mechanisms than the number of discernible DSPs. The cultivar AW488 was associated with high CAN-scores that identified both constitutive and induced resistance functions, and while associated with high numbers of RRI metabolites, had few RRC metabolites, based on univariate ANOVA. The cultivar Wangshubai showed high CAN-

scores, indicating constitutive resistance and had the greatest number of RRC metabolites. The cultivars BRS177 and Frontana had low CAN-scores, representative of constitutive resistance and high CAN-scores representative of induced resistance, respectively. While the former cultivar was associated with several RRI metabolites, the latter cultivar was not. Instead, it had moderate numbers of both RRC and RRI metabolites. Therefore, the mechanism of resistance appears to be complex. Not only may the number of RR metabolites play a substantial role in mechanisms of resistance but also their type and abundance.

An unsupervised classification of wheat cultivars using HCA and based on disease severity ratings grouped AW488, Wangshubai and Nobeoka Bozu as DSP1. Both Wangshubai and Nobeoka Bozu have an Asian origin and seem to be related to Sumai3. Wangshubai is a landrace from the Chinese province of Jiangsu. There is contradictory information about its resistance to FHB. Most likely Wangshubai and Sumai3 have the same major FHB resistance QTL on 3BS (Zhou et al., 2003). However, another study reported that Wangshubai had no alleles in common with Sumai3 (Gonzalez et al*.,* 2003). Nobeoka Bozu is highly resistant to FHB (Miedaner, 1997). It harbors three resistance genes, including two that are unique and a third that is identical to one in Sumai3 (Ban, 2003). In our study, very few RRC and RRI metabolites were detected in Nobeoka Bozu. Univariate ANOVA identified 14 RR metabolites in Wangshubai while only three RR metabolites (two of them the key RR metabolites *trans*-*p*-coumaric acid and myo-inositol) were found in Nobeoka Bozu.

Frontana and BRS177 originated from Brazilian germplasms used as a source of FHB resistance in Canadian winter wheat breeding programs (Gilbert and Tekauz, 2000). The two cultivars differ in their constitutive resistance despite sharing similar genetic background as a result of their common center of origin. Frontana possesses either type I, or type I and II resistance to FHB (Kolb et al*.,* 2001). Several studies have demonstrated the involvement of at least three additive genes in the resistance of Frontana to FHB (Siranidou et al*.,* 2002).

Several of the RR metabolites identified here have been reported to possess antimicrobial properties, to be involved in signaling pathways or the

synthesis of signal molecules. Different types of cinnamic acids, identified as RR metabolites, are precursors of lignin, a polymer known to reinforce cell walls and help the plant cell resist pathogens (Gogoi et al., 2001; Siranidou et al*.,* 2002). The greater abundance of phenolic compounds such as *p*-coumaric and ferulic acids in the glumes of the FHB resistant wheat cultivars (Siranidou et al., 2002) and their *in vitro* antifungal activity against *Fusarium* spp. (McKeen et al., 1999) have been documented.

Scopolin, classified as a RRI metabolite, has been detected in the highly resistant cultivar AW488. The compound 4H-1-benzopyran-4-one had high positive loadings on the pathogen-inoculated CAN1-vector but was not a RR metabolite. They belong to coumarins, a group of secondary metabolites with a 2H-1-benzopyran-2-one nucleus. These compounds have a broad spectrum of biological properties against various microorganisms (Daoubi et al., 2004). A negative correlation between coumarin content (especially scopolin) and *Sclerotinia* head rot severity of sunflowers has been reported (Parts et al., 2005).

Fatty acids are both antimicrobial compounds and precursors of signal molecules such as jasmonic acid. Myo-inositol, a sugar identified here as RR metabolite, is also an important sub-cellular signal molecule for jasmonic acid synthesis (Exton, 1996; Nelson et al*.,* 1998; Buchanan et al*.,* 2000).

Among organic acids with high loadings were the constitutive metabolites octadecanoic acid (73, 74) and 8, 11-octadecadienoic acid. These high molecular weight fatty acids, which are formed via the octadecanoid and hexadecanoid pathways, are also precursors of jasmonic acid (Farmer et al., 1998). Linolenic acid, a precursor of jasmonic acid, had high loadings to the water-inoculated CAN2-vector, consistent with its higher cellular demand in the synthesis of jasmonic acid. Jasmonic acid plays a key role in the salicylic acid independent pathways and activates multiple resistance mechanisms (Pieterse and Van loon, 1999). Other organic acids identified as RR metabolites were propanoic acid (Strobel, 2006) and butanedioic acid, both of which are part of the diterpenoids. These metabolites along with 4-aminobutyric acid are known to have antimicrobial activity (Walker et al., 2003; Xue et al., 2004).

Myo-inositol was detected as both a RRI and RRC metabolite. Myo-

inositol is involved in sub-cellular signaling, gene expression and the control of intracellular Ca^{2+} concentration (Exton, 1996). A positive correlation between a plant's disease resistance level and levels of this unique molecule has been demonstrated (Berridge and Irvine, 1989). The higher Ca^{2+} released in response to myo-inositol causes activation of protein kinases mediating activation of the NADPH oxidase complex, which in turn catalyses the production of superoxide ions from oxygen molecules dismutated to H_2O_2 by superoxide dismutase (Hammond-Kosac and Jones, 1996; Lamb and Dixon, 1997).

Two polyamines, cadaverine and 1, 4-butanediamine (putrescine), showed high loadings at homeostatic and induced states. These polyamines are synthesized through decarboxylation reactions of lysine and ornithine. These metabolites are involved in the regulation of DNA replication, cell division and the intrinsic signaling network of the extracellular matrix, all of which lead to a higher level of resistance. (Galston and Sawhney, 1990).

We have used a combination of three major criteria in selecting resistance-related biomarker metabolites for potential application in high throughput screening. These are: a) RR metabolites based on univariate ANOVA; b) metabolites with high loading to CAN-vectors associated with resistance functions through multivariate analysis; c) metabolites with known antimicrobial properties or those that are signaling molecules and precursor of these metabolites. This combination of criteria allowed the metabolites unrelated to resistance to be filtered out. The abundance of 41 RR metabolites identified in this study, especially the 27 that also denoted resistance functions, can be applied to high throughput screening of wheat breeding lines for FHB resistance. In future testing, the lines with higher abundance of RR metabolites can be considered as resistant lines. However, the identification of RR metabolites is based on the statistical association of metabolites with resistant cultivars and on their putative role as antimicrobial and signaling compounds. Further confirmation using near isogenic lines with resistant and susceptible alleles for a given metabolite is required to corroborate the involvement of these RR metabolites in plant defense (Schauer and Fernie, 2006). We have only demonstrated the mechanisms of FHB resistance in wheat based on metabolite profiling and statistical analysis.

Table 4.1. List of resistance-related (RR) metabolites in 6 cultivars of wheat (A=AW488, B=BRS177, F=Fontana, N=Nobeoka Bozu, W=Wangshubai, and C=CEP24) at 24 h after inoculation with *Fusarium graminearum* (P) or water (W), and the CAN-loadings of each metabolite to the first two CAN-vectors based on canonical discriminant analysis.

RN[b]	NIST/GMD metabolite names[c]	CAS[d]	A	B	F	N	W	P-Inoc. Can1[e]	W-inoc. Can2	Can1	Can2
162	EIQTMS_N12C_LESR_2707.8_04014A50 (g)	NA	I*	I*		I*	I*	0.25	-0.68		
126	*trans-p*-Coumaric acid (G)	10517303		I*	I*	I*	I	-0.69	**0.51**		
20	Alanine (Ng)	54986651		I	C	C	C*			-0.44	-0.12
14	2-Butenoic acid (Ng)	55044796	I*	I*	I		C	0.33	-0.01	**0.66**	0.72
60	Myo-Inositol (Ng)	2582798	CI*		C*	CI*	C*	-0.01	0.53	**0.40**	-0.58
19	Cadaverine (N)	462942	I	I			C	**0.57**	0.00	**0.42**	**0.82**
66	2-Propenoic acid (Ng)	40333082		I*	I*		CI*	0.39	-0.39	-0.57	-0.16
158	*trans*-Ferulic acid (2TMS) (G)	NA	I	I*				0.34	-0.44		
124	9-Hexadecenoic acid (N)	1120258			I		I	-0.82	-0.43		
15	Succinic acid (2TMS) (G)	NA			C*		C*			-0.63	**0.57**
28	Pentonic acid-1,4-lactone (3TMS) (G)	NA			C		C	**0.75**	0.28	**0.44**	**0.72**
16	Propanoic acid (Ng)	38191876		I*			C*	**0.48**	0.11	0.22	**0.88**
84	Benzoic acid (N)	75941843	I*	I*				**0.78**	-0.16	-0.68	0.09
21	Butanedioic acid (Ng)	NA	I*	I				**0.69**	0.08	-0.06	-0.18
118	Heptadecanoic acid (G)	NA	I*					**0.78**	0.32		
77	Scopolin (Ng)	EPA-108989	I					**0.74**	0.54		
82	Fructose (Ng)	39523074		I*				0.26	-0.52		
59	EIQTMS_N12C_LCHFR_4001.7_03363A10_	NA	I*					0.01	**0.58**		
61	Phenol, 2,5-bis(1,1-dimethylethyl)- (Ng)	5875456	I*					0.01	**0.58**		
95	Trehalose (8TMS) (G)	NA					C	0.19	**0.53**		
62	Cyclohexanone (Ng)	94318282	I*					0.20	**0.52**		
174	Cyclohexane (N)	55282025	I					0.07	0.36		
120	Cinnamic acid, *p*- (N)	27798692					I*	-0.43	0.34		
55	Glucopyranose (5TMS) (G)	NA	I*					0.16	-0.11		

74	Octadecanoic acid (G)	NA			**C**			**0.45**	**0.75**
71	EIQTMS_N12C_LPIL_1615.5_3268AU29 (g)	NA			**C**			0.28	**0.58**
68	Propanoic acid (N)	5129351		**C**				0.15	**0.71**
45	EIQTMS_N12C_LESFR_1893.7_3090AU02 (g)	NA			**C***			-0.01	**0.54**
48	EIQTMS_N12C_LJALD_2595.0_2236bg34 (g)	NA			**C***			0.03	**0.44**
37	EIQTMS_N12C_LCHFR_2275.9_03363A10 (g)	NA			**C**			-0.19	**0.41**
36	EITTMS_N12C_STUT_1852.9_1135ec30 (g)	NA			C*			-0.22	0.27
26	2,3,4-Trihydroxybutyric acid (Ng)	38191887		C				-0.28	0.24
3	Cyclohexanol (Ng)	27203925			C			-0.73	0.22
35	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Ng)	102608537			**C**	0.36	0.31	**0.53**	**0.76**
91	Melezitose(11TMS) (G)	NA	**I***			**0.71**	0.09	**0.51**	-0.09
135	8,11-Octadecadienoic acid (N)	56599587			**C***	-0.63	**0.44**	0.07	**0.92**
65	EIQTMS_N12C_LPAL_2715.8_3268AU16 (g)	NA			**C***	**0.44**	0.36	0.26	**0.77**
76	Cholan-16-one, 23-methyl-, (5, 20.xi.)- (Ng)	56143170			**C**	0.33	0.34	0.28	**0.74**
73	Octadecanoic acid (N)	74484778			**CI***	0.12	-0.13	0.17	**0.44**
67	Galactose (Ng)	120850886		**C***		**0.43**	-0.38	-0.58	-0.15
22	Aspartic acid (Ng)	15985054			C*	0.32	-0.02	-0.19	-0.17
53	Lyxofuranoside (Ng)	56390035				**0.83**	0.36		
33	Arabinofuranose (Ng)	55399490				**0.63**	0.33		
97	Sucrose (8TMS); alpha-D-Glc-(1,2)-beta-D-Fru (G)	NA				**0.62**	0.50		
99	Glucopyranoside (Ng)	60354430				**0.58**	**0.56**		
98	4H-1-Benzopyran-4-one, (N)	113486365				**0.59**	**0.56**		
136	9,12,15-Octadecatrienoic acid (N)	301008				-0.19	**0.52**		
107	EIQTMS_N12C_LHIL_1328.1_3268AU18 (g)	NA						**0.50**	**0.63**
88	Ribofuranose (Ng)	56271693						**0.47**	**0.76**
85	EIQTMS_N12C_LESL_2697.7_2352AI10 (g)	NA						**0.46**	**0.77**
75	Glucuronic acid (Ng)	55530808						**0.46**	**0.76**
72	Linolenic acid (G)	NA						0.31	**0.64**
47	EIQTMS_N12C_LJAF_1897.1_2236bg54 (g)	NA						0.01	**0.76**
10	Phosphoric acid (3TMS) (G)	NA						-0.72	**0.47**
13	Glycine (Ng)	5630820				0.37	0.22	**0.46**	**0.76**
29	1,4-Butanediamine (Ng)	39772639				-0.11	0.71	**0.45**	**0.66**

RRC metabolites: *P≤0.05 (P≤0.1)*	1(1)	0	3(5)	1(2)	12(22)
RRI-metabolites: *P≤0.05 (P≤0.1)*	10(14)	9(12)	2(6)	3(3)	4(6)
RR metabolites	14	12	11	4	26

[a] RR metabolites are constitutive (RRC = C = RW>SW); induced (RRI = I = RP>RW>SP) and both (CI) – details in text; letters with superscript star (*) are significant at $P \leq 0.05$ and the rest are borderline significance at $0.05 < P \leq 0.10$; the letters in bold. are RR metabolites with high loading (>0.4).

[b] RN = Metabolite reference number of 214 MSTs or metabolites tentatively identified in this study, RN= 1-100 and RN=101-214 are from Polar and Non-polar fractions respectively (see supplementary Table 4.1, Appendix 1).

[c] Metabolite name = short names of metabolites; the complete names are available in the NIST library by searching for the CAS number and details in the supplementary Table 4.1 (Appendix 1).

[d] CAS = Chemical abstract service number according to NIST.

[e] CAN = CAN-loadings of metabolites based on the canonical discriminant analysis of the abundance of metabolites with significant treatment effects, separately for water and pathogen inoculated spikelets.

Figure 4.1. Grouping of six wheat cultivars inoculated with *F. graminearum* (P) based on disease severity into disease severity phenotypes (DSPs). Dendrogram was generated based on hierarchical cluster analysis of AUDPCs. The scale represents the Euclidean distance in canonical space: A=AW488, W=Wangshubai, N=Nobeoka Bozu, B=BRS177, F=Frontana, C=CEP24, and the numbers following letters are the replicates.

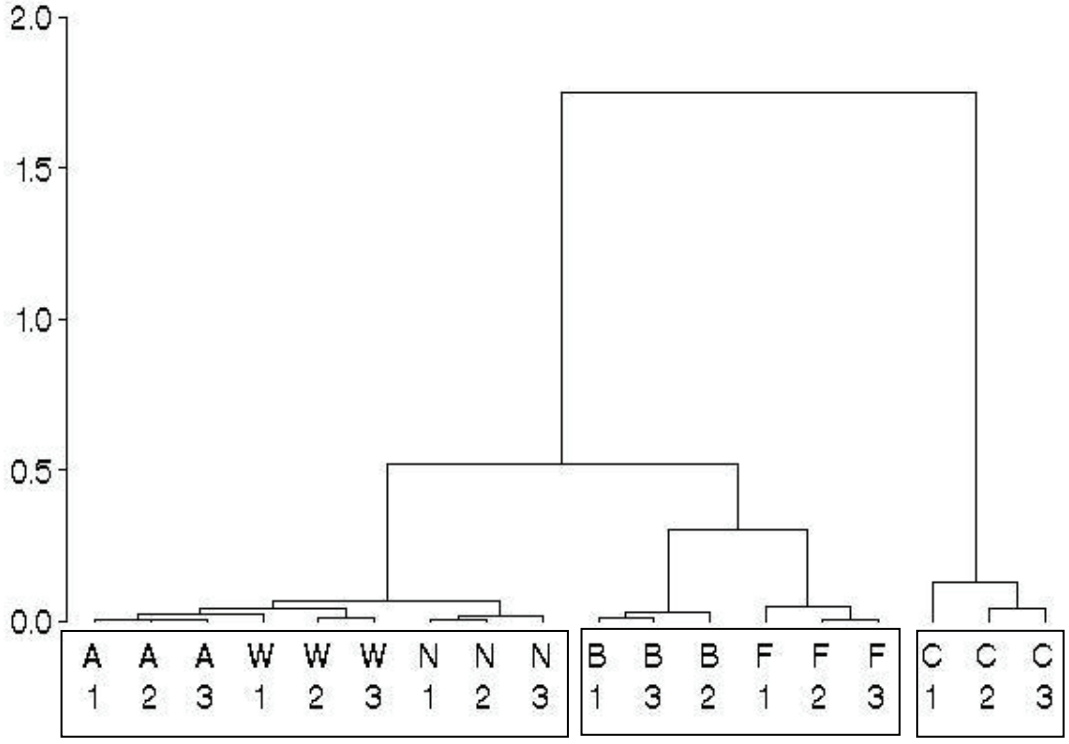
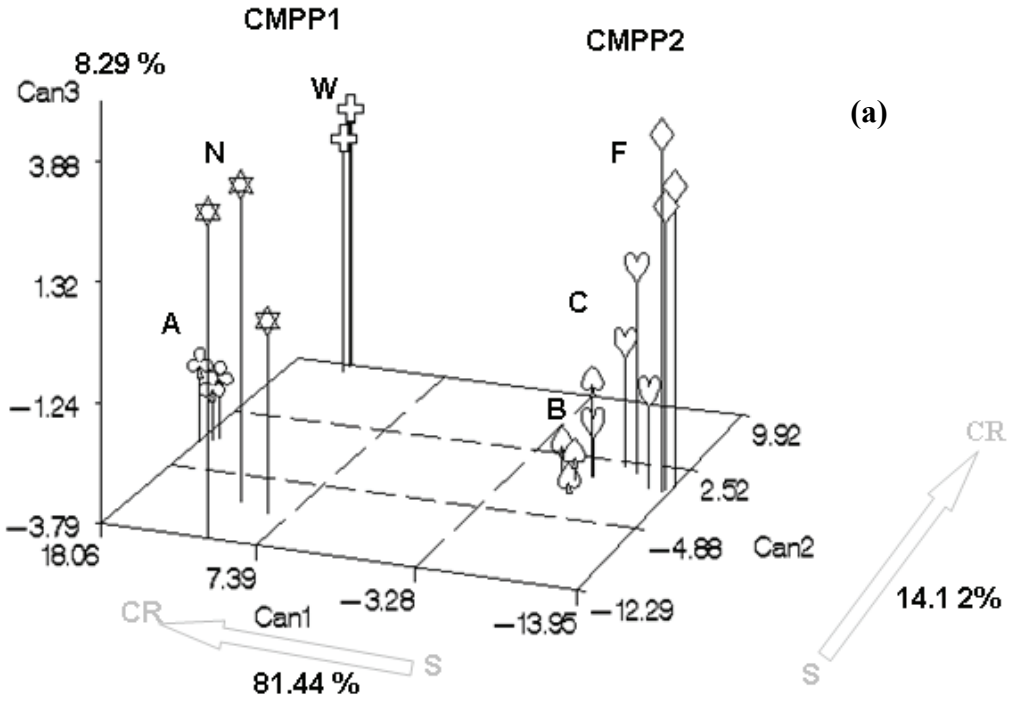
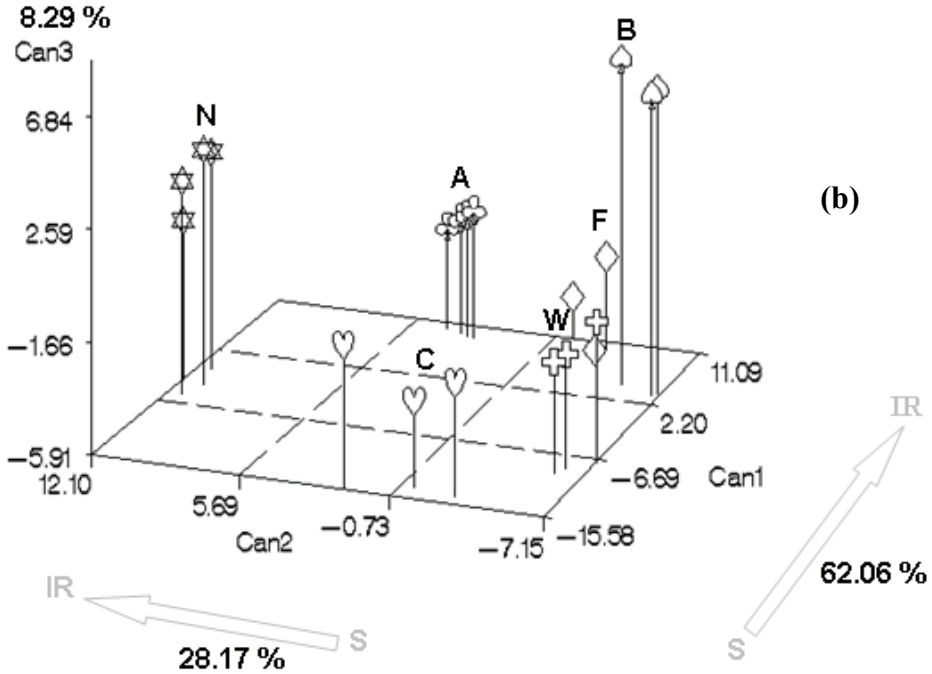

2.0
1.5
1.0
0.5
0.0
A A A W W W N N N B B B F F F C C C
1 2 3 1 2 3 1 2 3 1 3 2 1 2 3 1 2 3

Figure 4.2. Scatter plot, based on projections of the first three significant CAN-vectors of canonical discriminant analysis (CDA) of abundance of 46 and 56 metabolites in water-inoculated (a) and pathogen-inoculated (b), spikelets respectively. The symbols are A=AW488, B=BRS177, C=CEP24, F=Frontana, N=Nobeoka Bozu, W=Wangshubai, S=susceptibility, CR=constitutive resistance, and IR=induced resistance. The CDA identified two constitutive metabolic profile phenotypes (CMPPs) in the water-inoculated group of plants (a) and three constitutive and induced metabolite profile phenotype (CIMPPs) in the pathogen-inoculated plants (b).

