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

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Abstract

Metabolic profiles of spikelets of wheat cultivars, Roblin and Sumai3, respectively, susceptible and resistant to fusarium head blight (FHB) were analyzed using GC/MS to develop a technology to discriminate resistance. More than 700 peaks were detected and a total of 55 compounds were tentatively identified, of which 49 were induced/up-regulated following pathogen inoculation, including 23 in Roblin and 26 in Sumai3. However, only five metabolites were significantly different both between cultivars and inoculations. Metahydroxycinnamic acid, though was detected in all four treatments, exhibited a six-fold increase in abundance in Sumai3 following pathogen inoculation, with no corresponding change in Roblin. The abundance of myo-inositol in Sumai3 was higher than that in Roblin, and in both the abundance increased following pathogen inoculation. The compounds common to all treatments were subjected to factor analysis to identify groups of compounds, based on significant factor-loadings, associated with susceptibility or resistance against FHB. The treatment involving pathogen-inoculation of the resistant cv. Sumai3 was associated with the highest scores for the first and second factors that can be used for the discrimination of resistance against FHB. The first factor was associated with higher abundances of several fatty acids and aromatic compounds, while the second factor was associated with metabolites such as p- and m-coumaric acids, myo-inositol and other sugars, and malonic acid. The treatments involving pathogen-inoculation had higher factor scores for the third factor than the water inoculated, the highest being for the susceptible cultivar Roblin, and 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 various compounds detected in this study are discussed, in terms of their possible roles in plant defense against pathogen-stress, their metabolic pathways of synthesis, and their potential application for screening cultivars of wheat for resistance to FHB.

Keywords: *Gibberella zeae*; *Fusarium graminearum*; Metabolomics; Functional genomics; *Triticum aestivum*; Factor analysis; Wheat scab; Horizontal resistance

1. Introduction

Fusarium head blight (FHB; scab), caused by *Gibberella zeae* Schw. (Anamorph: *Fusarium graminearum* Schw.), is ranked as the number one disease of wheat in North America [7]. Under warm and humid conditions it can cause severe losses in yield and more importantly, it can reduce or destroy grain quality by producing many different mycotoxins [1,32]. Control of the disease by chemical, cultural and biological methods is very difficult [12,25–27] and host resistance is considered to be the most promising method [17]. The nature of FHB resistance in wheat is considered to be either passive associated with phenotypic traits or active associated with reduction of pathogen development or quantitative resistance [28]. Resistance mechanisms involved in wheat to FHB are not well understood, but quantitative trait loci associated with

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resistance have been identified [1,6,22]. Resistance to FHB is classified into five different types [18]: 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 (which is not a true resistance) and Type-V = resistances to mycotoxin accumulation. The first two Types have received the most research attention [28,40].

Wheat cultivars with improved resistance have been developed based on selection for low disease severity, often using simple disease ratings and without prior knowledge of mechanisms involved in resistance [11]. Thorough assessment of quantitative resistance parameters for FHB (e.g. infection efficiency, latent period, sporulation, disease progress over time, etc.) in large breeding populations would be useful in further improving resistance and in advancing understanding of resistance mechanisms, but would be prohibitively costly to conduct in greenhouse environments or in field environments over seasons and regions. Furthermore, breeders are looking for mechanisms of disease resistance genes to pyramid genes in to an elite cultivar. Thus, resistance screening methods that provide an understanding of the disease resistance mechanisms are needed 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, resulting in the detection of disease response genes and pathogenesis-related (PR) proteins that are induced following pathogen inoculation [29,34] and an observation that of a higher concentrations of free phenolic compounds (especially *p*-coumaric acid in glumes, lemmas and paleas) in resistant wheat cv. Frontana as compared to susceptible cv. Argent inoculated with *F. graminearum* [42]. In vitro, *p*-coumaric and ferulic acid have been observed to have synergistic effects in inhibiting mycelial growth of two isolates of *F. graminearum* [42].