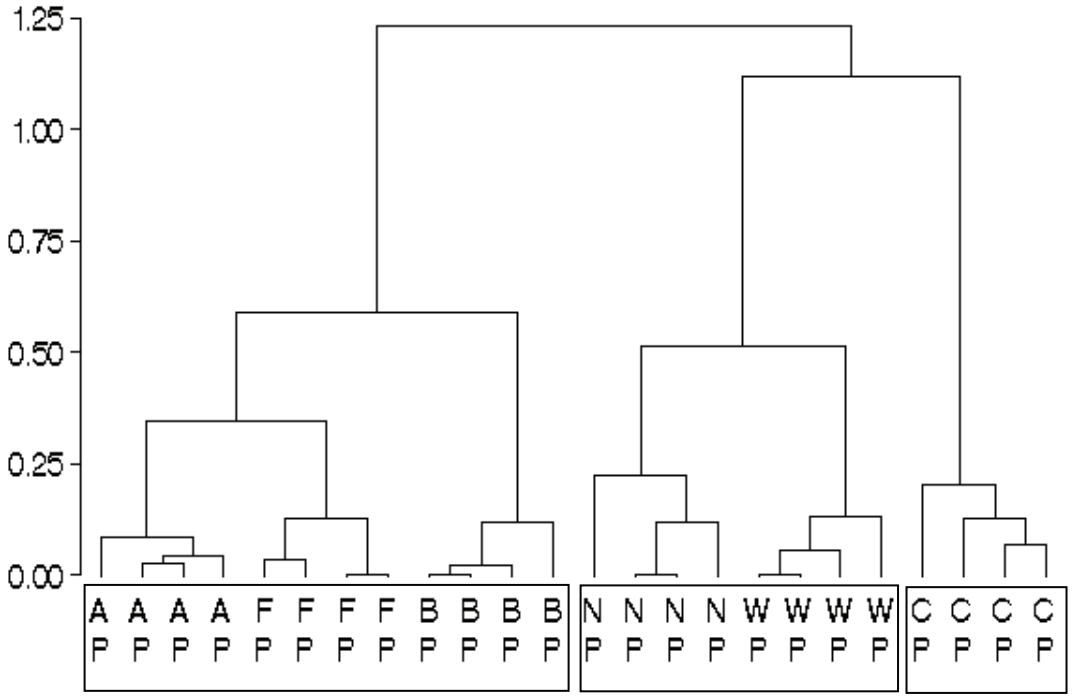

CMPP1
CMPP2
8.29 %
Can3
3.88
1.32
−1.24
−3.79
W
N
A
F
C
B
9.92
2.52
−4.88
Can2
18.06
7.39
−3.28
−13.95
−12.29
Can1
CR
S
81.44 %
CR
S
14.1 2%

(a)


8.29 %
Can3
6.84
2.59
−1.66
−5.91
N
A
B
F
W
C
11.09
2.20
−6.69
Can1
12.10
5.69
−0.73
−7.15
−15.58
Can2
IR
S
28.17 %
IR
S
62.06 %

(b)

Figure 4.3. Dendrogram based on hierarchical cluster analysis using the first three canonical variables (see Fig. 4. 2) from the canonical discriminant analysis of abundance of 54 metabolites detected in six wheat cultivars inoculated with *F. graminearum* (P). The scale represents the Euclidean distance in canonical space. The six cultivars were clustered into three constitutive and induced metabolic profile phenotypes (CIMPPs): (A=AW488, B=BRS177, C=CEP24 F=Frontana, N=Nobeoka Bozu, W=Wangshubai, and in all cases, the second letter P=pathogen and W=water-inoculated.


1.25
1.00
0.75
0.50
0.25
0.00
A A A A F F F F B B B B N N N N W W W W C C C C
P P P P P P P P P P P P P P P P P P P P P P P P

Chapter 5

Metabolite profiles of near isogenic lines of wheat with quantitative trait loci at chromosome 2DL conferring resistance or susceptibility to Fusarium head blight.

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Preface to Chapter 5

Chapter 5 is a manuscript submitted for publication by Hamzehzarghani, H., Paranidharan, V., Kushalappa, A.C., Mamer, O., and Somers, D. 2007 Metabolite profiles of two near isogenic lines of wheat with alternate alleles of the QTL for resistance or susceptibility to FHB at chromosome 2DL. Journal of Experimental Botany.

In the first (Chapter 3) and second (Chapter 4) studies, we established multiple criteria to discriminate FHB resistance in wheat. Metabolite profiling of wheat lines with alternate alleles of a known QTL conferring FHB resistance and relating such profiles to multiple metabolic criteria will streamline the process of discovering the mechanism of resistance. It will help breeders and metabolite engineers make intelligent decisions regarding which genes to use for pyramiding, so as to obtain greater levels of resistance, as controlled by different kinds of metabolites.

Many QTLs have been identified in various populations of crosses between wheat cultivars varying in resistance to FHB (Waldron et al., 1999; Feredric et al., 1999; Anderson et al., 2001; Kolb et al., 2001; Somers et al., 2003; Young et al., 2005). These QTLs have been molecularly mapped and their association with quantitative resistance to FHB has been studied. Study of the metabolic profiles of these QTLs or segregating populations could provide a better understanding of the mechanism of resistance to FHB.

In this study, both analytical instrument and software platforms underwent significant modification. The analytical software platform was improved by modifying it with the most recent software developed for metabolomics. A GC equipped with a time of flight (TOF) mass spectrometer (HP6890 GC-TOF-MS with an autosampler) was used, allowing faster, more sensitive and more reliable mass spectra than the quadrupole and ion trap mass spectrometers used in the last two studies. After baseline correction and mass spectral deconvolution using AMDIS, mass spectral abundance was quantified using MET-IDEA software. This

guaranteed a faster, easier and more consistent data generation and analysis step.

5.1. Abstract

The resistance to Fusarium head blight (FHB), one of the most destructive diseases of wheat, is controlled by several quantitative trait loci (QTLs), which are expressed as five various types of resistance. The objective of this study was to identify resistance-related metabolites associated with a QTL that confers resistance to FHB. To achieve this goal, two near isogenic lines (NILs), with alternate alleles for the FHB resistance/susceptibility QTL on chromosome 2DL, were grown under greenhouse conditions. At anthesis, four florets in each spike were inoculated either with a macroconidial suspension of *F. graminearum* or with water. Metabolites were extracted from the spikelets using a mixture of methanol-water and chloroform, and subsequently analyzed using GC-TOF-MS. Compound identification and quantification were achieved using AMDIS, the MSRI libraries, the NIST library (2005), and MET-IDEA as the software platform. A total of 182 components were detected, of which 127 metabolites were tentatively identified and 122 showed significant variations related to treatment effects. A Student's *t*-test of the quantities of these metabolites identified 27 resistance-related (RR) metabolites (greater abundance in the resistant NIL), including 22 constitutive (RRC) and 8 induced (RRI) RR metabolites. There were also three metabolites whose levels increased following pathogen inoculation (RRCI). Canonical discriminant analysis was used to classify treatments and identify metabolic functions. The putative mechanisms of FHB resistance are discussed based on the following RR metabolites: i) phenylpropanoid compounds including *p*-coumaric acid, cinnamic acid, several coumarins, benzoic acid and methyl vanillate; ii) important signal or signal-related compounds including myo-inositol and octadecanoic acid derivatives.

Key words: Canonical discriminant analysis, functional genomics, *Fusarium graminearum*, metabolomics, resistance-related (RR) metabolites, quantitative trait loci (QTL), wheat, wheat scab.

5.2. Introduction

Fusarium head blight (FHB), which is commonly caused by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch), is one of the most severe diseases of wheat in North America. It can cause huge annual losses (Leonard and Bushnell, 2003). The use of resistant cultivars is considered the most practical way to manage this disease. There are five types of resistance to FHB: resistance to initial infection (type I), resistance to spread of the pathogen within a spike (type II), resistance to kernel infection (type III), yield tolerance (type IV) and decomposition or non-accumulation of the fungal toxin deoxynivalenol (DON) (type V) (Mesterhazy, 2003). The most studied type of resistance, type II resistance is controlled by a few major genes (Bai et al., 2003b).

Wheat resistance to FHB is quantitative and its measurement is mainly based on the number of spikelets infected within a spike, the spread of disease within a spike and by the amount of mycotoxins in kernels (Leonard and Bushnell, 2003). A high resistance to initial infection has been described as a hypersensitive reaction involving type I resistance, but its association with single genes is yet to be demonstrated. Under field conditions, it is often difficult to differentiate type I resistance from disease escape. Because of this uncertainty, more efforts have been put towards analyzing type II resistance. There is ample evidence that resistance is strongly influenced by environmental factors. Type II resistance is controlled by several QTLs. QTLs conferring resistance to FHB in breeding lines originating from different parts of the world have been identified on chromosomes 2DL, 2DS, 3AS, 3BSc, 3BS, 4B, 4DL, 5AS, 6BS, and 7BL (Yang et al., 2005). In the Chinese spring cultivar Sumai3, the most widely exploited QTL in breeding for resistance to FHB is located on chromosome 3BS and explains 15-60% of the phenotypic variation under resistance type II (Yang et al., 2005). This cultivar also carries FHB resistance QTLs on 5A and 6BS. In order to enhance the level of resistance in an elite cultivar, the QTLs must be pyramided and this requires the identification of the functions of these QTLs. We hypothesize that wheat lines, which differ in QTLs conferring resistance against

FHB, vary in their metabolite profiles, and that the metabolites associated with these QTLs can explain the mechanisms of FHB resistance in wheat.

Several metabolites in wheat cultivars have been associated with resistance to disease stresses. The total phenolic acid fraction has been found to be associated with cultivars of wheat resistant to various diseases (Gogoi et al., 2001). The abundance of phenolic compounds, including *p*-coumaric and ferulic acids, has been reported to be greater in the glumes of FHB resistant cultivars of wheat (Siranidou et al., 2002). These compounds have shown antifungal activity against *Fusarium* spp. under *in vitro* conditions (McKeen et al., 1999). Using the metabolomics method, 55 metabolites varying in abundance were identified in resistant/susceptible wheat cultivars inoculated with *F. graminearum* (Hamzehzarghani et al., 2005).

Advances in metabolomics have considerably extended our abilities to describe functions of complex biological systems (Schauer and Fernie, 2006). We have applied metabolomics methodology to discriminate a plant phenotype resistant to a pathogen from that of a susceptible one (Hamzehzarghani et al., 2005). In that study, several metabolites were detected in higher abundance in the resistant wheat cultivar Sumai3 than in the susceptible cultivar Roblin, and most of these were constitutive metabolites. A higher abundance of several fatty acids, particularly stearic and palmitic acids, aromatic compounds such as *p*- and *m*-coumaric acids, and sugars such as myo-inositol were associated with the resistant cultivar Sumai3. To consider these compounds as resistance-related is risky, because the cultivars used in this study were genetically quite different. The use of near isogenic lines (NILs) can reduce complex whole genome epistatic interactions.

Consequently, the objective of the present study was to distinguish two wheat NILs, susceptible and resistant to FHB, based on their metabolic profiles. These NILs differed in having alternate resistance/susceptibility alleles for a QTL on chromosome 2DL that confers FHB resistance. The second objective of the study was to identify RR metabolites associated with the 2DL QTL, in order to characterize the mechanisms of FHB resistance in wheat.

5.3. Materials and methods

5.3.1. Plant production and inoculation

Seeds of NILs carrying alternate (FHB susceptible/resistant) alleles of the QTL on chromosome 2DL (Somers et al., 2003), were derived from the cross BW301 × HC374 (Somers et al., 2005), obtained through backcrossing. This QTL is considered to contain several genes, including FHB resistance genes. Line BW301 is a FHB susceptible hard red spring wheat adapted to western Canada, while line HC374, resistant to FHB, is derived from the cross Wuhan1 × Nyubai. Line HC374 carries the FHB resistance alleles of Wuhan1 located on 2DL QTL. These lines were genotyped with microsatellite markers and homozygous lines differing only in the alleles of the 2DL locus were selected and selfed to multiply seeds. The resistant NIL expressed high type II resistance, with no spread of disease beyond the inoculated spikelets and low DON concentrations, compared to the susceptible NIL. Seeds from these lines (obtained from Dr. D. Somers, AAFC, Winnipeg, MB) were sown in 6-inch pots, kept in a greenhouse maintained at 22±3 ^{o}C, and fertilized twice at the tillering and boot stages with a 0.2 percent solution of 20-20-20 NPK. Seven-day old cultures of *Fusarium graminearum*, isolate 99–15–35 (obtained from Dr. S. Rioux, CEROM, QC) were inundated with a 0.02% aqueous solution of Tween 80. The spore concentration was adjusted to 10^5 macroconidia ml^{-1}. Wheat spikes were area-source inoculated by putting 10 µl of macroconidial suspension (approximately 1000 spores/spikelet) into the middle florets of each of 4 mid-spike spikelets (Gary et al., 2000) for metabolite profiling. At anthesis (GS=60-69), a single mid-spike spikelet was point inoculated for subsequent disease severity assessment (Zadoks et al., 1974). Spikelets inoculated with 10 µl of distilled water containing 0.02% Tween 80 served as a control. The inoculated plants were covered with plastic bags, sprayed inside with water, and kept in the greenhouse at 22±3 ^{o}C for 24 h.

5.3.2. Disease severity assessment

The number of diseased spikelets in a spike was determined at 4 d intervals until day 20 after inoculation (dai), and from this various FHB severity parameters were calculated. These parameters were: a) number of spikelets diseased per spike on day 20; b) the proportion of spikelets diseased out of ten (around the spikelet that was inoculated) on day 20; c) the normalized area under the disease progress curve (normalized AUDPC), based on the proportion of diseased spikelets. The AUDPC was normalized by dividing it by the maximum possible total area of 20 (20 d × maximum disease severity, which was 1.0) (Hamzehzarghani et al., 2005).

5.3.3. Biochemical analysis

The spikelets were harvested at 24 hai by clipping the rachis on either side of the four inoculated spikelets in a spike. The spikelets were immediately crushed in liquid nitrogen to minimize any hydrolytic activity, freeze-dried and stored at -80^{o}C. Freeze-dried tissue (30 mg) was extracted with methanol-water and then with chloroform, according to the methods developed by Roessner et al. (2000) and Fiehn et al. (2000a, b), with minor modifications. Supernatants were separated from the residue using Ultrafree-CL 0.22 µl microfilters and the volume of polar and non-polar extracts reduced from 1 ml to 200 µl (Hamzehzarghani et al., 2005). Following this, the extracts were methoximated to stabilize and prevent cyclization of carbonyl moieties in the β-position of reducing sugars, and derivatized with MSTFA. At the outset, 50 µl of ribitol (0.2 mg ml^{-1} in water) and nonadecanoic acid (2 mg ml^{-1} in chloroform) were added, respectively, to the methanol-water and chloroform fractions, to serve as internal standards.

Aliquots of 1µl of the extracts were analyzed by electron ionization, using a Micromass GCT. The injection port temperature was maintained at 230 ^{o}C. A split ratio of 1:50 of the injected sample was used for chromatography with a HP-5 capillary column (0.25 µm film thickness, 0.25 mm in diameter and 25 m in length, Supelco, Canada) with a helium flow rate of 1 ml min^{-1} at 70.0 Kpa initial pressure. After 1 min at 70 ^{o}C, the column temperature was programmed to rise

by 5 ^{o}C min^{-1} up to 270 ^{o}C, followed by a 7 min bake out at 300 ^{o}C. The source temperature was 200 ^{o}C, and scans in the range of 70-600 *m/z* were done at a rate of 1.1 scans s^{-1}.

5.3.4. Chromatographic output data processing

The total ion chromatogram output from the GC-TOF-MS was converted into "net.cdf" files using the BRIDGE capability of MassLynx® to make them readable by the combination of software subsequently used. AMDIS (Automated Mass spectral Deconvolution and Identification System; Version 2.64, Davies, 1998) was used for the deconvolution of peaks, extraction of the baseline corrected pure mass spectra and identification of the retention time of every component. A mass spectral tag (MST) is a mass spectrum at a known retention time with a consistent fragmentation pattern over scans and replicates (Kopka 2006). MSTs were tentatively identified by searching MSRI libraries at GMD (Kopka et al., 2005) and the NIST library (version 2005, Palisade Corp., NY) to determine the best matches. The GC/MS output on scans, mass ions and their abundance was processed using the AMDIS software. The consistency of major fragments of mass spectrum for each peak across four replicates was manually verified. The MSTs observed in each GC/MS replicate analysis were compared with the five best matches in the NIST and MSRI libraries to select the best mass spectral match, therefore allowing the assignment of a name to a component. If a given spectrum did not suitably find a match in the libraries, the peak was designated as unidentified.