To further advance understanding of host-pathogen interactions, it may be beneficial to study the entire metabolome in a comprehensive manner [13,38]. No comprehensive metabolic profiling study has yet been reported on discrimination of disease resistance in wheat or any other plant species. Here, we hypothesize that wheat cultivars which differ in resistance to FHB will also differ in their metabolic profiles, inherently and/or in their early response (24 h) following 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 discriminate levels of resistance in wheat against FHB.

2. Materials and methods

2.1. Plant and pathogen production

Plants of spring wheat cultivars Roblin and Sumai3, susceptible [11] and resistant with a high level of Type-2 resistance to FHB [25,44], were grown in 15 cm pots and maintained in a greenhouse at 22 ± 3 °C. The plants were fertilized with 100 ml of a 0.2% solution of fertilizer Plant-Prod® twice, at growth stages: GS 25 (tillering stage) and GS 40 (booting stage) [47].

Seven day old cultures of *Fusarium graminearum* (teleomorph *Gibberella zeae*; isolate 99–15–35) were flooded with water, filtered through two layers of cheese cloth and spore suspensions were made in aqueous solutions of 0.02% Tween 80. The spore concentration was adjusted to 10^5 macroconidia ml^{-1}.

2.2. Inoculation and incubation

At anthesis (GS = 60–69) [47] the spikes were area-source inoculated by placing 10 μl of the macroconidial suspension into the middle floret of each of the four spikelets located about the middle length of spike [16]. Spikelets inoculated with 10 μl of distilled water containing 0.02% Tween 80 served as controls. After inoculation, the plants were covered with plastic bags sprayed inside with water to provide saturated atmosphere and kept in a greenhouse maintained at 20 ± 3 °C. The plastic bags were removed after 24 h.

2.3. Disease severity assessment

The spikelets with FHB symptoms were monitored after inoculation, at 4 d intervals, until 20 d after inoculation (dai). A spikelet showing discoloration, necrosis or visible mycelia was considered diseased. The FHB severity (y) was calculated as proportion of inoculated spikelets diseased (PISD) in a spike (y = number of spikelets diseased in a spike/number of spikelets inoculated). Several severity (PISD) values over time, the disease progress curve, were reduced to one value by calculating the area under the disease progress curve (AUDPC) using the formula [15,41]:

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

where y is the PISD, t is the time in days after inoculation, i is the ith observation and n is the total number of observations.

2.4. Metabolite extraction and GC/MS analysis

Sets of 16 inoculated spikelets, 4 inoculated spikelets from each of four spikes, were harvested at 24 h after inoculation (hai), and used as the sampling units for metabolite extraction and analysis. Immediately after harvesting these samples were crushed in liquid nitrogen to suppress probable hydrolytic activity. Metabolites were extracted following the methods developed by Fiehn et al. [13,14] with 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 12,000*g* for 5 min and the supernatant was decanted into a separate test tube to which 1.4 ml distilled water was added. To the pellets chloroform was added, shaken well and centrifuged at 12,000*g* for 5 min, and the supernatant was 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, 1 ml of the methanol fraction was removed, concentrated down to one fifth using SpeedVac concentrator, and then freeze dried. From the bottom portion, 1 ml of chloroform fraction was removed, 3% v/v H_2SO_4 in methanol was added to transmethylate fatty acids and lipids, washed 3 times with distilled water, dried by adding anhydrous Na_2SO_4, and concentrated by means of a SpeedVac concentrator. Since most compounds were non-volatiles, both the fractions were separately derivatized by adding *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) to volatilize them at GC oven temperatures. Methoxyamine hydrochloride in pyridine was added to the dried residue (methoximation) because direct derivatization of sugars such as fructose cause confusing peaks related to hexoses [37].

One micro-liter aliquots of spikelet extracts from each of methanol and chloroform were injected into the port of GC/MS (GC 3400×c with Voyager® ion trap mass analyzer; Varian®, Que., Canada) equipped with an auto sampler. The injection port temperature was maintained at 230 °C. A capillary column DB-5MS (0.25 μm film thickness, 0.25 mm in 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 as 70 °C for 5 min, followed by temperature ramping at the rate of 5 °C min^{-1} up to 280 °C, then held at 280 °C for 3 min. 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}. The GC/MS output consisted of scans and abundances for ions 46–600 *m*/*z* for each scan.

2.5. Metabolite identification and quantification

The GC/MS output on scans and mass abundances were processed using Saturn Lab software and the compounds were identified using NIST library mass spectrum search program (version 1.6). For each peak the consistency of major fragments of spectrum across the four blocks (replicates) were manually investigated, using Pivot Table feature of Microsoft Excel to sort data based on retention time. The observed spectra of each peak for different blocks were compared with ten topmost choices in NIST to confirm the identity of a compound 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 in further analysis. The peaks, especially with low abundances, were inconsistent across blocks, as they were close to noise level. In the table of compounds automatically generated by the Saturn software, when peaks were detected in only two or three blocks of a treatment, the corresponding retention-time regions of the chromatographs were inspected for all blocks. When peaks, similar in spectra, were detected across all the four blocks, the identities and abundances of compounds were determined using the automated component table builder of the Saturn lab software. For a given spectra of peaks, when no suitable match in the NIST hits was found, 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 the four treatments (SW, SP, RW, RP) were considered discriminatory.

2.6. Experimental design and statistical analysis

The experiment was designed as a randomized complete block, with four treatments (of two cultivars, Roblin and Sumai3; two inoculations, water and pathogen), and the evaluations were conducted at four different times, thus four blocks. The experimental unit for metabolic profiling consisted of a sample pool of 16 inoculated-spikelets (four inoculated spikelets harvested from four spikes of four plants) harvested 24 h after inoculation. In addition, four spikes from four pathogen-inoculated plants of each cultivar, for each of the four blocks, were used for disease severity assessment, where the FHB symptoms on the spikelets were assessed non-destructively over a period of 20 d after inoculation.

The disease severity (PISD) and the AUDPC were subjected to ANOVA, using SAS (21). The data on metabolic profiles, which consisted of several compounds (up to 55 that were consistent among replicates) and their relative abundances (the ion-trap mass analyzer output from the GC/MS analyses, were subjected to univariate analysis using SAS (21), to identify metabolites with significant differences between cultivars and between inoculations. The metabolites that were significantly up-regulated in their abundances following pathogen inoculation (and also novel metabolites) based on univariate analysis, were designated as pathogenesis related (PR−) metabolites (SP > SW and RP > RW). The abundances of compounds that occurred in all the treatments (48 compounds = 31 from methanol and 17 from chloroform extracts of spikelets) were subjected to factor analysis following FACTOR procedure, using principal components, of SAS [19–21], to identify the contribution of individual metabolites (based on their factor-loadings) to a treatment, and the interrelationship among treatments (factor-scores). The factor-scores explained the spatial location of treatments, indicating the relationships among treatments, where a positive factor-score was associated with a positive factor-loading for metabolites. The values of factor-loadings increased with an

Table 1

Table1 List of compounds (abundance = $\times 10^6$; rounded-off) detected in Roblin and Sumai3 wheat cultivars, at 24 h after inoculation with *Fusarium graminearum* or water (control). Eigenvectors and Eigenvalues for each the first three factors calculated by factor analysis of normalized abundances of 49 metabolites (32 from Methanol 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). Higher factor-loadings show higher contributions of the corresponding metabolites in variability of the factor (Factor scores contributing to different treatments are shown in Fig. 1).

RT (mm:ss) range	Compound name	M/C[a]	UR-/PR-metabolite	Chem group	RP	RW	SP	SW	F1	F2	F3
5.70–.75	1,2-Ethanediamine, *N*,*N*,*N*′,*N*′-tetramethyl, (161)[b]	M	S > R (NS;0.09)[c]	AM[f]	0.23	0.19	0.27	0.18	0.64*[e]	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	45	123	70	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 ester, (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 ester, (356)	C	S > R	FA	0.71	0.70	0.87	0.45	0.96*	0.05	0.29
26.73–.80	Pentadecnoic acid, 14-methyl-, methyl ester, (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-dimethylethyl)-, (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