The processed data from AMDIS, saved as "elu" and "fin" files were loaded into MET-IDEA (METabolomics Ion-based Data Extraction Algorithm; version 1.2.0) to calculate the component abundance and to align the peaks across samples (Broeckling et al., 2006). MET-IDEA processed one representative total ion chromatogram (TIC) from the data folder and extracted the ions and retention time markers (IRTs) for each component. The generated IRT list was saved as an "ion" file, and subsequently MET-IDEA executed a calibration step, scanned the data folder and recognized all "net.cdf" files (samples) to apply a directed extraction of the ion intensity values based on a list

of IRT-values. The results were organized into a single tab-delimited text file with compound identifiers as column headings and sample names in rows with a user-designated filename.

The data was loaded into a MS-EXCEL spreadsheet and, using the Pivot Table procedure, the peaks with a signal to noise ratio <20, a peak purity <20%, as well as peaks from the column bleed and those not appearing in all four replicates were rejected from further consideration. Finally, the peak areas of the metabolites and their retention times across the study were corrected for inter-sample variations relative to the internal standards. The polar and non-polar sample profiles were combined and designated as a metabolic profile.

5.3.5. Experimental design and statistical analysis

The experiment was designed as a completely randomized block with two NILs with alternate alleles of QTL on chromosome 2DL: resistance (R) and susceptibility (S). Treatments were inoculations of spikelets with the pathogen (P) or with water (W), which served as a control in four replicates. Each experimental unit consisted of a pooled sample of 16 spikelets (the rachis with four inoculated spikelets harvested from each of four spikes from four plants). For each experimental unit a combined metabolite profile (polar and non-polar) was established.

The data on i) disease severity (number of diseased spikelets per spike; proportion of spikelets diseased; normalized area under the disease progress curve) and ii) metabolite profiles (abundance of compounds) were subjected to ANOVA using SAS (SAS Institute, 1999). Only the metabolites with potential significant ($P \leq 0.20$) treatment effects were considered for multivariate and *t*-test analyses. A *t*-test of mean values of the data associated with each treatment was used to identify RR metabolites (metabolites with significantly ($P \leq 0.05$) greater abundance in resistant than in susceptible NILs). To identify metabolite phenotypes and to investigate the concealed biological functions, the levels of metabolites, with significant treatment effects for the two NILs irrespective of inoculum, were also subjected to canonical discriminant analysis using the CANDISC procedure of SAS. CANDISC developed linear combinations of the

metabolites (CAN-vectors) and summarized between-class variation. The CAN-scores of individual samples were plotted as coordinates on the axes of a three-dimensional scatter plot and a graphical illustration of the relationship between samples (metabolite profile) in a CAN space was generated. The separation of clusters of samples in such a plot illustrates the differences among distinct metabolic systems (Morris et al., 2004; Roessner et al., 2001). The CAN-vector that classified the metabolite clusters/phenotypes was considered to identify a biological system. This was investigated by relating the metabolite phenotypes to disease severity phenotypes. The metabolites with high loadings to the CAN-vector that identified the resistance function were considered to explain the resistance functions. A higher loading for a metabolite indicated a bigger weight of the metabolite on the magnitude of the CAN-score along the corresponding CAN-vector. Additional unsupervised classification of treatments based on their metabolite profiles (using canonical scores) was performed with the cluster procedure of SAS to identify the pattern similarity of their grouping into metabolite phenotypes.

5.3. Results

5.3.1. Disease severity

The two NILs, with alternate alleles for the FHB resistance QTL on chromosome 2DL, differed in their disease severity. All the pathogen-inoculated spikelets had visible disease-associated discoloration and/or necrosis. At 20 dai, the mean number of diseased spikelets per spike was 1.0 and 2.75 for the resistant and the susceptible NILs, respectively, with the proportion of diseased spikelets per spike being 0.100 and 0.275. This indicates that the disease spread beyond the inoculated spikelet in the susceptible NIL, but not in the resistant NIL, confirming the absence and presence of type II resistance, respectively. The means of disease severity assessed as the normalized AUDPC in the resistant and susceptible NILs were 0.023 and 0.047, respectively. The resistant NIL had significantly (p<0.001) lower disease severity than the susceptible one, based on both the proportion of spikelets diseased and the normalized AUDPC.

5.3.2. Metabolic profiles

GC/MS analyses of the plant extracts yielded 262 and 297 chromatographic peaks in the polar and non-polar fractions, respectively. Several steps of discrimination analysis in the chromatographic data confirmed the detection of 182 components or metabolites of which 84 were found in the polar fraction and 98 in the non-polar fraction (the mass spectra are available at http://www.metabolomics.mcgill.ca). Of 182 metabolites, only 127 metabolites were tentatively identified and the remaining 55 unidentified metabolites corresponding with those classified as 'unidentified' in MSRI libraries (Kopka et al., 2005). When the levels of the 182 metabolites were subjected to ANOVA, only 122 metabolites demonstrated significant changes related to the treatment ($P \leq 0.20$). Only these 122 metabolites were further subjected to *t*-tests and canonical discriminant analysis in order to classify the type of resistance with which they were associated.

5.3.2.1. Resistance-related (RR) metabolites

The metabolites that were in significantly ($P \leq 0.05$) higher abundance in the resistant NIL than in the susceptible NIL were designated as resistance-related (RR) metabolites (Table 5.1). The RR metabolites were further classified into three groups: **i) RRC metabolites**: RR metabolites that were constitutive, based on a homeostatic state or on the water-inoculated treatment (*i.e.*, with significantly higher level in the water-inoculated resistant NIL than the susceptible NIL). **ii) RRI metabolites**: RR metabolites that were induced under the stressed/pathogen-inoculated state. In other words, the abundance of a RRI metabolite was significantly greater in both the resistant water-inoculated and susceptible pathogen-inoculated treatments compared to the pathogen-inoculated resistant NIL. **iii) RRCI metabolites**: RR metabolites that were both constitutive and induced; these were constitutive RR metabolites that were further significantly induced in the resistant NIL, following pathogen inoculation.

Out of 122 metabolites showing significant variation with treatment, 27 were identified as RR metabolites, including 22 RRC metabolites and 8 RRI

metabolites, with three that were common to both (*i.e.*, RRCI metabolites) (Table 5.1).

The 22 RRC metabolites identified in this study belonged to seven different chemical groups: seven organic acids (5, 11, 12, 18, 33, 35, and 36); three amino acids (3, 6, and 17); one aromatic (34); eight from unknown groups (2, 4, 8, 13, 15, 19, 32, and 37) and three from other groups (7, 14, and 21) (Table 5.1). Metabolites that had moderately higher abundance in the resistant NIL were myo-inositol, proline, isoleucine, threonine, asparagine, octadecanoic acid, pentanedioic acids, and γ-aminobutyric acid (Table 5.1). Benzoic acid was only a borderline significant ($P \leq 0.1$) metabolite, but its abundance in the water-inoculated resistant NIL was twice that of the susceptible NIL.

The eight RRI metabolites identified belonged to five chemical groups: one phenolic: *p*-coumaric acid; two amino acids: proline and serine; one sugar: myo-inositol; one other group: 1-Palmitoylglycerol; and three unidentified from GOLM (16, 22, and 27). The levels of all these metabolites, except for myo-inositol, were at least two fold greater in the resistant NIL than the susceptible NIL (Table 5.1).

The three RRCI-metabolites identified in this study belonged to three different chemical groups: sugar: myo-inositol; amino acid: proline; alcohol: 1-Palmitoylglycerol.

5.3.2.2. Identification of biological functions based on CANDISC analysis and metabolite loadings

To sort metabolites according to treatments (RW, RP, SW, and SP), and to identify their possible biological functions, canonical discriminant analysis was applied to the 122 metabolites showing significant variation with treatment. The first two CAN-vectors accounted for 98% of the variance in the abundance of the 122 metabolites (CAN1=85% CAN2=13%; Fig. 5.1). Each CAN-vector is a linear combination of 122 metabolites and summarizes part of the variance observed in the metabolite profiles of the two NILs. The CAN1-vector grouped the two NILs into two separate clusters related to the constitutive resistance function, as the pathogen and water inoculations showed little difference in the CAN1-score. The CAN2-vector, on the other hand, classified the inoculation treatments. The

inoculation treatments were farther apart in the FHB resistant NIL than the susceptible NIL; therefore, the CAN2-vector was considered to explain the induced resistance function.

The metabolites with high loadings to the CAN-vectors that identify resistance functions were ranked in a descending order of loading within CAN1 and CAN2 (Table 5.1). Of 122 metabolites twenty-five and sixteen highly loaded (L>0.5) to the CAN1 and CAN2-vectors, respectively, with eight being common to both. Twenty-one of the 25 metabolites with high loading to the CAN1-vector associated with constitutive resistance were also RR metabolites (based on univariate ANOVA), of which nineteen and five were RRC and RRI metabolites, respectively, including three RRCI metabolites that were common. Some of the important metabolites with very high loadings to CAN1 were asparagine; proline; γ-aminobutyric acid; isoleucine; threonine; benzoic acid; 1-palmitoylglycerol; 2-propenoic acid; tetroquinone; myo-inositol; hexadecanoic acid and the unidentified compounds (2, 4, and 13) (Table 5.1).

Among the 16 metabolites significantly loaded to the CAN2-vector, eight were RR metabolites, of which seven were RRI and three were RRC, including 2 RRCI that were common to both. Some of the important metabolites with high loadings were serine; *p*-coumaric acid; xylose; benzoic acid; cinnamic acid and myo-inositol.

5.5. Discussion

Of the 182 metabolites detected in wheat spikelets in the present study, 127 were tentatively identified (*i.e.,* 55 unidentified) and 122 showed significant variation with treatment. A student *t*-test identified 27 RR metabolites, of which 24 and 8, respectively, were constitutive and induced RR metabolites, including three common to both (*i.e.*, RRCI metabolites). Canonical discriminant analysis of the abundance of the 122 significant metabolites discriminated the FHB resistant NIL (bearing resistance alleles at a QTL on 2DL) from that of the FHB susceptible NIL (with alternate susceptibility alleles at a QTL on 2DL). Almost all the RR metabolites had high (L≥0.5) CAN-loadings (Table 5.1). These RR metabolites can be used to differentiate the NIL with resistance alleles at a QTL on 2DL from

that of the NIL with susceptible alleles. The remaining metabolites, even though they had high loadings to resistance functions (identified by CAN1 and CAN2), were not identified as RR metabolites due to high variances. This is the first study where metabolic profiles were used to discriminate resistance in NILs with alternate alleles of a QTL for disease resistance. All of the 182 metabolites detected in this study, whether identified or unidentified, were consistently detected in replicates and as a result represented the metabolic profiles of the NILs studied here (Kopka, 2006).

The disease severity, measured as the number of diseased spikelets per spike, in the NIL with FHB resistant alleles at a QTL on 2DL was 1.0, compared to 2.75 for the susceptible NIL. In the resistant NIL disease did not spread beyond the spikelet that was inoculated, confirming type II resistance. This NIL also exhibited type II resistance under greenhouse conditions (Somers et al., 2003).

Using a metabolomics approach, we consistently detected 182 metabolites, of which 127 were tentatively identified. This is not considered comprehensive, as several metabolites that may have been present in the samples were either not extracted with the solvents used, were not capable of being extracted efficiently, or were too non-volatile to be gas chromatographed (Sumner et al., 2003; Dunn et al., 2005). However, significant biological information had previously been reported based on only a few metabolites (Hamzehzarghani et al., 2005; Schauer et al., 2006). Manual processing of GC/MS output leads to erroneous dataset generation therefore, we applied AMDIS and MET-IDEA to process our GC/MS data. AMDIS performs noise analysis, component perception, spectral deconvolution and compound separation, to provide optimal data for library searching. MET-IDEA was used to improve the sensitivity of detection and gas chromatogram alignment and quantification (Broeckling et al., 2006). We have used a non-targeted approach to detect metabolites. While the RR metabolites reported here were identified using comparisons with mass spectral libraries, confirmation by comparisons with authentic standards is still required.

In metabolomics, both biological and technical variations are generally high. We eliminated the metabolites that were not significant at $P \leq 0.2$ based on univariate ANOVA from further analysis. RR metabolites and respective resistance functions were identified based on a student *t*-test and multivariate analysis. The RR metabolites were identified based on a *t*-test with a varying $P \leq 0.05$ level of significance. The biological variability may be related to the variation in the physiological stage of the spikelets at the time of pathogen inoculation and to the use of a mixture of plant parts (the rachis, rachilla, lemma, palea and reproductive parts) in one sample. Technical errors may include variable metabolite extraction efficiencies.

Another problem in metabolomics, as in other 'omics', is data visualization. The ANOVA reduced the number of compounds submitted for further analysis. The *t*-test enabled identification of RR metabolites. The CANDISC analysis reduced the dimensionality of the data space and improved the data visualization by maximizing the resolution power. In addition, this analytical scheme enabled us to illuminate the mechanisms of resistance to FHB in wheat NILs by identifying biological functions and associated metabolites.

Several of the RR metabolites identified here, even though based on statistical analyses, have already been reported as antimicrobial compounds, signal molecules or their precursors. Plant phenylpropanoids are involved in signal transduction, the synthesis of several defense-related metabolites and the development of physical barriers (Dixon et al., 2002). The metabolite *p*-coumaric acid has been reported in wheat (Wu et al., 2001; McKeen et al., 1999). It has been found to be in greater abundance in wheat cultivars resistant to FHB than those, which are not (Siranidou et al., 2002; Hamzehzarghani et al., 2005). It was identified in this study as an RRI metabolite. It is a precursor for the synthesis of other defense-related phenolics such as coumarins and monomers of lignin such as *p*-coumaryl, conferyl and sinapyl alcohols. These lignin monomers are used by plants for the rigidification of cell walls in order to prevent the spread of the pathogen (Fig. 5. 3) (Dixon et al., 2002).

The compound γ-aminobutyric acid, identified here as a RRC metabolite, has antimicrobial activity (Walker et al., 2003). It has been shown to accumulate in the leaves of *Cistus ladanifer* after treatment with salicylic acid (Chaves et al., 2001). It was reported to protect *Arabidopsis* against the Oomycete pathogen *Peronospora parasitica* through activation of the natural defense mechanisms of the plant such as callose deposition, the hypersensitive response, and the formation of trailing necroses (even in mutants lacking salicylic acid, jasmonic acid, and ethylene signaling pathways) (Zimmerli et al., 2000).

Several of the RR metabolites identified in this study were linked to each other through different metabolic pathways leading to the production of defense-related metabolites. The metabolites *p-coumaric* and benzoic acids, identified here as RRI and RRC metabolites, respectively, are produced following deamination of phenylalanine (Fig. 5.3). Even though phenylalanine was identified here as an RRC metabolite, its abundance in the resistant NIL was significantly reduced following pathogen inoculation, suggesting that in the resistant NIL it is depleted by other biosynthetic shunts more rapidly than in the susceptible NIL (Fig.5.3). Benzoic acid is a precursor for salicylic acid and a key signaling molecule implicated in both local and systemic induced resistance responses (Pieterse and Van loon, 1999). A constitutive role in defense against rice blast (*Magnaporthe grisea*) has been attributed to salicylic acid in rice (*Oryza sativa* L.) (Silverman et al., 1995). Salicylic acid has also been implicated in the resistance response of wheat against Russian wheat aphids (Mohase and van der Westhuizen, 2002). The use of salicylic acid-like compounds such as benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) has been shown to induce systemic acquired resistance of wheat to powdery mildew (Gorlach et al., 1996). Salicylic acid was not detected in our study. However, a significantly greater abundance of benzoic acid in the resistant (*vs.* susceptible) NIL (RRC metabolite) indicates the existence of a possible array of precursors for salicylic acid (Fig. 5.3). In addition, benzoic acid and its derivatives have antifungal activity (Bénigne-Ernest et al., 2002).

Palmitic and stearic acids were identified as RR metabolites. They are required for the synthesis of jasmonic acid that plays a key role in the salicylic acid independent pathways and activates multiple resistance mechanisms (Pieterse and Van loon, 1999). Jasmonic acid has been detected in the shoots and roots of wheat seedlings (Dathe et al., 1994). It increases the synthesis of phenolic compounds by stimulating the phenylpropanoid pathway (Gundlach et al., 1992; Buchanan et al., 2000). We have detected linolenic acid (18:3) which had moderate negative loading to CAN1 (L1=-0.40). However, it was not identified in this study as a RR metabolite. Farmer et al. (1998) have reported the importance of the hexadecanoic and octadecanoic acid pathways in the synthesis of jasmonic acid via synthesis of linolenic acid (Fig. 5.4).

Plants with higher levels of resistance to diseases have higher levels of myo-inositol (Berridge and Irvine, 1989; Hammond-Kosac and Jones, 1996; Lamb and Dixon, 1997). Myo-inositol was identified here as a RRC metabolite. It is synthesized through the conversion of glucose (Loewus and Murthy, 2000). This pathway is implicated in cellular signaling, gene expression and intracellular calcium (Ca^{2+}) concentration control mediated by ion channel regulators (Nelson et al*.,* 1998; Exton, 1996). It has been shown that the defense response genes coding the ion channel regulator are located on the 2D and 3B chromosomes of wheat (Li et al., 1999).

Resistance to FHB in wheat is controlled by several FHB-QTLs (Somers et al., 2003; Bai et al., 2003a) The QTL on chromosome 2DL, used in this study, has been identified in Wuhan1 (Somers et al., 2003). The 2DL-QTL has also been identified in the Chinese landrace Wangshubai (Mardi et al., 2005), but not in Sumai3. Several, though not all, of the RR metabolites identified in this study in the NIL with a FHB resistance QTL on 2DL chromosome had also been identified as metabolites related to the resistance function, based on the factor analysis in our previous study on Sumai3, even though it lacks the QTL on chromosome 2DL (Hamzehzarghani et al., 2005). Some of these were phenylpropanoid related metabolites, signal compounds such as myo-inositol, fatty acids of the octadecanoid/hexadecanoid pathway, and amino acids. These

RR metabolites were common to both Sumai3 and the resistant NIL with QTL on 2DL from Wuhan1. This means that other FHB-QTLs (3BS and 6BS QTL of Sumai3) are also capable of producing these RR metabolites. Alternatively, not all the metabolites or their abundance were common to Sumai3 and the NILs tested here. Therefore, cultivars such as Sumai3 with several known QTLs seem to produce an array of RR metabolites. Unfortunately, because the analytical protocol between our two studies was different, a more precise comparison of the abundance of metabolites was not possible. A comprehensive study to identify the RR metabolites of NILs with different QTLs would be crucial to elucidate the specific roles of these QTLs in FHB resistance.

A QTL is a polymorphic site on a chromosome comprising alleles that differentially control the expression of a continuously distributed trait. However, each QTL may have several genes and each gene may have several alleles, occurring in varying combinations in different genotypes, wherein lies the polymorphism in wheat resistance to FHB. It is possible that several wheat QTLs or genes conferring resistance to FHB, expressed phenotypically as different types of resistance, vary in the RR metabolites produced. Additionally, within each of these resistance QTLs, the number of alleles determines the abundance of the respective RR metabolites they produce. In this study, we have identified several RR metabolites associated with the FHB resistance QTL on 2DL chromosome. The presence of such a large number of RR metabolites can be explained only by the presence of several genes within this QTL on chromosome 2DL. Metabolic profiling of NILs containing QTLs with fewer or single resistance genes and their alleles could lead to the identification of gene specific metabolite(s), and the reason for their variation in abundance. This may enable a better understanding of resistance mechanisms. Studies based on such a hypothesis have recently been carried out on introgression lines (ILs) of tomato in order to identify the genomic regions associated with changes in tomato fruit metabolites (Schauer et al., 2006). Schauer et al. (2006) did a comprehensive metabolic profiling, along with phenotyping of plant characters and a genotyping that allowed the development of a cartographic network based on correlation

analysis. This led to the identification of nearly 900 quantitative fruit metabolic loci, as well as the identification of associated genes. However, this was a long-term study. Alternatively, similar studies on NILs with different FHB-QTLs can identify sets of RR metabolites, the variation in which can explain the mechanisms involved in the resistance to FHB.