Table 1 (*continued*)

RT (mm:ss) range	Compound name	M/C[a]	UR-/PR-metabolite	Chem group	RP	RW	SP	SW	F1	F2	F3
24.52–.57	1,2,3-Propanetricarboxylic acid, 2-[(TMS)oxyl]-, tris(TMS) ester 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) ester, (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 = Malic acid (tms), (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*-(trimethylsiloxy)-, 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)-, TMS ester, (273)	M	S	AA	0.41	0.50	0.25	0.22	0.33	*−0.93**	−0.13
34.95–.99	Myristic acid, 2,3-bis(trimethylsiloxy) 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

Table 1 (*continued*)

RT (mm:ss) range	Compound name	M/C[a]	UR-/PR-metab-olite	Chem group	RP	RW	SP	SW	F1	F2	F3
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	Ribofura-nose-1,2,3,5-tetraTMS, (438)	M	S	SU			0.41				
25.12–.19	Tetradeca-noic 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*-butylbenze-nethiol, (278)	C	R > S	AR	0.18	0.14	0.15	0.15	−0.07	−0.24	0.97*
29.90–.98	8,11-Octade-cadienoic 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 ester, (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*

Table 1 (*continued*)

RT (mm:ss) range	Compound name	M/C[a]	UR-/PR-metabolite	Chem group	RP	RW	SP	SW	F1	F2	F3
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-dimethylethyl)-, (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(trimethylsiloxy)-, (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 ester, (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 ester, (554)	M	R	OA	0.04		0.05	0.06			
33.95–.99	Eicosanoic acid, methyl ester, (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 ester, (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) ester, (480)	M	(0.01;NS)	OA	0.66	0.66	1.03	1.03	−0.18	0.97*	−0.13

Table 1 (*continued*)

RT (mm:ss) range	Compound name	M/C[a]	UR-/PR-metabolite	Chem group	RP	RW	SP	SW	F1	F2	F3
20.47–.50	Arabinoic acid, 2,3,5-tris-*O*-(TMS)-,. gamma lactone, (364)	M		OA				0.37			
23.93–97	Cinnamic acid, *p*-(trimethylsiloxy)-, methyl ester, (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 acid tetra TMS, (424)	M		OA	0.01	0.02	0.01	0.02	−0.16	−0.03	*0.99**
R = R-UR = nique Roblin-UR metabolites = RP > RW	12[f]			Eigenvalues					19.79	18.43	12.78
S = S-UR = Unique Sumai3-UR metabolites = SP > SW	14			%Variance explained					0.39	0.36	0.25
R > S; S > R = Common-UR metabolites = common R > S-UR; S > R-UR	23			Cumulative % of explained variance					0.39	0.75	1
Total R/R > S-UR-metabolites = (Unique and Common R-UR) = 12 + 11	23										
Total S/S > R-UR-metabolites = (Unique and Common S-UR) = 14 + 12	26										

[a] M, from methanol fraction and C, From chloroform fraction of the extract of spikelets.

[b] Relatively high factor-loading of metabolites to the respective factor in the column; * = higher levels of factor-loading of the corresponding compound to the factor in the column; the factor-scores for treatments are given in Fig. 1.

[c] Significance level based on univariate analysis (in parenthesis the first number is between cultivars and the second number is between inoculations); values $\rho \leq 0.01$ is highly significant, $\rho \leq 0.05$ is significant, $\rho \leq 0.10$ is borderline significant.

[d] Number of up-regulated UR-metabolites in different categories mentioned; PR-metabolite is pathogenesis related metabolite = UR-metabolites that are significantly up-regulated.

[e] Molecular Weight;

[f] AA = amino acid; AM = amine; AR = aromatic; FA = fatty acid; OA = organic acid; SU = sugar; UN = unidentified.

increase in the abundance of metabolites that loaded to a given factor.