The conventional method of screening wheat-breeding lines for resistance to FHB involves the visual assessment of spikelet disease incidence, disease severity, kernel damage, DON content in grains and grain yield. These parameters are also used in identifying the types of resistance. The classification of the type of resistance is vague and overlapping. The RR metabolites, which were identified in this study, along with their putative roles in plant defense, demonstrate the potential of metabolic profiling to explain the mechanism of resistance by relating metabolites to resistance QTLs. However, a clear proof of the metabolic function of a given metabolite would require similar studies based on NILs with single genes or knockout mutants. The physical, chemical and biological environmental factors significantly influencing resistance under field conditions can be simulated under controlled greenhouse conditions, and their effects can be quantified based on the level of RR metabolites. Such an approach, can save time involved in the screening of breeding lines based on types of resistance, under different geographical locations over years. When most significant RR metabolites in selected sources of breeding materials are identified, they can be used as biomarker metabolites in screening other breeding lines and segregating populations. A greater abundance of RR metabolites, relative to a standard(s), can be selected for and further used in breeding, pyramiding genes or gene discovery (Schauer et al., 2006).

The QTL on chromosome 2DL, used in this study, is one of several FHB-resistance QTLs studied in wheat, and explains only approximately 13% of the phenotypic variation in resistance to FHB (Yang et al., 2005). More than ten QTL conferring significant levels of resistance to FHB have been identified (Mesterhazy 2003). Similar studies involving several other QTLs are required to develop a more comprehensive technology to use metabolomics as a screening

tool. The knowledge base generated here on RR metabolites, after confirmation of their identities, can also be used to enhance the resistance level in elite cultivars. This would be based on metabolic engineering to overexpress RR metabolites, and based on gene pyramiding by accumulating suitable genes with known functions.

Table 5.1. A selected list of tentatively identified metabolites from a total of 182 consistently found in replicates, including RR metabolites of two wheat NILs with resistance (R) or susceptibility (S) alleles for a QTL on 2DL chromosome, at 24 h after inoculation with *F. graminearum* (P) or water (W). The RR metabolites, based on the results of *t*-test, are constitutive RR (RRC, where RW>SW) or induced RR (RRI, where RP>RW>SP). In addition, the metabolites with high levels of loadings, in descending order, to two significant CAN-vectors explain 97 percent of the variance in the abundance of 122 metabolites, based on canonical discriminant analysis.

No[1]	Ref. No[2]	NAME[3]	CAS[4]	CAN1	CAN2	RR-M[5]
1	30	Asparagine	EPA41391	**0.99**	0.11	
2	170	EITTMS_N12C_NTAL_1636.8_1135ec07	NA	**0.93**	-0.37	C
3	22	Proline	30274772	**0.87**	0.46	CI
4	176	EIQTMS_N12C_LPIL_2255.3_3268AU29	NA	**0.83**	0.43	C
5	24	γ-Aminobutyric acid	NA	**0.83**	0.29	C
6	9	Isoleucine	15985010	**0.82**	**0.50**	C
7	160	1-Palmitoylglycerol	NA	**0.80**	**0.60**	CI
8	150	EIQTMS_N12C_LJAN_2420.0_2233bg14_	NA	**0.80**	0.38	C
9	109	Benzoic acid	23676097	**0.78**	0.46	
10	3	Tetroquinone	319891	**0.75**	**0.66**	
11	163	2-Propenoic acid	EPA221713	**0.75**	**0.60**	C
12	158	Hexadecanoic acid	53212934	**0.74**	-0.15	C
13	164	EIQTMS_N12C_LPAL_1128.1_3268AU16_	NA	**0.73**	-0.03	C
14	76	Myo-Inositol	33910064	**0.72**	**0.53**	CI
15	62	EIQTMS_N12C_LPEFR_2093.3_03363A03_	NA	**0.71**	0.08	C
16	14	EIQTMS_N12C_LJALM_1344.9_2236bg40_	NA	**0.70**	**0.70**	I
17	8	Threonine	7536825	**0.69**	0.44	C
18	26	Glutaric acid	60022879	**0.69**	-0.56	C
19	174	EITTMS_N12C_STUO_2121.9_1135ec12_	NA	**0.67**	0.37	C
20	25	EIQTMS_N12C_LESFR_1555.8_3090AU05_	NA	**0.62**	-0.58	
21	79	Adenosine	53294330	**0.61**	0.27	C
22	84	EIQTMS_N12C_LPIFR_2730.9_03363A05_	NA	0.27	**0.96**	I
23	5	Serine	NA	0.34	**0.93**	I
24	119	*p*-coumaric acid	27798692	0.28	**0.90**	I
25	12	EITTMS_N12C_ATHL_1326.9_1135ec24_	NA	-0.44	**0.89**	
26	77	EIQTMS_N12C_LESFR_2525.2_3090AU09_	NA	0.22	**0.88**	
27	51	EITTMS_N12C_NTAL_1932.2_1135ec26_	NA	**0.56**	**0.83**	I
28	132	Cinnamic acid	27798705	0.21	**0.79**	
29	100	6,7-Dimethyl-3H-isobenzofuran-1-one	343852506	-0.61	**0.77**	
30	166	β-Monostearin	621614	0.50	**0.63**	
31	33	Xylose	NA	-0.31	**0.57**	
32	45	EIQTMS_N12C_LPIFR_1891.4_03363A05_	NA	0.33	0.48	C

33	162	Methyl tricosanoate	2433978	**0.54**	0.43	C
34	108	Methyl vanillate	3943746	0.42	0.41	C
35	140	Stearic acid	57114	0.49	0.06	C
36	142	Pentadecanoic acid	111615	0.13	-0.05	C
37	75	EIQTMS_N12C_LJALD_2396.6_2236bg30_	NA	**0.56**	-0.59	C

[1] Compound serial number.

[2] Compound reference number (see supplementary Table 5.1, Appendix 2).

[3] Compound names, or unidentified but detected in GOLM metabolome database (Kopka et al., 2005), according to NIST® 2005 and MSRI libraries.

[4] Chemical abstract number based on NIST 2005, NA=not applicable.

[5] RR metabolites: Metabolites with significantly ($P \leq 0.05$) higher abundance in the resistant allele; C = Constitutive = RRC; I = Induced = RRI, IC = RRCI.

Figure 5.1. Scatter plot, based on projections of first three significant CAN-vectors of canonical discriminant analysis (CDA) of the abundance of 122 metabolites for water- and pathogen-inoculated NILs with resistance and susceptibility QTL alleles on 2DL. The arrows along CAN1- and CAN2-vectors show the direction of increase in resistance (the resistance measured as disease severity is associated with CAN-scores) with CAN-scores. CAN1 (constitutive) and CAN2 (induced) explained 98 percent of the total variance in metabolite profiles.

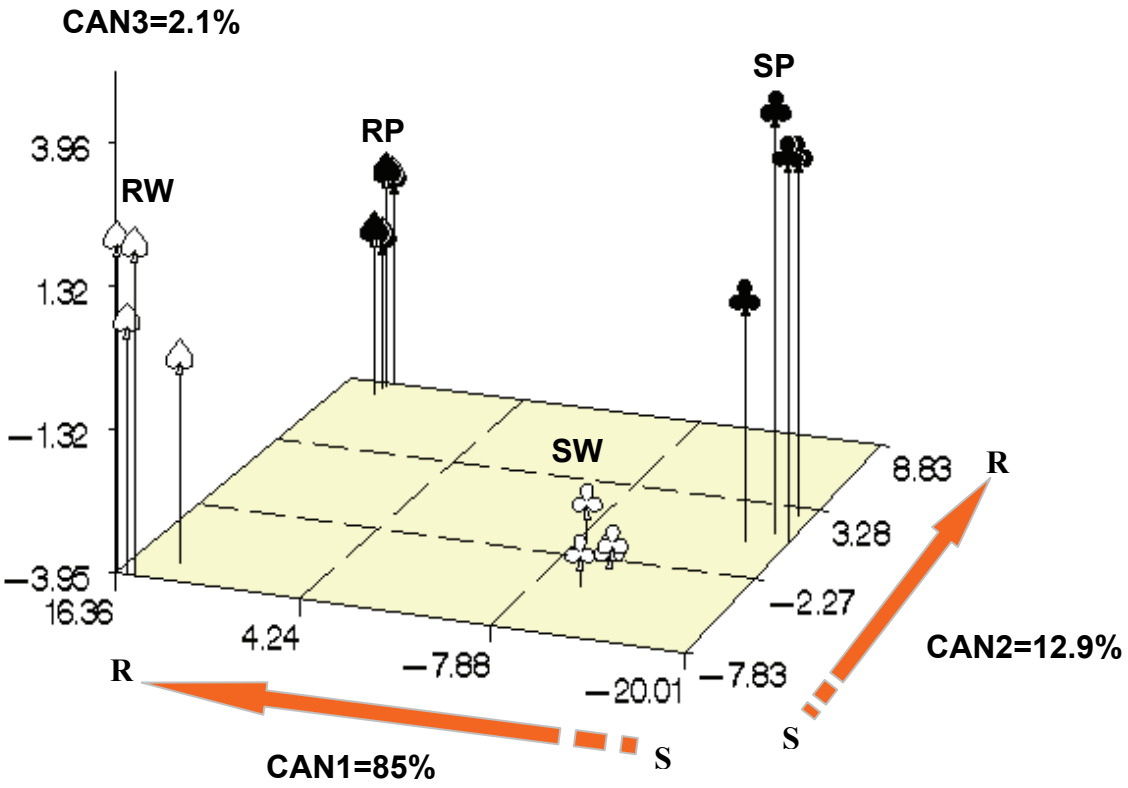

CAN3=2.1%
SP
RP
RW
3.96
1.32
−1.32
−3.95
16.36
4.24
−7.88
−20.01
SW
8.83
3.28
−2.27
−7.83
R
S
CAN2=12.9%
R
S
CAN1=85%

Figure 5.2. Cluster tree generated based on hierarchical cluster analysis using the first three canonical variables from the canonical discriminant analysis of the abundance of 120 metabolites detected in the two wheat NILs (Q and N representing NILs with alternate alleles of QTL on chromosome 2DL, respectively) inoculated with *F. graminearum* (P) or water (W). The scale represents the Euclidean distance in canonical space.

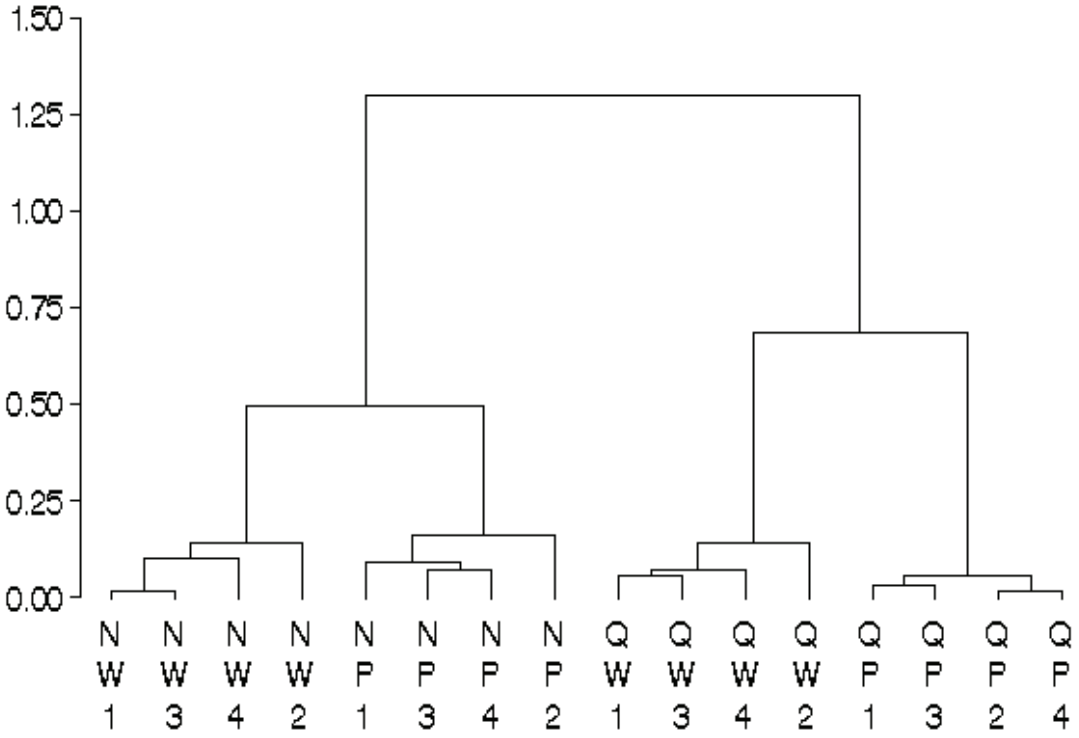

1.50
1.25
1.00
0.75
0.50
0.25
0.00
N W 1
N W 3
N W 4
N W 2
N P 1
N P 3
N P 4
N P 2
Q W 1
Q W 3
Q W 4
Q W 2
Q P 1
Q P 3
Q P 2
Q P 4

Figure 5.3. Phenylpropanoid pathway in plants drawn from Dixon et al. (2002); BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; CA, *tran*-cinnamic acid; 4-CA, 4-coumaric acid; CA2H, cinnamate 2-hydroxylase; PAL, phenylalanine ammonia-lyase; L-phe, L-phenylalanine; ShA, shikimic acid; The pathways are trimmed. L1 and L2 are loadings of the metabolite on CAN1- and CAN2-vectors, respectively.

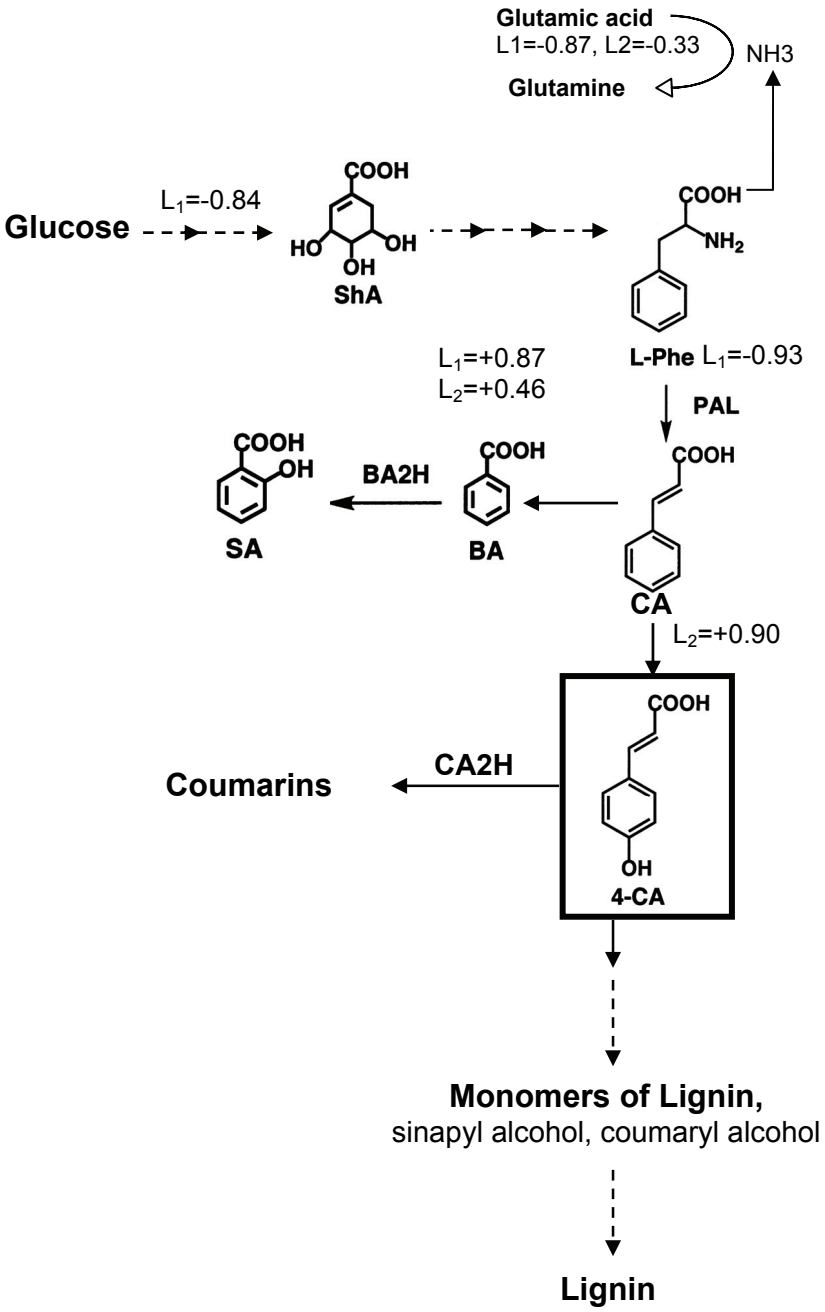
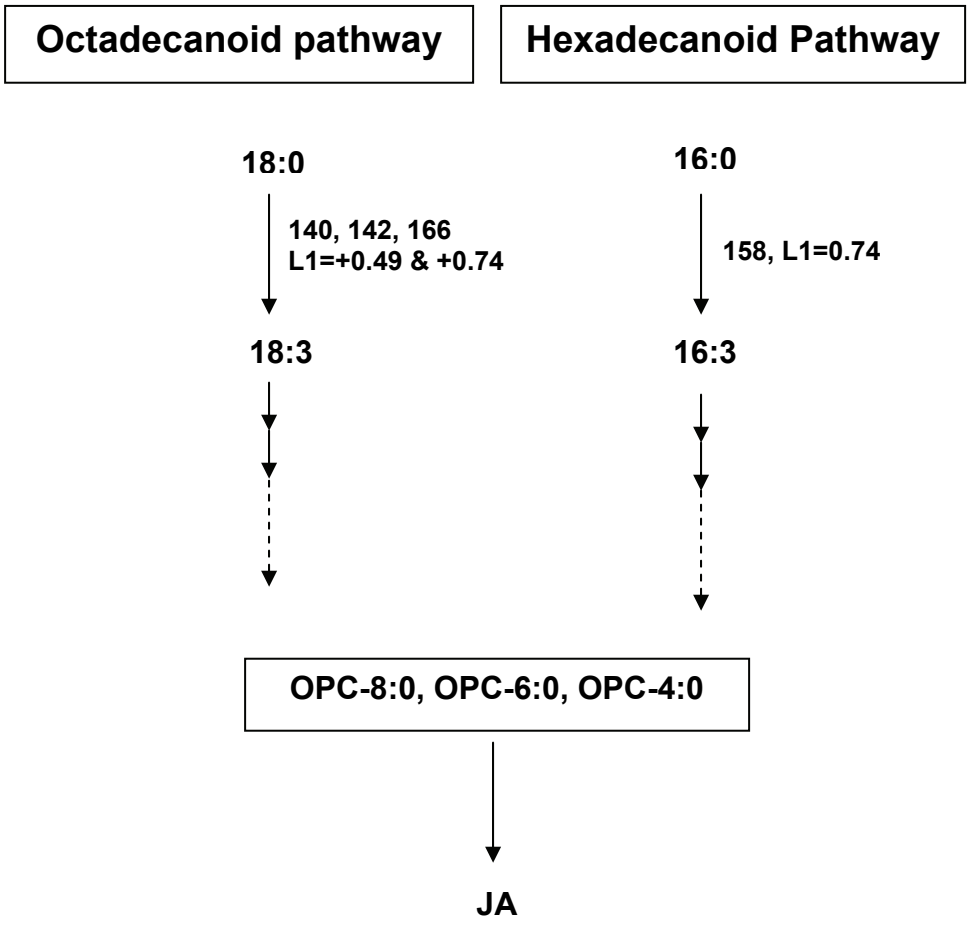

Glutamic acid
L1=-0.87, L2=-0.33
Glutamine
NH3
Glucose
L1=-0.84
COOH
HO
OH
OH
ShA
COOH
NH2
L-Phe L1=-0.93
L1=+0.87
L2=+0.46
PAL
COOH
COOH
OH
SA
BA2H
COOH
BA
COOH
CA
L2=+0.90
COOH
OH
4-CA
CA2H
Coumarins
Monomers of Lignin,
sinapyl alcohol, coumaryl alcohol
Lignin

Figure 5.4. A simplified biosynthetic pathway of jasmonic acid (JA) in *Arabidopsis* showing the importance of octadecanoic and hexadecanoic acid pathways in JA biosynthesis modified from Farmer et al. (1998), 16:0=hexadecanoic acid; 18:0= octadecanoic acid; 16:3=7,10,13-hexadecatrienoic acid; 18:3=9,12,15-octadecatrienoic acid and OPC 4, 6, and 8 are oxo(pentenyl) cyclopentane 4, 6, and 8, respectively. L1 and L2 are loadings of the metabolite on CAN1 and CAN2-vectors, respectively; numbers=names of the metabolites, full names are given in Table 5.1.


Octadecanoid pathway
Hexadecanoid Pathway
18:0
140, 142, 166
L1=+0.49 & +0.74
16:0
158, L1=0.74
18:3
16:3
OPC-8:0, OPC-6:0, OPC-4:0
JA

Chapter 6

General summary and suggestions for further research

6.1. General summary

Fusarium head blight (FHB), caused mainly by *F. graminearum,* is the principal disease of wheat in Canada. The damaging effects of the disease include yield and qualitative losses, the latter due to the production of several mycotoxins in wheat kernels (Bai and Shaner, 1994; Parry et al., 1995). Among the different methods of controlling the disease, breeding for resistance remains one of the most promising (Bai and Shaner, 1994).

Complete resistance in adapted elite cultivars is far from attainable, especially in certain cultivars of winter and durum wheat. The disease has been challenging breeders for decades and many breeding programs have attempted to develop new scab resistant varieties with good agronomic traits.

One of the most important steps of breeding for FHB resistance is screening wheat genotypes for resistance. Resistance is mainly evaluated by screening wheat cultivars and lines based on a disease severity scale and/or DON content rating of the grain. However, a high spatiotemporal variability of FHB symptoms hinders the visual assessment of FHB resistance (Parry et al., 1995). Furthermore, visual evaluation of FHB resistance is quite time consuming and expensive. Development of new high throughput techniques for a more accurate, rapid, and less expensive evaluation of FHB resistance in wheat germplasm is a top priority of breeding programs. Besides their application in screening for FHB resistance, such methods develop our knowledge of the functions of the FHB resistance genes and help pyramid suitable FHB-resistance alleles into elite cultivars.

The new era of high throughput functional genome analysis of plants aims to assign functions to unknown genes. To help achieve this goal, methods such as transcriptomics, proteomics, and metabolomics can be employed. Spatiotemporal analysis of the qualitative and quantitative changes of mRNAs,

proteins and metabolites can generate the most comprehensive gene expression database regarding unknown cell functions.

Metabolite profiling is a high throughput and unbiased analysis of a wide range of metabolites through a single measurement. It is considered a rough biochemical snapshot of the cellular metabolome recorded by sophisticated hyphenated technologies such as GC/MS. The most attractive feature of metabolite profiling is direct detection of a biochemical phenotype through the process of relating, combining, and coupling chemical analysis with genetic analysis (Holtorf et al., 2002; Bino et al., 2004). Metabolites are the final products of gene expression. Therefore, metabolite profiling can be used independently to identify and phenotype a trait. As an instrumental platform, spectroscopy-based techniques (mainly GC/MS) have recently shown a wide range of applications for the profiling of small molecules. This is due to their relatively rapid and easy extraction methods, along with simple and cost effective separation protocols. Metabolite profiling using GC/MS technology has been used quite effectively for profiling various biological systems (Fiehn, 2000a, b; Roessner et al., 2000, 2001; Fiehn, 2002). However, despite its high sensitivity, it suffers from a lack of the ability to detect non-volatile metabolites (Johnson et al., 2003).

This study investigated the potential of metabolite profiling technology for studying plant disease resistance, using wheat-fusarium head blight as a model system. The study was not only undertaken to discriminate wheat genotypes varying in resistance to FHB, but also to look at the possible biochemical mechanisms of resistance and the association of certain metabolic pathways to resistance to FHB.

It was hypothesized that wheat genotypes varying in resistance to FHB carry different genes/QTLs for resistance, and vary in their metabolic profiles. The variations in metabolic profiles were subjected to univariate and multivariate statistical analyses to define and classify the biochemical phenotypes of the genotypes. The metabolite phenotypes were related to the respective disease severity phenotypes to identify biological functions. The metabolites loaded to

these functions were subsequently used to explain possible mechanisms of resistance.

Knowledge based on plant defense pathways can be further exploited through metabolic engineering. Wheat defense genes to FHB can be identified by relating resistance-correlated metabolites to databases of resistance gene expression, including transcriptome and proteome databases (Muthukrishnan et al., 2001). This knowledge can then be used to pyramid resistance genes into an elite cultivar.

Three independent pot experiments were designed: (i) using two cultivars Sumai3 (FHB resistant) / Roblin (FHB susceptible); (ii) six cultivars – Wangshubai, AW488, Nobeoka Bozu, BRS177, Frontana, CEP24 (in descending order of FHB resistance), and (iii) two NILs with alternate (resistant and susceptible) alleles on the 2DL QTL.

In the first study, (Chapter 3) the GC/MS outputs of total ion chromatograms were processed using Saturn Lab software. The metabolites were identified using a MS search program and the NIST library (version 2.0). Peak identification was carried out by manual inspection of the consistency of retention times as compared to internal standards and major fragments of the mass spectra across the replicates. The observed mass spectra of each peak for different replicates were compared with the ten best choices in the NIST library to confirm the identity of a component using match factors. Only the metabolites that appeared in all four replicates were considered for further analysis. The metabolite profiles of different treatments consisted of the names of the components, their respective retention times and the areas under the peaks (designated as the abundance of metabolites).

The data of the second study (Chapter 4) was originally processed manually, in a manner very similar to the data analysis of the first study. However, it was then reanalyzed with a more advanced analytical platform used for the third study (Chapter 5). The GC/MS outputs on total ion chromatograms of the second study (Varian Saturn ”sms” file format) were converted into a universal “net.cdf” file format using the mass spectral file translator (MassTransit

version 3.0.1.16, Palisade Corp., NY). The amount of background noise in some chromatograms was quite high and required noise reduction prior to any further analysis. The chromatograms were subsequently analyzed using the MetAlign software package to correct the baseline or the background noise (Tikunov et al., 2005). The baseline-corrected chromatograms were then analyzed using AMDIS (Version 2.64) and MET-IDEA for identification and quantification of mass spectral tags as indicated in the third study (see below).

In the third experiment (Chapter 5), due to the emergence of advanced data analysis software, an easier, faster, and more reliable platform for data processing was employed. The total ion chromatogram output from GC-TOF-MS was converted into “net.cdf” files using the BRIDGE capability of MassLynx® to analyze them by AMDIS (Automated Mass spectral Deconvolution and Identification System; Version 2.1, DTRA/NIST 2000) (Davies, 1998). The AMDIS output was used for the extraction of the baseline-corrected pure mass spectra and correction of the retention time of each component. The NIST library (version 2.0) was then searched with the mass spectra of each component, to find the best 10 matches (Halket et al., 1999). The processed data saved as ”elu” and “fin” files, were loaded to MET-IDEA (METabolomics Ion-based Data Extraction Algorithm; version 1.2.0) (Broeckling et al., 2006).

MET-IDEA aligned and quantified the peaks and recorded the abundance of each peak in a tab-delimited file format that was loaded into an EXCEL spreadsheet. Using the Pivot Table procedure, peaks were filtered for signal noise ratio (S/N<20), peak purity (<20%), peaks from column bleed, and peaks not appearing in all four replicates. The metabolite profiles of different samples consisted of retention times and areas under the peaks, designated as the abundance of metabolites. This data was submitted to statistical analysis. The areas under the peaks were then adjusted for internal variations by dividing them by the abundance of the internal standards.

The first study reported on the identification of several metabolites relating to FHB resistance. In this study, many of the 55 plant-pathogen interaction metabolites that were tentatively identified, had already been reported to have

known significant roles in plant defense (Fiehn et al., 2000b; Roessner et al., 2000 and 2001; Dixon et al., 2002).

The metabolites 4-hydroxycinnamic acid (*p*-coumaric acid) and *m*-coumaric acid, two important PAL pathway metabolites, had an up to six-fold increase in abundance in the resistant cultivar Sumai3 following pathogen inoculation (SP/SW>6). No such change in these two metabolites was observed in the susceptible cultivar Roblin. The PAL pathway converts phenylalanine to *trans*-cinnamic acid, a precursor for salicylic acid. The enzyme cinnamate 4-hydroxylase then hydrolyses *trans*-cinnamic acid into *p*-coumaric acid, a key metabolite that may serve as precursor of the other phenolic metabolites and monomers utilized for reinforcement of the cell wall (Blechert et al., 1995; Blount et al., 2000; Buchanan et al., 2000; Dixon et al., 2002). The metabolite 4-hydroxycinnamic acid has also been reported as a precursor of a group of phytoalexins in oat (*Avena sativa* L.) leaves infected with the rust disease caused by *Puccinia coronata* f.sp. *avenae* (Muthukrishnan et al., 2001; Okazaki et al., 2004). Following inoculation with *F. graminearum* the resistant wheat cultivar Frontana exhibited higher concentrations of free phenolic metabolites than did the similarly inoculated susceptible cultivar Argent (Siranidou et al., 2002). One such metabolite was *p*-coumaric acid, which increased in the glumes, lemmas, and paleas.

Benzoic acid (BA) was induced in SP (Sumai3 pathogen-inoculated), but not detected in RW (Roblin water-inoculated). However, it was found in lower levels in RP (Roblin pathogen-inoculated). The decarboxylation of *trans*-cinnamic acid to BA and 2-hydroxylation of BA to salicylic acid (SA) have been reported by Leon et al. (1995). Aromatic metabolites such as BA and SA, in addition to their intra- and inter-cellular signal transduction roles, are antimicrobial (Hammond-Kosack and Jones, 1996). The quantity of myo-inositol in Sumai3 was significantly greater than in the susceptible cultivar, Roblin, and the level increased in both cultivars following pathogen inoculation.

A factor analysis of metabolites common to all treatments (SP/SW/RP/RW), based on their factor loadings, classified the metabolites into groups

according to their association with susceptibility or resistance to FHB. The highest scores for the first and second factors (Fig.3.1) were associated with the pathogen-inoculated spikelets of the resistant cultivar Sumai3. The first factor was associated with a higher abundance of several fatty acids and aromatic metabolites, and the second with metabolites such as *p*- and *m*-coumaric acids, and myo-inositol.

Following pathogen inoculation, the amounts of certain fatty acids increased in both cultivars, but to higher levels in Roblin. When both cultivars were inoculated with the pathogen (RP and SP), they demonstrated higher F3 scores; however, that of RP was significantly greater than that of SP (Table 3.1), pointing to the importance of the JA signal transduction system in Roblin. The hexadecanoid/octadecanoid pathway has close links to the JA-pathway, and plays a vital role in regulating secondary pathways (Blechert et al., 1995). However, despite the rapid increase in the production of JA-related fatty acids in the susceptible cultivar, Roblin, the pathogen still invaded this cultivar.

To investigate the throughput of metabolite profiling and potential application of the technology for cultivar screening, an experiment was performed with a set of six spring wheat cultivars varying in their level of resistance. These included the cultivars AW488, Nobeoka Bozu, Wangshubai, Frontana, BRS177, and CEP24 (listed in decreasing order of FHB resistance). This second study involved the classification of six wheat cultivars varying in their quantitative levels of FHB resistance, based on their metabolic profiles, and the assignment of potential resistance biomarker metabolites via the identification of RR metabolites and FHB resistance biomarker metabolites.

As the six cultivars had smaller differences in their levels of resistance than existed between cultivars Sumai3 and Roblin, this experiment presented more of a challenge for the use of metabolite profiling as a tool for differentiating wheat genotypes resistant to FHB.

Univariate ANOVA and HCA of the disease severity of the six cultivars classified them into three disease severity phenotypes (DSPs): (i) DSP1 (resistant), cvs. Wangshubai, AW488, Nobeoka Bozu; (ii) DSP2 (moderately

resistant), cvs. Frontana, BRS177; and (iii) DSP3 (susceptible or slightly resistant), cv. CEP24 (Fig. 4.1). The classifiers, resistant, moderately resistant, and slightly resistant were chosen arbitrarily. The wheat cultivars Sumai3 and its derivatives, Nobeoka Bozu, Wangshuibai, and Frontana (Chinese, Japanese and Brazilian source of FHB resistance, respectively), are considered as worldwide sources of FHB resistance. Progenies from the crosses to such Brazilian, Chinese and Japanese cultivars have shown promise (Comeau et al., 2005). Unlike Sumai3 and Frontana that possess only two to three resistance genes, Nobeoka Bozu and Wangshubai have five to six dominant FHB resistance genes with polygenic inheritance. The latter two cultivars have many forms varying in resistance (Mesterhazy, 2003).

Attempts to find any reports on the relative ranking of the six cultivars in their resistance to FHB failed. The percentages of FHB measured (based on four isolates and three replicates) for Sumai3, Nobeoka Bozu, and Frontana were 2.00, 4.96, and 7.36, respectively (Mesterhazy, 2003). In another study, a greater disease severity (DS), and incidence (DI) was reported for the cultivar CEP24 (DS/DI=6.5/42), compared to Frontana and BRS177 (5.1/35 and 4.6/35, respectively) (Del Ponte et al., 2005). The disease severity measured in these two studies was assessed under different environmental conditions and on a different scale, than in the present studies. Therefore, the comparison of the absolute values of the cultivars' disease severities may not be valid. It is still possible to have a relative ranking of the cultivars in descending order of FHB resistance according to the results of ratings drawn from the abovementioned studies. The cultivars in descending order of resistance can be ranked as follows: Sumai3, Nobeoka Bozu, BRS177, Frontana, and CEP244. This is very similar to the ANOVA and HCA based classification of the six cultivars in the second study (Chapter 4). In the absence of any numeric records for Wangshuibai and AW488, it is not possible to assign a ranking to them. However, they have been consistently reported as highly FHB resistant or as resistant as Sumai3 (Mesterhazy, 2003; Comeau et al., 2005; Ma et al., 2006).

In the second study, of several hundred detected peaks, 79 metabolites

were tentatively identified in plant extracts of the pathogen-/water-inoculated spikes of the six wheat cultivars (Table 4.1). Univariate ANOVA identified 41 RR metabolites with significant cultivar effect in homeostatic (RRC) and induced (RRI) states. An array of the metabolites very similar to those identified in the first study, including myo-inositol, some phenolic metabolites and some fatty acids were defined as RR metabolites. A metabolite was determined to be a RR metabolite if its abundance was significantly ($P \leq 0.05$) greater in a resistant cultivar than in the control cultivar CEP24.

One would expect to identify the same set of metabolites as detected in the first study, because a similar analytical method was pursued with some minor modifications. As the number of cultivars tested increased in the second study, modification of analytical platform towards detection of more metabolites for a larger coverage of the metabolome was required. This was necessary to increase the odds of detecting more potential RR metabolites. On the other hand, analytical platform uniformity was essential to keep different related studies comparable. In the absence of a comprehensive analytical method to profile and determine the metabolite identity of a plant, the technical uniformity requirement contradicts the versatility of the experimental technique to guarantee the detection of as many metabolites relating to FHB resistance as possible. The development of a comprehensive and universal method for extraction, identification, and quantification of plant metabolites therefore remains a challenge for plant metabolomics (Bino et al., 2004). This limitation is one of main obstacles of relating experimental results form various studies and/or different laboratories.

The canonical discriminant analysis identified two constitutive (Fig 4.2, a) and three induced-constitutive metabolic profile phenotypes (Fig 4.2, b) that were not identical to the disease severity phenotypes. This may indicate that additional types of resistance mechanisms are involved and that they may overlap in different DSPs. Cultivar AW488 was associated with high CAN-scores that identified both constitutive and induced resistance functions. However, it was associated only with a high number of RRI, but not RRC, metabolites based on

univariate ANOVA. The cultivar Wangshubai was associated with high CAN-scores that identified constitutive resistance and had the greatest number of RRC metabolites. The cultivars BRS177 and Frontana had low CAN-scores associated with constitutive resistance and high CAN-scores that identified induced resistance. While the former was associated with several RRI metabolites, the latter was not. Instead, it had moderate levels of both RRC and RRI metabolites. The mechanism of resistance appears to be complex. Not only does the number of RR metabolites play a substantial role in the mechanism of resistance to FHB, so may the types of metabolites and their levels. The amount of some critically important metabolites such as myo-inositol and some phenolic compounds may play key roles in the expression of resistance. If this assumption is correct, in the absence of key RR metabolites, even a high level of other RR metabolites may not significantly change the defense status of the plant.

Resistance to FHB in wheat is controlled by several FHB-QTLs (Anderson et al., 2001; Bai et al., 2003; Somers et al., 2003). Each QTL may be involved in the activation of the metabolic pathways leading to shifts in the levels of certain combinations of RR metabolites. The cultivars used for the first and second studies have different sets of multiple FHB-QTLs. In the first two studies, in spite of the confounding effects of wide differences in genetic background, a multivariate statistical approach was able to separate resistant genotypes from susceptible ones. Metabolite profiling of several FHB resistance QTLs can reveal the biochemical basis of gene expression in FHB resistance.

The third study provided a unique opportunity to approach the goal of assigning function(s) through the use of two susceptible and resistant near isogenic lines with alternate alleles on the 2DL QTL. The results of the statistical approach and knowledge regarding metabolic pathways were combined to characterize constitutive and induced resistance-related metabolites. The near isogenic lines had practically identical genetic backgrounds and only varied in the 2DL QTL. The objective of the third study was to investigate the possibility of discriminating a resistant NIL from a susceptible one and relating the RR metabolites to a QTL.