3. Results

3.1. Disease progress

Fusarium head blight symptoms developed in all the pathogen-inoculated spikes, but in not all of pathogen-inoculated spikelets. At least 2 out of 4 pathogen-inoculated spikelets, in a spike, were diseased in Sumai3 but the number diseased was higher for Roblin. Within a spike, the infection spread from the pathogen-inoculated spikelets to non-inoculated spikelets in the susceptible cv. Roblin but not in the resistant cv. Sumai3, meaning the latter expressed Type-II resistance. The ratios of diseased spikelets to inoculated spikelets (PISD) at 4, 8, 12, 16 and 20 d 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 PISD >1.0 means the infection has spread to spikelets beyond those that were inoculated. The area under the disease progress curve (AUDPC) was 7.50 in Sumai3 and 39.00 in Roblin, meaning the cultivar Sumai3 was more resistant than the Roblin to FHB.

3.2. Metabolic profiles

3.2.1. Metabolites of wheat-FHB system

More than 700 peaks were detected in the wheat-FHB pathosystems tested here, out of which a total of 55 compounds were tentatively identified, including 38 and 17 compounds from methanol-water (hydrophilic) and chloroform (lipophilic) fractions of plant extracts, respectively (Table 1). Out of 55 compounds detected here, a total of 48 compounds were common to all treatments. Among the three treatment-discriminatory metabolites three metabolites, Arabinoic acid, 2,3,5-tris-*O*-(TMS)-,gamma lactone; Ribofuranose-1,2,3,5-tetraTMS and D-Ribose, 2,3,4,5-tetrakis-O were unique to Sumai3, and the remaining 4 were common to ≤ 3 treatments. A total of only 11 and nine compounds were significantly different between cultivars and between inoculations, respectively, including five compounds that were significantly different both between cultivars and inoculations (Table 1). The abundances of cinnamic acid, *m*-(trimethylsiloxy)- were highly significant both between cultivars and inoculations. The metabolites detected here belonged to diverse functional groups such as fatty acids, sugars, aromatics, amino acids and phenolics (Table 2).

3.2.2. Factor analysis of metabolites

The 48 compounds that were common to all the treatments, from both methanol and chloroform fractions (Table 1; Section 3.2), were subjected to factor analysis. The first three factors accounted for 100% (F1 = 39%; F2 = 36%; F3 = 25%) of the variances in the selection of compounds with significant factor-loadings (Table 1). The factor-scores showing the interrelationship among treatments are presented in Fig. 1, and the factor-loadings of metabolites contributing to factor-scores in Table 1. The metabolites with significant factor-loadings to each of the first three factors are listed in Fig. 1 (see caption). The scatter plot of factor-scores of treatments, in a three dimensional space (F1 × F2 × F3), showed clustering of treatments.

3.2.3. Wheat cultivar-related metabolites

Among the 55 metabolites identified here 54 were detected in Sumai3 and only 49 in Roblin inoculated with water (Table 1), including 11 metabolites that significantly varied in abundances between cultivars, irrespective of inoculations. The abundances of 24 metabolites were higher in Roblin water-inoculated than in Sumai3 water-inoculated, of which 2 were significant, namely L-proline, 1-(TMS)-TMS ester and butanedioic acid, [(TMS)oxy]-, bis(TMS) ester (=Malic acid (TMS)). On the other hand, the abundances of 27 metabolites were higher in SW than in RW, among which eight were significant, including butandioic acid bis(TMS) ester; cinnamic acid, *m*-(trimethylsiloxy)-, methyl ester 1,2,3-propanetricarboxylic acid, 2-[TMS)oxyl]-, tris(TMS) ester; D-fructose, 1,3,4,5,6-pentakis-*O*-(TMS)-, *O*-methyloxime; D-fructose, 1,3,4,5,6-pentakis-*O*-(TMS)-, *O*-methyloxime RT2; glucose 2,3,4,5,6-pentakis-*O*-(TMS)-, *O*-methyloxime RT2; myo-Inositol, 1,2,3,4,5,6-hexakis-*O*-(TMS) and alpha-D-glucopyranoside,1,3,4,6-tetrakis-*O*-(TMS)-. Five metabolites were detected only in SW but not in RW, such as propanoic