In this study, the analytical platform was improved by shifting from the ion trap MS to a time of flight MS that is more sensitive and records ion counts more accurately. A more precise and significantly faster automated computation of component abundance was done by implementing the MET-IDEA program in the software platform to advance the GC/MS output processing. The analytical approach remained the same as in the previous studies (see Appendix 3 for analytical approach). The data processing involved data filtration based on the signal/noise ratio (the signal to noise value of the peak) and the purity of the peaks (percentage of the total ion signal at the peak maximum scan belonging to the deconvoluted peak). Any metabolite with a signal to noise ratio and purity less than 20 was filtered out. In a second step, any metabolite eluting during column bleeding, and hence not from the plant, was removed from further consideration. The final filter was a statistical univariate ANOVA filtration in which only metabolites that were different between resistant and susceptible genotypes at $P<0.20$ level were retained for multivariate analysis. The value of $P<0.20$ as a level of significance was used in a manner similar to stepwise discriminant analysis (STEPDISC). In STEPDISC, the significance levels for both the addition of variables in the forward selection mode (SLE) and the retention of variables in the backward elimination mode (SLS) is 0.15 (Johnson and Wichern, 2002). The researcher may increase SLE and reduce SLS to ensure that no potentially important variable that can be used for discrimination is erroneously removed from the dataset. Therefore, this univariate ANOVA filtration was applied to enhance the chance of including as many potential metabolites in CANDISC as possible and simultaneously to eliminate as many metabolites with large variability from dataset as possible.

The RR metabolites were selected based on a *t*-test significant at the $P\leq0.05$ level. In order to distinguish it from RR metabolites, a metabolite was considered borderline significant when it was only significant at the $P\leq0.10$ level.

The third experiment provided a complete differentiation of resistant and susceptible NILs with alternate alleles on the 2DL QTL. With the use of canonical discriminant analysis, many putative resistance biomarker metabolites were

assigned to the 2DL QTL. The grouping of the pathogen- and water-inoculated NILs was verified by a separate classification of the wheat varieties using hierarchical cluster analysis.

Of several hundred detected peaks 120 metabolites were tentatively identified, including 27 resistance-related (RR) metabolites with a significantly ($P \leq 0.05$) greater abundance in the resistant NILs, including 22 constitutive (RRC) and 8 induced (RRI) metabolites, of which three were common, and also increased following pathogen inoculation.

Canonical discriminant analysis of the abundance of the 120 significant metabolites discriminated FHB resistance in the resistant and susceptible NILs. A total of 25 and 16 metabolites accounted for the constitutive and induced resistances, respectively.

The first two CAN-vectors accounted for 98% of the variance in the abundance of the 120 metabolites (CAN1=85% and CAN2=13%) (Fig.5.1). The CANDISC of metabolite profiles was applied to distinguish the two NILs with alternate alleles on the 2DL QTL, as well as to investigate the metabolic function underlying the clustering pattern of the genotypes. The CAN1- and CAN2-vectors classified four populations of P- and W-inoculated plants in each of the two NILs into separate clusters in a scatter plot and in a dendrogram (Fig.5.1 and 5.2, respectively). The CAN1-vector classified the two NILs into separate clusters with little difference in CAN1-scores between the P- and W-inoculated plants of each NIL. This CAN-vector was considered a measure of the constitutive resistance function, as the inoculations (W/P) had little effect upon it. The CAN2-vector classified the W- and P-inoculated plants; therefore, it was considered a measure of the induced resistance function.

The CAN-vectors were screened for metabolites with high loadings and ranked in ascending order, along CAN1- and CAN2-vectors (Table 5.1). Out of 120 metabolites, 22 and 16 metabolites highly loaded (L>0.5) to CAN1- and CAN2-vectors, respectively, with eight that were common to both.

It is significant to note that all 21 metabolites that highly loaded to the CAN1-vector (measuring the constitutive resistance), and 25 of the metabolites

that highly loaded to the CAN2-vector (explaining the induced resistance), were RR metabolites identified based on the *t*-tests.

The phenylpropanoid metabolites, including *p*-coumaric acid, were shown to be IRR metabolite markers, while benzoic acid and methyl vanillate were constitutive RR marker metabolites. Butanoic acid was identified as a RRC metabolite. Its accumulation in the leaves of *Cistus ladanifer* after treatment with SA has been demonstrated (Chaves et al., 2001). It has also been shown to have strong antimicrobial activity (Walker et al., 2003). Myo-inositol was significantly higher in the NIL with resistance alleles on the 2DL QTL. This compound also had a high loading to both CAN1- and CAN2-vectors. Therefore, it was determined to be an induced and constitutive RR metabolite.

Significantly higher levels of different forms of octadecanoic, hexadecanoic, and other fatty acids were identified as RRC metabolites. The importance of the hexadecanoic (16:0) and octadecanoic (18:0) acid pathways in the synthesis of jasmonic acid (JA), through synthesis of linolenic acid (18:3), has been documented, and both 16:0 and 18:0 fatty acids have been shown to be the original precursors of JA (Fig. 5.4) (Farmer et al., 1998).

JA is one of the most important fatty acid-derived signal molecules involved in plant defense responses. It induces the synthesis of SA-non-inducible PR genes and enhances the synthesis of SA (Bohland et al., 1997; Pieterse and Van loon, 1999). It also mediates the induction of defense-related genes coding for small antimicrobial proteins called plant defensins. JA is known to increase the formation of phenolic metabolites by stimulating the phenylpropanoid pathway (Gundlach et al., 1992; Buchanan et al., 2000).

Throughout the three independent studies, a number of important RR metabolites, such as the PAL metabolites (cinnamic acid, coumaric acid, isoferulic acid, coumarins, and benzoic acid), myo-inositol, the hexadecanoid/octadecanoid fatty acids, and butanoic acid, were consistently detected in close association with FHB resistance. This consistency in the detection of resistance-related metabolites was irrespective of the continuous modifications the experimental platform had to undergo. These RR metabolites

can be considered as potential biomarker candidates for screening wheat genetic material for FHB resistance. However, verification of their identities after spiking with authentic standards is of crucial importance and is emphasized.

Many of the metabolites identified consistently as FHB resistance-related are also well known for their roles in plant defense (Whetten and Sederoff, 1995; McKeen et al., 1999; Wu et al., 2000, 2001; Dixon et al., 2002; Siranidou et al., 2002; Daoubi et al., 2004). Some examples of these metabolites were coumarins (Berenbaun and Zangerl, 1996); benzoic acid (Bénigne-Ernest et al., 2002); butanoic acid (Chaves et al., 2001; Walker et al., 2003); and important signal molecule such as myo-inositol (Berridge and Irvine, 1989; Smart and Flores,1997; Nelson et al., 1998); and the JA-related fatty acids such as octadecanoic, hexadecanoic and linolenic acid (Gundlach et al., 1992; Dathe et al., 1994; Pieterse and Van loon, 1999; Bohland et al., 1997).

In metabolomics automatic peak detection and identification is necessary to perform large-scale metabolite profiling (Duran et al., 2003). The metabolite identification in the three studies was tentative. Spiking with authentic standards, or the application of other hyphenated instruments such as tandem mass spectrometry MS/MS and NMR, are required to validate the chemical identity of a metabolite. In the second and third studies, MSRI libraries (GOLM metabolome database; Kopka et al., 2005) and NIST library (version 2.0d, 2005, Palisade Corp., NY) were used. The former contains the mass spectra of metabolites principally of plant origin, and in the new version of the NIST library, there are more mass spectra of plant metabolites. This reduced the chance of misidentification of metabolites. However, it does not remove the need for more rigorous identification of *de novo* and RR metabolites that are proposed as resistance biomarkers.

Regardless of the crucial importance of metabolite standards, in many cases they are difficult to obtain. To overcome the challenge, it is suggested that a system be initiated which assists the exchange of purified or synthetic reference metabolites between research laboratories. The reference material is a

purified fraction from a plant extract that must be authenticated by NMR and MS/MS.

Yet such a database of authentic annotated plant metabolites has not been developed for metabolomics and remains one of the most important limiting factors of metabolomics studies. As an example, *Arabidopsis* has been one of the most extensively studied plants by functional genomics researchers (Bino et al., 2004). Yet of the estimated 5000 metabolites in a typical *Arabidopsis* leaf, only approximately 10% have been annotated using current technologies.

Metabolomics, while still in its infancy, must still overcome certain limitations such as a lack of comprehensiveness (lack of full coverage of the plant metabolome), the need to facilitate comparison of the results between laboratories and experiments, and the absence of bioinformatic methods to integrate metabolomic data with other functional genomic information. The definition of common criteria in community-based works, the foundation of concerted action directed towards the release of standard reference materials, the invention of combined metabolite libraries, and the construction of metabolite-specific data management systems are practical objectives for metabolomics to follow to become a fully accepted branch of science (Bino et al., 2004).

This study was able to differentiate wheat genetic material varying in resistance to FHB and identify resistance biomarker metabolites. However, despite what the metabolite profiling technique was able to accomplish in this study, when coupled with the data management protocols developed here, the method is still in its development phase.

6.2. Suggestions for future research

1. In this study, several RR metabolites were identified. However, the identification of the metabolites is not conclusive. For confirmation of the identity of these metabolites, the authentic samples must be spiked, and matched.
2. If the match of an unknown metabolite does not coincide with that of the authentic sample, the metabolite is probably a novel one. Other hyphenated technologies, such as nuclear magnetic resonance (NMR), GC/MS/MS, etc. will be required to verify the chemical identity of the metabolite.
3. In our study, biological variation was significant. To reduce this error it is recommended to grow plants in a more controlled environment. This includes the measurement of soil conditions, watering volume and frequency, and the quantity and frequency of fertilization. A non-fertilized control must also be included in all experiments. Steps should be undertaken to ensure greater flowering uniformity at anthesis. This will reduce the error significantly because it will assure a higher degree of metabolic uniformity in the spikelets at the time of inoculation, and therefore less discrepancy in spikelet metabolite profiles.
4. There was some technical error in some of the studies. This was mainly because of the split GC/MS analyses of samples from the same experiment. The samples must be randomized in the autosampler. It is strongly recommended to inject all the samples of the same experiment at once. For example, we assume a runtime of 1 hour for each sample in a small experiment of 24 samples. The waiting time between the first and the last samples would be 24 h at ambient temperature.
5. The metabolites were related to QTL in an attempt to explain possible resistance functions. Similar studies with more NILs, each carrying a different QTL or knockout gene studies will help to investigate the role of each QTL in the resistance of wheat to FHB.

6. The QTLs often carry multiple genes, each with several alleles. Genes may work qualitatively and alleles may be responsible for quantitative changes of metabolites. Some of these resistance QTLs have known pleiotropic effects and therefore have multiple effects. Metabolic profiles of lines with different genes in segregating populations varying in alleles for resistance to FHB would enable the identification of specific functions of these genes.
7. Several RR metabolites were detected, most of which either were precursors of antimicrobial metabolites or linked to these metabolites in their synthetic pathway. Integration of these metabolites to other omics', in a single study, can significantly increase the chance of identifying the genes and enzymes involved in the production of these metabolites. To facilitate such a functional genomic project, simultaneous extraction of mRNA, proteins, and metabolites is recommended as both Fiehn (2001) and Fiehn et al. (2001) have suggested.

Chapter 7

Contributions to knowledge

The main goal of this study was to investigate the possibility of discriminating plant disease resistance using the metabolite profiling technique. Metabolite profiling technology was coupled with univariate/multivariate statistical modeling. This led to the identification of an association between some metabolites and: (a) their metabolic pathways and (b) the quantitative resistance of some wheat varieties to FHB. The contributions to knowledge of this study were:

1. Metabolic phenotyping of wheat cultivars resistant/susceptible to FHB is possible through intensive chromatographic and univariate/multivariate statistical techniques.
2. Metabolite profiling can be a potentially high throughput, and relatively rapid and easy tool for the automation of screening wheat genotypes for FHB resistance.
3. This study reports on the detection and tentative identification of biomarker RR metabolites from different chemical groups such as phenolics, fatty acids, amino acids, and sugars. These chemical metabolites have the potential application for discriminating different wheat cultivars and breeding lines for resistance.
4. PR and RR metabolites were defined based on univariate ANOVA and multivariate statistical methods. The same statistical criteria were applied to classify RR metabolites into constitutive (CRR) and induced (IRR) RR metabolites.
5. Metabolic profiling was used to differentiate resistant and susceptible wheat NILs varying in the alleles of just one resistance QTL at both homeostatic and induced states.
6. There was evidence of a statistical association ($P \leq 0.05$) between metabolites related to the PAL and hexadecanoid/octadecanoid pathways with FHB resistance in wheat.

7. Several RR metabolites that have known antimicrobial properties such as *p*-coumaric acid, isoferulic acid, cinnamic acid, benzoic acid, aminobutyric acid, propanoic acid and other fatty acids were identified.
8. This study reports on the application of MetAlign, AMDIS and MET-IDEA as an analytical software platform to automate the tasks of mass spectral deconvolution, baseline correction and quantification of MS abundance of metabolites in wheat.

Chapter 8

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Chapter 9
Appendices

Appendix 1: Supplementary Table 4.1. Name (based on NIST or MSRI libraries), CAS number and retention time of the metabolites detected in spikelets of six wheat cultivars, varying in level of resistance to FHB. CAS=chemical abstract service number according to NIST, NA=not applicable.

ID	NIST/GOLM	RT	CAS
1	D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-O-(trimethylsilyl)-, cyclic methylboronate)	5.07	54477019
2	1H-Thiopyrano[3,4-c]pyridine-5-carbonitrile, 3,4-dihydro-6-isopropylthio-8-morpholino-3,3-dimet	5.09	EPA277000
3	Cyclohexanol, 2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-, trans-()-)	5.75	27203925
4	DL-Arabinopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-)	5.75	56271648
5	2-[2,N-Dimethyl-2-aminoethyl]benzofuran)	5.84	EPA256879
6	N,O-Bis-(trimethylsilyl)alanine	7.29	2899447
7	2-(p-Anisidino)tropone	8.06	118818410
8	N-(4-Benzyloxy-phenyl)-acetamide	9.23	EPA300719
9	N,O-Bis-(trimethylsilyl)valine)	10.11	15984937
10	EIQTMS_N12C_LJALM_1281.6_2236bg40_Phosphoric acid (3TMS))	11.60	NA
11	Palmitic acid, 2-(1-octadecenyloxy)ethyl ester, (E)-)	11.63	30760044
12	Trimethylsilyl ether of glycerol)	11.64	6787106
13	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	12.42	5630820
14	2-Butenoic acid, 3-methyl-2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	12.50	55044796
15	EIQTMS_N12C_LJARP_1327.0_2236BN20_Succinic acid (2TMS)	12.78	NA
16	Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	13.10	38191876
17	Methylamine, N,N-bis(N.-decyl)-	13.11	EPA237658
18	p-Octylacetophenone)	14.65	10541567

19	Cadaverine, N,N,N',N'-tetrakis(trimethylsilyl))	15.60	65898762
20	L-Alanine, N-methyl-N-(trifluoroacetyl)-, 1-methylpropyl ester, (S)-)	16.31	54986651
21	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester)	17.12	38166119
22	N,N,o'-Tris-(trimethylsilyl)aspartic acid)	17.87	15985054
23	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester	17.90	30274772
24	EIQTMS_N12C_LJARP_1530.2_2236BN20_4-Aminobutyric acid (3TMS))	18.02	NA
25	Pyridine, 2,2'-methylenebis-)	18.66	1132372
26	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.)	18.79	38191887
27	p-Octylacetophenone)	19.48	10541567
28	EITTMS_N12C_ATHR_1749.6_1135EC44_[Pentonic acid-1,4-lactone (3TMS)]	20.49	NA
29	1,4-Butanediamine, N,N,N',N'-tetrakis(trimethylsilyl)-	22.58	39772639
30	3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]-, (R*,S*)-	22.72	25258020
31	Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester)	23.25	57197350
32	D-Ribofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-)	23.26	56271693
33	Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-)	23.42	55399490
34	Mannose, 6-deoxy-2,3,4,5-tetrakis-O-(trimethylsilyl)-, L-	24.26	19127152
35	3,7,11,15-Tetramethyl-2-hexadecen-1-ol)	24.92	102608537
36	EITTMS_N12C_STUT_1852.9_1135ec30	25.00	NA
37	EIQTMS_N12C_LCHFR_2275.9_03363A10_	25.09	NA
38	Ethanesulfonic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester)	25.17	67557588
39	2H-Quinolizine-1-methanol, octahydro-)	25.42	10159792
40	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	25.46	56196146
41	3,10B-Dihydrofluoranthene)	25.47	37980077
42	(-)-Norreticuline, 6'-bromo-N-formyl-)	25.66	56196146
43	EIQTMS_N12C_ATHL_1883.9_3161BN26_Fructose methoxyamine {BP} (5TMS))	25.66	NA
44	EIQTMS_N12C_SD1_1887.9_3035BB03_D-Gluconic acid-1,5-lactone (4TMS))	25.81	NA
45	EIQTMS_N12C_LESFR_1893.7_3090AU02_	25.86	NA
46	Methyl 2-(1,1,2-trifluoro-2-nitroethyl)tetrafluorobenzoate)	25.92	128278502
47	EIQTMS_N12C_LJAF_1897.1_2236bg54_	25.94	NA
48	>EIQTMS_N12C_LJALD_2595.0_2236bg34_	26.28	NA

49	d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-)	26.30	128705737
50	EIQTMS_N12C_SD1_1945.7_3184BF20_Coniferylalcohol (2TMS))	26.97	32342011
51	Gulonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone)	27.19	55528751
52	DL-Lyxofuranoside, methyl 2,3,5-tris-O-(trimethylsilyl)-)	27.29	56390035
53	à-l-Galactofuranoside, methyl 6-deoxy-2,3,5-tris-O-(trimethylsilyl)-	27.32	56390035
54	1-Propene, 1-methoxy-)	27.35	7319166
55	EIQTMS_N12C_ATHL_1984.6_3161BN26_Glucopyranose (5TMS)	27.67	NA
56	2-O-Glycerol--d-galactopyranoside, hexa-TMS)	28.59	EPA-98090
57	EIQTMS_N12C_LPIL_2050.1_3268AU05_	28.97	NA
58	Hexadecanoic acid, trimethylsilyl ester)	28.98	55520893
59	EIQTMS_N12C_LCHFR_4001.7_03363A10_	29.54	NA
60	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	29.55	2582798
61	Phenol, 2,5-bis(1,1-dimethylethyl)-)	29.56	5875456
62	Cyclohexanone, 2-[(4-methoxyphenyl)-1-piperidinylmethyl]-)	29.58	94318282
63	EIQTMS_N12C_SD1_2094.6_2081BN06_Sinapyl alcohol (2TMS)	29.72	NA
64	Galactose oxime hexakis(trimethylsilyl))	29.89	120850886
65	EIQTMS_N12C_LPAL_2715.8_3268AU16_	30.20	NA
66	2-Propenoic acid, 2-methyl-3,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester)	30.32	40333082
67	Galactose oxime hexakis(trimethylsilyl)	30.41	120850886
68	Propanoic acid, 2,2-dimethyl-, propyl ester)	30.95	5129351
69	2,2-Dimethyl-1,2,3,4-tetrahydro-ç-carboline	31.22	22315680
70	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	31.91	56259075
71	EIQTMS_N12C_LPIL_1615.5_3268AU29_	32.01	NA
72	EIQTMS_N12C_SCA_2219.1_3147BF25_9,12,15-(Z,Z,Z)-Octadecatrienoic acid (1TMS)	32.02	NA
73	Octadecanoic acid, 11-methyl-, methyl ester)	32.28	74484778
74	EIQTMS_N12C_SD1_2247.0_1267BK12_Octadecanoic acid (1TMS)	32.48	NA
75	D-Glucuronic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester)	34.59	55530808
76	Cholan-16-one, 23-methyl-, (5,20.xi.)-)	34.96	56143170
77	Scopolin, tetra(trimethylsilyl)-)	36.97	EPA108989
78	D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)--D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimeth	37.01	39523074