Table 2
Total abundances ($\times 10^6$) of different functional groups of metabolites detected in Roblin (R) and Sumai3 (S) cultivars, inoculated with pathogen (P) or water (W)

Chemical group	RP	RW	SP	SW
Amine	0.35	0.3	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	14.7	16.51	7.66
Organic acid	1.87	1.83	2.31	2.53
Sugar	107.8	70.6	181.46	119.6
Unidentified	0.64	1.03	0.83	1.17

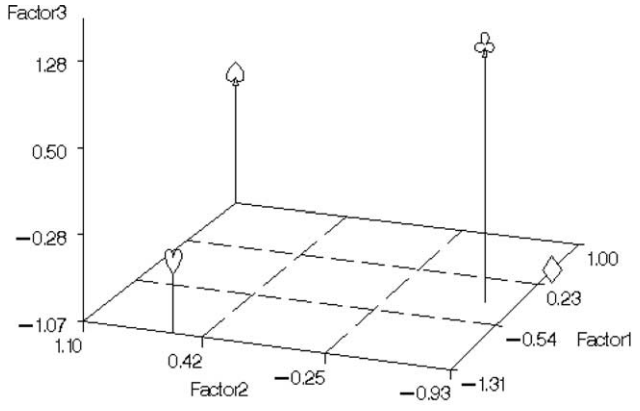

Fig. 1. Scatter plot, based on projections of three factor scores, of factor analysis of abundances of 49 metabolites (32 methanol fraction and 17 chloroform fraction compounds) that were common to all the treatments: Sumai3 pathogen (SP = ♠), Sumai3 water (SW = ♥), Roblin pathogen (RP = ♣) and Roblin water (RW = ♦) inoculated. Plausible hidden functions explained were: (a) *Defense/resistance function*: the treatments with F1 scores in descending order were: SP, RW, RP, SW; the treatments with F2 scores in descending order were: SP, SW, RP, RW; SP had the highest scores for both F1 and F2 as compared to RP, meaning correlation between higher resistance, and also with metabolites with high factor-loadings on F1 and F2; (b) *Pathogenesis/susceptibility function*: the treatments with F3 scores in descending order were: RP, SP, SW, RW; RP had the highest score, meaning Roblin being more susceptible/pathogenic. The factor-loadings of metabolites, for different factors, are shown in Table 1. The metabolites with significant factor-loadings to the first three factors are (the complete names of these compounds are in Table 1): F1 = tetradecanoic acid; pentadecnoic acid; heptadecanoic acid; hexadecanoic acid; octadecanoic acid; myristic acid; monostearin; benzene (1-butylopentyl)-; F2 = *m*-coumaric acid; *p*-coumaric acid; myo-inositol; fructose; galactose and glucose; propanetricarboxylic acid; α-*d*-glucopyranoside; malonic acid; butandioic acid; F3 = 8,11-octadecadienoic acid; phenol, 2, 4-bis; Tri-*t*-butylbenzenethiol; glutamine; L-alanine and Tri-*t*-butylbenzenethiol.

acid, 2,3-bis[(TMS)oxyl]-,TMS ester; D-ribose, 2,3,4,5-tetrakis-*O*; arabinoic acid, 2,3,5-tris-*O*-(TMS)-,gamma lactone; D-glucuronic acid, 2,3,4,5-tetrakis-*O*-(TMS)-, TMS ester and benzoic acid, 3-methoxy-.alpha.,4-.