79	3H-Imidazole-4-carboxylic acid, 3-methyl-5-[(pyridin-4-ylmethyl)amino]-, (4-tert-butylphenyl)ami	37.07	EPA302355
80	Hexopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-)	37.17	55521158
81	1,1'-Biphenyl, 2,3,4,4'-tetramethoxy-5,6'-dihydroxycarbonyl-)	37.22	123705895
82	D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-à-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimet	37.25	39523074
83	Ethanol, 2-(3-trifluoromethyl-1,2,4-triazolo[4,3-b]pyridazin-6-yl)amino-	37.29	309741262
84	Benzoic acid, 4-(4-ethylcyclohexyl)-, 4-butoxy-2,3-dicyanophenyl ester)	37.34	75941843
85	EIQTMS_N12C_LESL_2697.7_2352AI10_	37.41	NA
86	EITTMS_N12C_NTAR_2690.8_1135EC08_Lactose methoxyamine (8TMS); beta-D-Gal-(1,4)-D-	37.56	NA
87	Benzo[1,2-c:3,4-c':5,6-c'']tris[1,2,5]oxadiazole)	37.84	16279155
88	D-Ribofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-)	37.86	56271693
89	Benzoic acid, 4-(4-ethylcyclohexyl)-, 4-butoxy-2,3-dicyanophenyl ester)	37.88	75941843
90	EIQTMS_N12C_OARPLAS_2603.6_3350DF06_[872; 1-Monohexadecanoylglycerol (2TMS)]	37.92	NA
91	EITTMS_N12C_SD1_3463.2_1288EC31_Melezitose(11TMS));alpha-D-Glc-(1,3)-beta-D-Fru-(2,1)-al	37.93	NA
92	EITTMS_N12C_ATHR_2996.4_1135EC25_[890; Galactinol (9TMS); alpha-D-Gal-(1,3)-myo-Inositol	38.12	NA
93	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-, didehydro deriv.)	38.14	11051277
94	L-Alanine, N-[N-[N-(3-pyridinylmethylene)-L-valyl]-L-isoleucyl]-, ethyl ester)	38.22	37580328
95	EIQTMS_N12C_NID1_3067.4_1334BV27_[924; Trehalose (8TMS)]	38.34	NA
96	EIQTMS_N12C_LJARL_2652.6_2236BN14_Sucrose (8TMS); alpha-D-Glc-(1,2)-beta-D-Fru	38.40	NA
97	EIQTMS_N12C_SD1_2653.9_1267BK12_Sucrose (8TMS); alpha-D-Glc-(1,2)-beta-D-Fru	38.43	NA
98	4H-1-Benzopyran-4-one, 2-(2-naphthalenyl)-)	38.44	113486365
99	D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)--D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimet	38.50	60354430
100	D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-à-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimet	38.62	39523074
101	Per-O-trimethylsilyl-(3-O-á-d-mannopyranosyl-d-glucitol)	5.04	EPA-72629
102	Benzene, (1-butylpentyl)-	7.35	20216880
103	Unknown	7.71	NA
104	EIQTMS_N12C_ATHL_1257.2_3161BN27_[939; 1,3-Di-tert-butylbenzene]	11.13	NA
105	EIQTMS_N12C_LJARL_1284.6_2236BN14_Glycerol	11.64	NA
106	Hexane, 3,3-dimethyl-	11.72	563166
107	EIQTMS_N12C_LHIL_1328.1_3268AU18_	12.40	NA
108	2-Decene, 4-methyl-, (Z)-	12.63	74630301

109	Cyclohexane, (1,2-dimethylbutyl)-	12.84	61142378
110	Unknown	13.03	NA
111	1,3-Dioxolane, 2-tert-butyl-2-phenyl-)	13.46	6135600
112	2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	16.88	77822572
113	1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-	16.89	6754661
114	2-Hexyl-1-octanol)	17.07	19780791
115	EIQTMS_N12C_NID1_1542.3_1267BK12_[786; 2,4,6-Tri-tert.-butylbenzenethiol])	18.26	NA
116	>Dinaphtho(1,2-b:2',1'-d)thiophene	21.51	239725
117	Unknown	22.31	NA
118	>EIQTMS_N12C_SCA_2033.4_3147BF22_[906; Heptadecanoic acid methyl ester]	22.60	NA
119	Unknown	23.36	NA
120	Cinnamic acid, p-	23.95	27798692
121	Tetradecanoic acid, 12-methyl-, methyl ester,	24.72	62691058
122	Thiophen-2-methylamine, N,N-dinonyl-)	25.07	EPA310361
123	EIQTMS_N12C_SCA_1852.6_3147BF23_[931; Tetradecanoic acid	25.14	NA
124	9-Hexadecenoic acid, methyl ester,	26.64	1120258
125	EIQTMS_N12C_SCA_2033.4_3147BF22_[906; Heptadecanoic acid methyl ester]	26.74	NA
126	EIQTMS_N12C_SD1_1943.0_1340AU11_trans-p-Coumaric acid	26.96	NA
127	Hexyl octyl ether	27.15	17071544
128	Cinnamic acid, 3-methoxy-4-	27.19	27798705
129	Unknown	27.39	NA
130	Unknown	27.42	NA
131	EIQTMS_N12C_SCA_2033.4_3147BF22_[906; Heptadecanoic acid methyl ester]	28.67	NA
132	3,3,5,5-Tetramethylcyclohexanol)	28.74	2650400
133	Hexadecanoic acid, trimethylsilyl ester	28.96	55520893
134	Benzene, 1,1'-(1,5-hexadiene-1,6-diyl)bis-	29.68	4439456
135	8,11-Octadecadienoic acid, methyl ester)	29.91	56599587
136	9,12,15-Octadecatrienoic acid, methyl ester,	30.02	301008
137	9-Octadecenoic acid, methyl ester, (E)-	30.13	1937628
138	EIQTMS_N12C_SCA_2132.6_3147BF22_[934; Octadecanoic acid methyl ester]	30.51	NA

139	Oxacyclotetradec-5-yn-2-one, 8-[[	31.48	110242983
140	Unknown	31.75	NA
141	Unknown	32.04	NA
142	Octadecanoic acid, trimethylsilyl ester	32.48	18748919
143	p-Terphenyl)	33.87	92944
144	9,10-Secocholesta-5,7,10	34.88	55759949
145	Phthalic acid, di(3-methylphenyl) ester	34.88	EPA315373
146	Myristic acid, 2,3-bis	34.97	1188734
147	Pregnane-3,20-diol, 14,18-[4-methyl-3-oxo-1-oxa-4-azabutane-1,4-diyl)]-, diacetate)	36.47	EPA194303
148	EIQTMS_N12C_SD1_1775.1_1324BK24_L-Glycerol-3-phosphate (4TMS)	36.48	NA
149	Heptadecane, 9-hexyl-)	36.65	55124793
150	Docosanoic acid, methyl ester)	37.15	929771
151	Dehydrocholic acid)	37.16	81232
152	Dihydromorphine , di(trimehylsilyl) ether	37.43	EPA308804
153	2-Monopalmitin trimethylsilyl ether)	37.45	53212978
154	EIQTMS_N12C_OARPLAS_2603.6_3350DF06_[872; 1-Monohexadecanoylglycerol (2TMS)]	37.93	NA
155	EIQTMS_N12C_MAC_3288.8_3157BF09_Stigmasterol (1TMS)	38.11	NA
156	EIQTMS_N12C_NLA_3337.3_3146BF10_[835; Stigmasterol (1TMS)]	38.17	NA
157	Dihydromorphine , di	39.01	EPA308804
158	EITTMS_N12C_ATHR_2101.7_1135EC06_trans-Ferulic acid (2TMS)	39.07	NA
159	Carda-16,20	39.08	29428873
160	Cholest-5-en-3-one)	39.34	EPA153632
161	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-)	39.61	55282343
162	EIQTMS_N12C_LESR_2707.8_04014A50_)	39.63	NA
163	Prosta-5,13-dien-1-oic acid, 9,11,15-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, (5Z,9à,11à,13E	39.86	50669959
164	>EITTMS_N12C_ATHR_1662.4_1135EC25_[Dodecanoic acid (1TMS)]	39.88	NA
165	Morphinan, 7,8-didehydro-4,5-epoxy-3,6-bis[	39.89	55319885
166	Cyclohexane, 1,4-dimethyl-2-octadecyl-)	39.94	55282025
167	Tungsten, dicarbonyl-	39.96	75857760
168	EITTMS_N12C_ATHR_1357.1_1135EC06_[Picolinic acid	39.98	NA

169	Isoxazole, 5-[3,3-dicyano-1-cyclohexylidene-2-morpholino-prop-2-enyl)]-3-p-methoxyphenyl-)	40.02	EPA154150
170	3-Desoxo-3,16-dihydroxy-12-desoxyphorbol 3,13,16,20-tetraacetate)	40.16	EPA194807
171	Carda-16,20	40.19	29428873
172	9-Desoxo-9x-hydroxy-7-ketoingol 3,8,9,12-tetraacetate)	40.21	EPA203030
173	2-Monostearin trimethylsilyl ether)	40.22	53336133
174	Cyclohexane, 1,4-dimethyl-2-octadecyl-)	40.24	55282025
175	Cyclohexane, 1,4-dimethyl-2-octadecyl-)	40.25	55282025
176	3'H-Cycloprop[1,2]androsta-1,4,6-triene-3,17-dione, 1'-carboethoxy-1'-cyano-1,2-dihydro-)	40.37	75857760
177	Morphinan, 7,8-didehydro-4,5-epoxy-3,6-bis[	40.42	55319885
178	Ruthenium, tricarbonyl[	40.51	118772386
179	EITTMS_N12C_SD1_2765.9_1344EC78_Maltose methoxyamine {BP} (8TMS); alpha-D-Glc-(1,4	40.51	NA
180	Ethyl iso-allocholate)	40.55	EPA-43053
181	EIQTMS_N12C_OARPLAS_2783.6_3350DF06_[924; 1-Monooctadecanoylglycerol (2TMS)]	40.70	NA
182	EIQTMS_N12C_MAC_3353.5_3157BF09_beta-Sitosterol	40.83	NA
183	á-Sitosterol trimethylsilyl ether	40.84	NA
184	á-Sitosterol acetate	40.87	NA
185	EIQTMS_N12C_SD1_3353.2_3035BB09_beta-Sitosterol (1TMS)	40.89	NA
186	Cholest-5-en-3-ol, 4,4-dimethyl-, (3á)-	40.97	1253889
187	1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,5,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]	41.00	EPA210500
188	Benzoic acid 3-methyl-4-(1,3,3,3-tetrafluoro-2-methoxycarbonyl-propenylsulfanyl)-phenyl ester	41.10	85485138
189	4à-Methylcholest-7-en-3-one	41.15	13490578
190	3-Hydroxy-4-decenoic acid, 9-(tetrahydropyran-2-yl)oxy-, 2-(trimethylsilyl)ethyl ester	41.17	EPA196053
191	Propanoic acid, 2-(3-acetoxy-4, 4,14-trimethylandrost-8-en-17-yl)-	41.19	EPA194012
192	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3á,5Z,7E)-	41.20	40013874
193	3H)-Furanone, 5-ethyldihydro-)	41.23	695067
194	Benzazepin, 3,4-dihydro-1-[2-[3,4,5-trimethoxyphenyl]ethyl]-7,8-dimetho	41.24	113193901
195	1-Nickela-2-azonia-3-azacyclopentadieno[a]naphthalene, 2-	41.26	EPA191058
196	Cholestan-7-amine, N,N-dimethyl-	41.27	55331890
197	EITTMS_N12C_ATHL_3035.3_1135EC05_[952; Sucrose (8TMS)]	41.33	NA
198	Stigmastanol	41.45	19466478

199	Morphinan, 7,8-didehydro-4,5-epoxy-3,6-bis[	41.51	55319885
200	1H-[1]Benzoxepino[2,3,4-ij]isoquinoline, 2,3,12,12a-tetrahydro-6,9,10-trimethoxy-1-methyl-,	41.63	EPA103003
201	Furo[2,3-H]coumarine, 1-	41.64	EPA270163
202	Cyclopropa[3',4']benz[1',2':4,5]azuleno[1,8a-d]-1,3-dioxole-5b,7,7a-triol, 3a,5a,6,7,8,8a,8b,11-oct	41.69	77573387
203	Cyclohexanol, 4-ethyl-4-methyl-3-(1-methylethenyl)-, (1à, 3à, 4á)-	41.76	56272083
204	Stigmastane	41.79	601581
205	1,8-Dihydroxyanthraquinone, O,O'-bis(trimethylsilyl)-	41.94	7336687
206	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-)	42.01	55282343
207	2-Azetidinone, 3,3-dimethyl-1,4-diphenyl-)	42.06	30365652
208	5-Cholestan-7-amine, N,N-dimethyl-)	42.09	1254019
209	EIQTMS_N12C_OARPLAS_3040.3_3350DF05_[Hexacosanoic acid (1TMS)]	42.14	NA
210	Cyclohexane, 1,4-dimethyl-2-octadecyl-)	42.19	1975344
211	2H-Pyran, 2-[[(5à, 17á)-androstan-17-yl] oxy] tetrahydro-	42.20	80988290
212	Spiro[1,3-dioxolane-2,2'-bicyclo[2.2.2]oct-5'-ene], 4,5-bis(ethoxycarbonyl)-7'-syn-hydroxy-	42.26	EPA153147
213	1,8-Dipropyl-3,6-diazahomoadamantan-9-ol)	42.28	138023275
214	I-22,23-Dihydrostigmasterol)	42.43	EPA214826

Appendix 2: Supplementary Table 5.1. Names of metabolites, CAS number and retention time of metabolites detected in spikelets of wheat near isogenic lines with alternate resistant/susceptible alleles on QTL-2DL (profiling based on GC-TOF-MS). CAS=chemical abstract service number according to NIST

ID	NAME (NIST/GOLM)	RT	CAS
1	l-Alanine, N-(TMS)-, TMS ester)	5.65	27844071
2	3-Hydroxycotinine, TMS ester	5.82	EPA74612
3	Tetroquinone	7.12	319891
4	L-Valine, N-(TMS)-, TMS ester)	8.35	7364445
5	Serine, bis(TMS)-)	9.35	NA
6	L-Norleucine, N-(TMS)-, TMS ester	9.82	55320111
7	Glycerol (3TMS)	10.04	NA
8	l-Threonine, O-(TMS)-, TMS ester)	10.33	7536825
9	N,O-Bis-(TMS)isoleucine	10.37	15985010
10	4-Aminobutyric acid (2TMS)	10.43	NA
11	2-Methylacetoacetic acid, di(TMS) deriv.	10.6	98779023
12	EITTMS_N12C_ATHL_1326.9_1135ec24_	10.62	NA
13	1,3-Cyclopentadiene, 1,2,3,5-tetramethyl-4-(pyrrolyl-2-ethyl)-)	10.65	EPA161273
14	EIQTMS_N12C_LJALM_1344.9_2236bg40_	11.35	NA
15	Butanedioic acid, bis(TMS) ester)	10.82	40309577
16	Propanoic acid, 2,3-bis[(TMS)oxy]-, TMS ester)	11.5	38191876
17	L-Serine (3TMS)	12.26	NA
18	L-Threonine (3TMS)	12.96	NA
19	EITTMS_N12C_STUT_1518.0_1135ec30_	15.23	NA
20	Malic acid, O-(TMS)-, bis(TMS)ester)	15.56	107241827
21	Benzoic acid, 4-ethoxy-, ethyl ester)	15.86	23676097
22	L-Proline, 5-oxo-1-(TMS)-, TMS ester)	16.03	30274772
23	Pyrazole-1-carboxylic acid, 5-[5-(2-thienyl)-2-thienyl]-, ethyl ester	16.29	EPA266463
24	4-Aminobutyric acid (3TMS)	16.3	NA
25	EIQTMS_N12C_LESFR_1555.8_3090AU05_	16.4	NA
26	Pentanedioic acid, 2-(methoxyimino)-, bis(TMS) ester)	17.64	60022879
27	N,O-Bis(TMS)-L-phenylalanine	18.55	7364514

28	L-Glutamic acid (3TMS)	18.65	NA
29	Benzimidazole, 5-tert-butyl-2-ethyl-	19.54	5805630
30	Asparagine tri-TMS)	19.82	EPA141391
31	EIQTMS_N12C_LPAL_1691.6_3268AU28_	20.23	NA
32	D-Fructose, 1,3,4,5,6-pentakis-O-(TMS)-, O-methyloxime	20.34	56196146
33	Xylose methoxyamine (4TMS)	20.41	NA
34	EIQTMS_N12C_NID1_1710.6_1248BK08_[532;1H-Indole-2,3-dione,1-(tert-butyldimethylsilyl)-5-isopropyl-,3-	20.53	NA
35	EIQTMS_N12C_LESR_2114.0_04014A49_	21.08	NA
36	EIQTMS_N12C_LESFR_1784.6_3090AU10_	22.07	NA
37	Phosphoric acid, bis(TMS) 2,3-bis[(TMS)oxy]propyl ester)	22.17	31038116
38	Pentanoic acid, di(isopropyl)silyl ester	22.27	EPA279480
39	2-Keto-l-gluconic acid, penta(O-TMS)-	22.34	EPA59824
40	2-(4-Methoxyphenyl)-2-(4-trimethoxysilyloxy)propane	22.37	EPA283555
41	1,2,3-Propanetricarboxylic acid, 2-[(TMS)oxy]-, tris(TMS) ester)	23.33	14330973
42	1,2,3-Propanetricarboxylic acid, 2-[(TMS)oxy]-, tris(TMS) ester)	23.43	14330973
43	EIQTMS_N12C_LJALD_1866.5_2236bg30_	24.07	NA
44	EIQTMS_N12C_LPEFR_1889.3_03363A02_	24.66	NA
45	EIQTMS_N12C_LPIFR_1891.4_03363A05_	24.68	NA
46	EITTMS_N12C_ATHR_1870.4_1135ec25_	24.76	NA
47	Fructose oxime hexaTMS)	24.93	120788241
48	EIQTMS_N12C_LCHL_1899.1_3268AU37_	25.16	NA
49	EIQTMS_N12C_LESFR_2341.5_3090AU13_	25.66	54548117
50	TMS ether of glucitol)	25.95	14199805
51	EITTMS_N12C_NTAL_1932.2_1135ec26_	26.03	NA
52	D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(TMS)--D-glucopyranosyl]-1,4,5,6-tetrakis-O-(TMS)-)	26.23	39523074
53	EIQTMS_N12C_LPEFR_1978.7_03363A02_	26.76	NA
54	D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(TMS)--D-glucopyranosyl]-1,4,5,6-tetrakis-O-(TMS)-)	26.83	39523074
55	EIQTMS_N12C_LCHL_2979.4_3268AU37_	26.91	NA
56	EIQTMS_N12C_LESFR_2016.7_3090AU05_	27.15	NA
57	EIQTMS_N12C_LESL_1963.7_2352AI10_	27.22	NA
58	Glucaric acid, 2,3,4,5-tetrakis-O-(TMS)-, bis(TMS) ester)	27.42	38165967
59	Phosphonic acid, methylenebis-, tetrakis(TMS) ester)	27.5	53044261
60	D-Gluconic acid, 2,3,4,5,6-pentakis-O-(TMS)-, TMS ester)	27.51	34290523

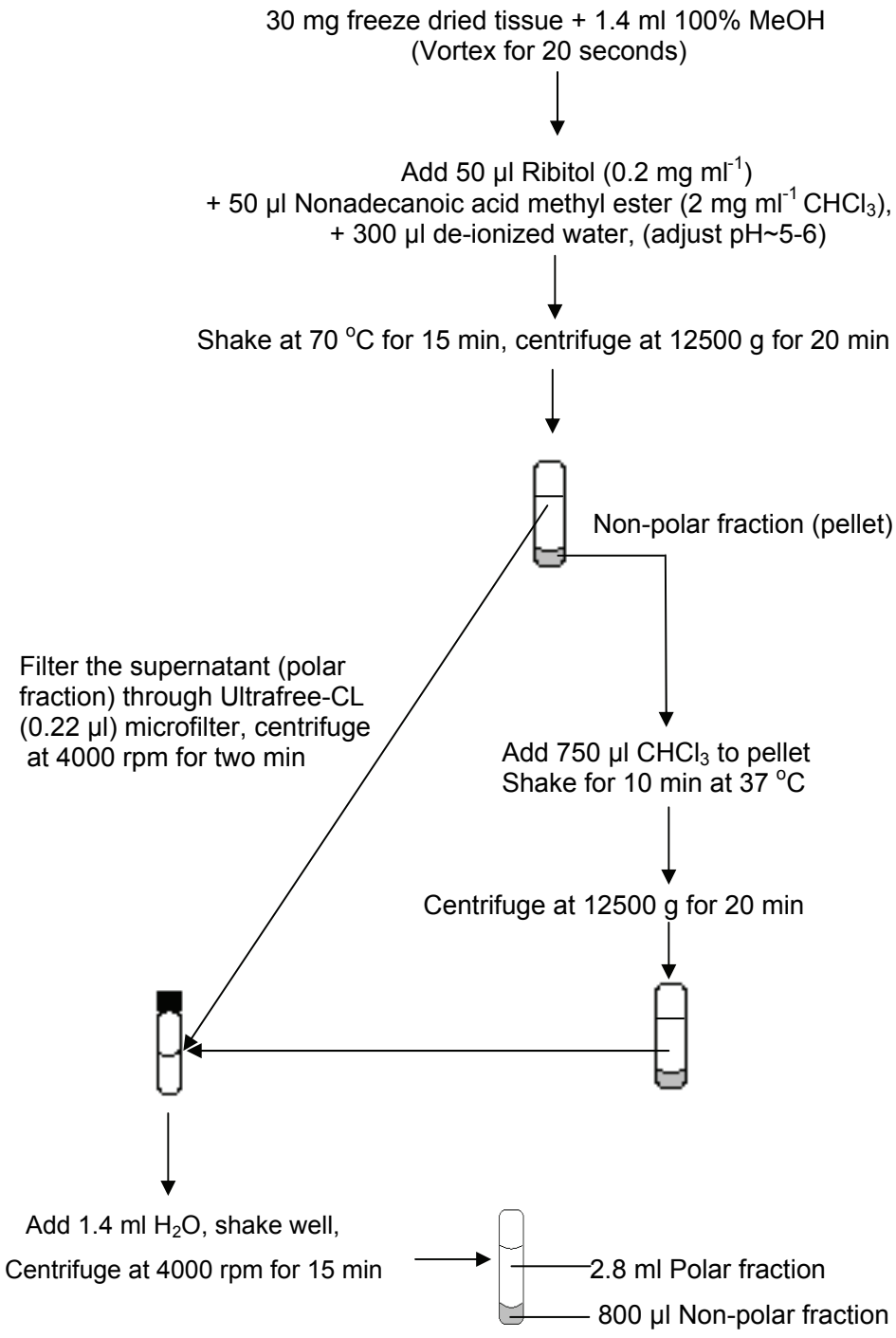
61	Sinapyl alcohol (2TMS)	28.25	NA
62	EIQTMS_N12C_LPEFR_2093.3_03363A03_	28.79	NA
63	EIQTMS_N12C_LPIL_2960.7_3268AU29_	29.15	NA
64	2H-1-Benzopyran-2-one, 7-(2H-naphtho[1,2-d]triazol-2-yl)-3-phenyl-)	29.37	3333628
65	Glucose oxime hexaTMS)	29.38	120850897
66	N,N',o-Tris-(TMS)tryptophane	30.77	7537044
67	Mannose, 6-deoxy-2,3,4,5-tetrakis-O-(TMS)-, L-)	30.86	19127152
68	Octadecanoic acid, TMS ester	30.97	18748919
69	EIQTMS_N12C_LHIFR_2288.0_03363A09_	32.52	NA
70	EIQTMS_N12C_LESR_2298.0_04014A49_	33.02	NA
71	EIQTMS_N12C_LESR_2329.5_04014A50_	33.15	NA
72	D-Mannose, 2,3,4,5-tetrakis-O-(TMS)-, O-methyloxime, 6-[bis(TMS) phosphate])	33.28	55530762
73	D-Mannose, 2,3,4,5-tetrakis-O-(TMS)-, O-methyloxime, 6-[bis(TMS) phosphate])	33.55	55530762
74	EIQTMS_N12C_LESFR_2370.4_3090AU09_	33.92	NA
75	EIQTMS_N12C_LJALD_2396.6_2236bg30_	34.17	NA
76	Myo-Inositol, 1,3,4,5,6-pentakis-O-(TMS)-, bis(TMS) phosphate)	34.72	33910064
77	EIQTMS_N12C_LESFR_2525.2_3090AU09_	36.48	NA
78	EIQTMS_N12C_LCHL_3403.1_3268AU31_	36.48	NA
79	Adenosine (4TMS)	37.89	NA
80	EIQTMS_N12C_LPIFR_2656.2_03363A04_	38.50	NA
81	EIQTMS_N12C_LESFR_2651.6_3090AU13_	38.56	NA
82	2-Monostearin TMS ether	39.35	53336133
83	1-Monooctadecanoylglycerol (2TMS)]	39.84	NA
84	EIQTMS_N12C_LPIFR_2730.9_03363A05_	40.39	NA
85	Dodecane	5.56	112403
86	Benzeneacetic acid, methyl ester)	7.16	101417
87	Benzaldehyde, 2,4-dimethyl-)	8.03	15764166
88	EIQTMS_N12C_LHIL_1211.9_3268AU06_	8.32	NA
89	Dodecane, 4,6-dimethyl-)	8.79	61141728
90	1,3-Di-tert-butylbenzene	9.03	NA
91	Decane, 5-ethyl-5-methyl-)	9.91	17312742
92	Decane, 5-ethyl-5-methyl-)	10.05	17312742
93	EIQTMS_N12C_LESR_1547.4_04014A49_	10.25	NA

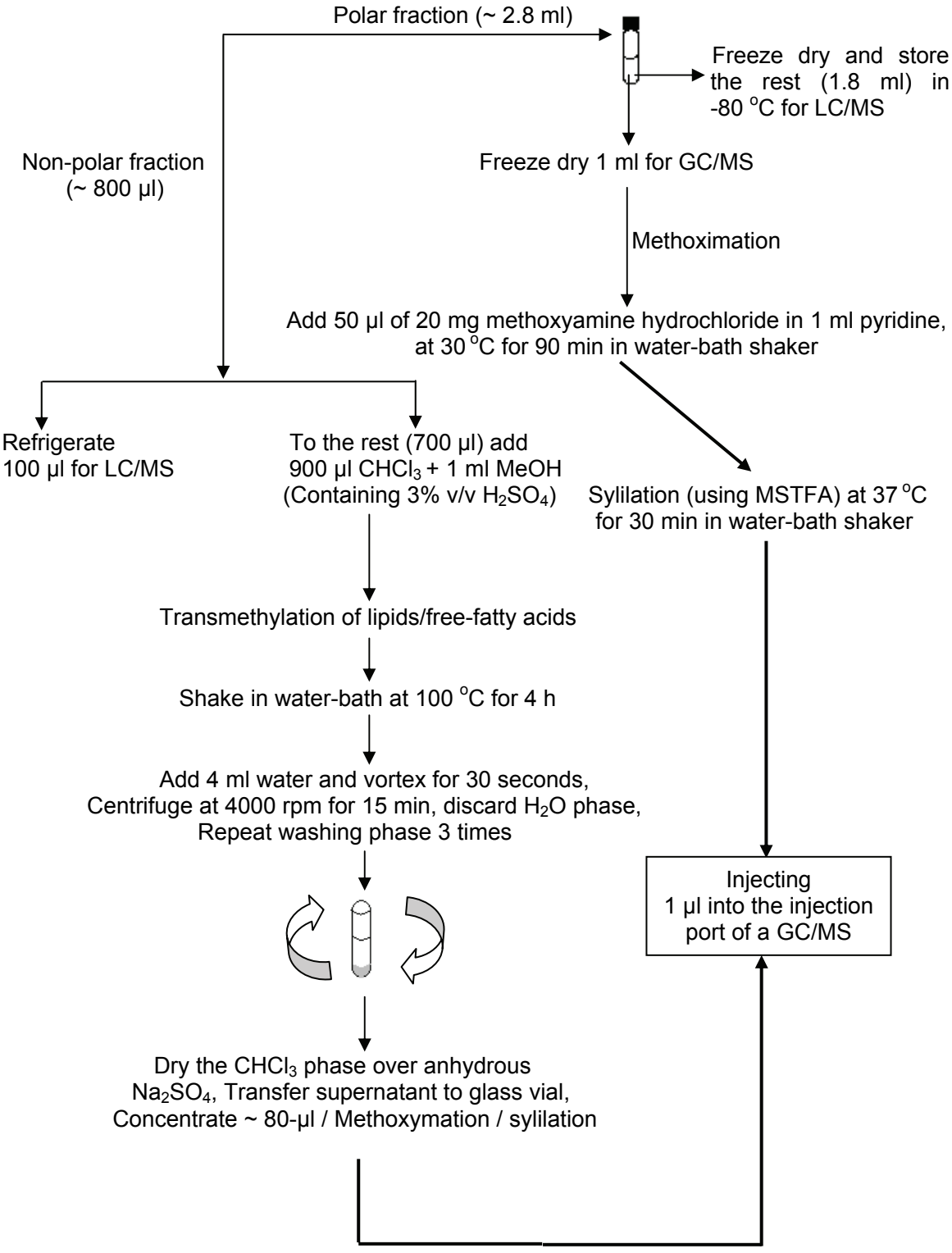
94	EIQTMS_N12C_LHIL_1307.8_3268AU06_	10.35	NA
95	1-Nonadecene)	10.35	18435455
96	1-Heptadecene)	10.78	6765395
97	Hexadecane, 2,6,11,15-tetramethyl-	10.87	504449
98	EIQTMS_N12C_LHIL_1331.4_3268AU18_	11.12	NA
99	Pentadecane	11.36	629629
100	6,7-Dimethyl-3H-isobenzofuran-1-one	11.7	343852506
101	2,5-di-tert-Butyl-1,4-benzoquinone	14.4	2460777
102	Decane, 2,2-dimethyl-)	14.79	17302373
103	EIQTMS_N12C_LESR_2342.3_04014A50_	15.02	17312742
104	EIQTMS_N12C_LESFR_1752.1_3090AU02_	15.16	NA
105	Decane, 3-ethyl-3-methyl-)	15.26	17312662
106	EIQTMS_N12C_LESFR_2341.5_3090AU09_	15.39	NA
107	Phenol, 2,4-bis(1,1-dimethylethyl)-	15.57	96764
108	Benzoic acid, 4-hydroxy-3-methoxy-, methyl ester	15.65	3943746
109	Benzoic acid, 4-ethoxy-, ethyl ester)	15.85	23676097
110	EIQTMS_N12C_LESFR_1751.9_3090AU09_	16.26	NA
111	EITTMS_N12C_CONTR_1743.6_1135ec03_	16.76	NA
112	EIQTMS_N12C_LESFR_2103.5_3090AU05_	19.67	NA
113	Hexadecane, 2,6,10,14-tetramethyl-)	20.22	638368
114	EIQTMS_N12C_LPIL_2342.6_3268AU05_	20.41	NA
115	EITTMS_N12C_CONTR_1743.6_1135ec03_	20.85	NA
116	Heptadecane, 2-methyl-)	21.18	1560890
117	EITTMS_N12C_CONTR_1743.6_1135ec03_	21.48	NA
118	Cyclohexane, 1-ethyl-2-propyl-)	21.69	62238339
119	Cinnamic acid, p-(trimethylsiloxy)-, methyl ester	21.93	27798692
120	EITTMS_N12C_CONTR_1743.6_1135ec03_	22.22	NA
121	EIQTMS_N12C_LESR_2596.1_04014A49_	22.65	NA
122	Pentadecanoic acid, methyl ester	22.75	7132641
123	4-n-Dodecylresorcinol	23.22	24305564
124	Tetradecanoic acid, TMS ester)	23.35	18603173
125	4-n-Dodecylresorcinol	23.68	24305564
126	2-Heptadecanone)	24.33	2922512

127	Methoxsalen	24.56	298817
128	Tricyclo[3.3.1.1(3,7)]decan-6-one, 2,2,7-trimethyl-5-propyl-1,3-diaza-	24.77	EPA305746
129	EIQTMS_N12C_LCHL_2166.8_3268AU37_	24.79	NA
130	Hexadecanoic acid, methyl ester	24.87	112-39-0
131	Vinyltributoxysilane)	25.27	18402294
132	Cinnamic acid, 3-methoxy-4-(trimethylsiloxy)-, methyl ester	25.38	27798705
133	EIQTMS_N12C_LESFR_2341.9_3090AU02_	26.4	NA
134	1-Octadecanol)	27.92	112925
135	1-[(TMS)oxy]-2-methylanthraquinone	28.14	EPA103735
136	9,11-Octadecadienoic acid, methyl ester, (E,E)-	28.15	13038476
137	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	28.28	301008
138	9-Octadecenoic acid, methyl ester,	28.37	1937628
139	Octadecanoic acid methyl ester	28.79	NA
140	Octadecanoic acid	29.46	57114
141	EIQTMS_N12C_LPEL_2166.7_3268AU15_	29.72	NA
142	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	30.02	1001805
143	EIQTMS_N12C_LESFR_2341.5_3090AU13_	30.38	NA
144	Manganese, bis[1,3-bis[2-(dimethylamino)ethyl]indenyl]-	30.63	EPA160407
145	2-Naphthalenecarboxaldehyde, oxime, (E)-	30.84	51873984
146	2,5-Cyclohexadien-1-one, 4-[3,5-bis(1,1-dimethylethyl)-4-oxo-2,5-cyclohexadien-1-ylidene]-2,6-bis(1,1-dime	30.95	2455143
147	Eicosanoic acid methyl ester	32.37	NA
148	EIQTMS_N12C_LESFR_2341.5_3090AU13_	32.74	NA
149	4H-1-Benzopyran-4-one, 2-[3,4-bis[(TMS)oxy]phenyl]-3,5,7-tris[(TMS)oxy]-	33.20	4067667
150	EIQTMS_N12C_LJAN_2420.0_2233bg14_	33.38	NA
151	EIQTMS_N12C_LJAN_2420.0_2233bg14_	33.67	130090185
152	Myristic acid, 2,3-bis(trimethylsiloxy)propyl ester	33.85	1188734
153	3,5-Bis(2,5-dimethylphenyl)- 2,3-dihydro-1H-inden-1-one	34.07	357941130
154	Cholest-7-en-3-ol, trifluoroacetate,(3)-	34.17	55515225
155	Hexadecanoic acid, 2-methylpropyl ester	35.52	110349
156	Cholestan-7-ol, 8,9-epoxy-3-(phenylmethoxy)-, (3,5,7,8)-	35.67	74420818
157	Tris(3-phenyl-2,4-pentanedionato)aluminum(iii)	35.87	15750739
158	Hexadecanoic acid, 1-[[(trimethylsilyl)oxy]methyl]-1,2-ethanediyl ester	36.31	53212934
159	Acetic acid, 7,7,10a,12a-tetramethyl-1,5-dioxo-1,2,3,4,4a,4b,5,7,8,9,10,10a,10b,11,12,12a-hexadecahydro	36.38	EPA194639

160	1-Monohexadecanoylglycerol (2TMS)]=1-Palmitoylglycerol	36.94	NA
161	EIQTMS_N12C_LJAN_3594.3_2233bg14_	37.01	NA
162	Tricosanoic acid, methyl ester)	37.28	2433978
163	2-Propenoic acid, 3-[3,4-bis[(tert-butyldimethylsilyl)oxy]phenyl]-, tert-butyldimethylsilyl ester	37.86	EPA221713
164	EIQTMS_N12C_LPAL_1128.1_3268AU16_	38.33	NA
165	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	38.59	621613
166	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	38.72	621614
167	Tetracosanol (1TMS)	39.11	EPA221689
168	(2-Methoxy-1,3,2-thiozin)[5,10,9]androstan-17-ol-3,11-dione, acetate	39.2	EPA128145
169	5à-Cholestan-7á-amine, N,N-dimethyl-	39.49	1254019
170	EITTMS_N12C_NTAL_1636.8_1135ec07	39.52	NA
171	1-Monooctadecanoylglycerol (2TMS)	39.85	NA
172	1-Monooctadecanoylglycerol (2TMS	39.86	NA
173	1,1'-Bicyclohexyl, 4-methyl-4'-pentyl-	40.32	92263428
174	EITTMS_N12C_STUO_2121.9_1135ec12_	40.49	NA
175	D-Glucopyranoside, 5-(acetyloxy)-7-[(acetyloxy)methyl]-1,4a,5,7a-tetrahydrocyclopenta[c]pyran-1-yl, 2,3,4-tr	40.88	75228205
176	EIQTMS_N12C_LPIL_2255.3_3268AU29	41.02	NA
177	1,8-Dihydroxy-3-methylanthraquinone, O,O'-bis(trimethylsilyl-)	41.13	7336723
178	delta-Tocopherol (1TMS)	41.39	NA
179	Tetracosanol (1TMS)	41.54	NA
180	3-Acetyl-1-(3,4-dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-3H-2,3-benzodiazepine	41.72	90140651
181	Phthalic acid, 3,5-dimethylphenyl 4-formylphenyl ester	41.85	EPA315738
182	Hexacosanol (1TMS)	41.97	NA

Appendix 3. Analytical method for metabolic profiling of wheat spikelets (modified from Fiehn et al., 2000a, b)




Polar fraction (~ 2.8 ml)
Freeze dry and store the rest (1.8 ml) in -80 °C for LC/MS
Non-polar fraction (~ 800 µl)
Freeze dry 1 ml for GC/MS
Methoximation
Add 50 µl of 20 mg methoxyamine hydrochloride in 1 ml pyridine, at 30 °C for 90 min in water-bath shaker
Refrigerate 100 µl for LC/MS
To the rest (700 µl) add 900 µl $CHCl_3$ + 1 ml MeOH (Containing 3% v/v H_2SO_4)
Sylilation (using MSTFA) at 37 °C for 30 min in water-bath shaker
Transmethylation of lipids/free-fatty acids
Shake in water-bath at 100 °C for 4 h
Add 4 ml water and vortex for 30 seconds, Centrifuge at 4000 rpm for 15 min, discard H_2O phase, Repeat washing phase 3 times
Injecting 1 µl into the injection port of a GC/MS
Dry the $CHCl_3$ phase over anhydrous Na_2SO_4, Transfer supernatant to glass vial, Concentrate ~ 80-µl / Methoxymation / sylilation