3.2.3.1. Factor loadings of metabolites and cultivar discrimination. The first and second factors differentiated the cultivars. The cv. Roblin had a positive factor-score for F1 and a negative factor-score for F2, while the cv. Sumai3 had the opposite for both (Fig. 1). The metabolites with significant factor-loading to thee factors can be used in the discrimination of cultivars (Table 1). The cultivars Roblin which had significant positive factor-score for F1 had significant factor-loading for fatty acids, while the Sumai3 had negative factor-score for F2 had significant factor-loading for cinnamic acid, *p*-(trimethylsiloxy)-, TMS ester; galactose oxime hexaTMS and an unidentified peak (retention time 23.39–23.43 mass spectra 217, 73, 218, 147, 219, 45, 305), thus discriminating the two cultivars. However, there were metabolites with significant negative factor-loadings for both F1 and F2. Alpha-linolenic acid, TMS ester; an unidentified peak (retention time 23.39–23.43 mass spectra 95, 67, 123, 81, 82, 69, 55); benzene, 1, 3-bis (1, 1-dimethyethyll)- *N*, *N*-bis [2-trimethylsiloxylethyl] ethaneamine; L-valine, *N*-(TMS)-, TMS ester; octadecanoic acid, trimethylsilyl ester; 9,12-octadecadienoic acid (*Z*,*Z*)-, TMS ester and butanoic acid, 4-[bis(TMS)amino-, TMS ester had significant negative loadings for F1, indicating higher abundances of these metabolites in Sumai3. Metabolites with significantly higher abundances in Roblin like L-proline, 5-oxo-1-(trimethysilyl)-, TMS ester; 8, 11-octadecadienoic acid, methyl ester; glycine, *N*, *N*-bis (TMS)-, TMS ester; phosphoric acid, bis(TMS)monomethyl ester; an unidentified peak (retention time 25.14–25.18 mass spectra 345, 73, 255, 147, 346, 347, 45) had negative factor-loadings for F2.

3.2.4. Wheat-FHB-related metabolites (UR/PR-metabolites)

The metabolites induced, novel compounds or up-regulated in abundance, following pathogen inoculation were grouped into: (a) R-UR-metabolites that were unique to susceptible cv. Roblin (RP > RW); (b) S-UR-metabolites that were unique to resistant cv. Sumai3 (SP > SW); and (c) RS-UR-metabolites that were common to both cultivars (RP > RW and SP > SW), and the common ones were regrouped into R > S-UR-metabolites (= RP > SP), and S > R-UR-metabolites (= SP > RP) (Table 1). There were 49 UR-metabolites, including 12, 14, and 23, R-UR, S-UR and RS-UR-metabolites. Among the common metabolites, 11 and 12 were R > S-UR and S > R-UR-metabolites. The FHB resistance response can thus be discriminated into susceptible and resistant based on a total of 23 Roblin (R/R > S-UR) and 26 Sumai3 (S/S > R-UR) metabolites induced following pathogen-inoculation.

The UR-metabolites also varied in their extent of regulation following pathogen inoculation. The compounds that were more up-regulated in Sumai3 than in Roblin were:

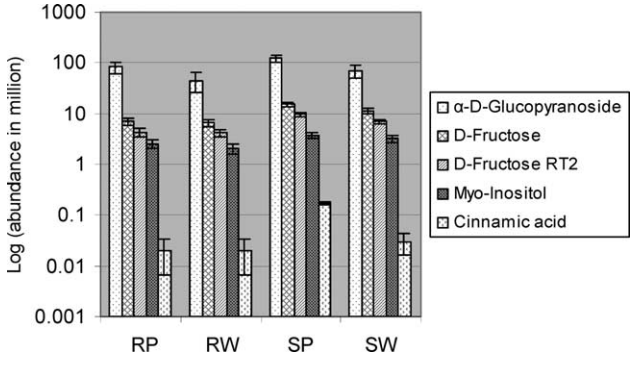

Fig. 2. Bar graph of the abundances (logarithmic scale) of five PR-metabolites, significantly discriminating treatments (RP = Roblin-Pathogen inoculated, RW = Roblin-Water inoculated, SP = Sumai3-Pathogen inoculated and SW = Sumai3-water inoculated). Error bars are ± root mean square of error (root MSE). The complete names of metabolites included here are given in Table 1. Among 9 PR-metabolites (= significant UR-metabolites) only 5 varied significantly among cultivars are shown here.

α-D-glucopyranoside,1,3,4,6-tetrakis-*O*-(TMS)-; hexadecanoic acid; octadecanoic acid; pentadecanoic acid; cinnamic acid, m-(trimethylsiloxy)-; heptadecanoic acid; hexadecanoic acid; myristic acid; tetradecanoic acid; etc. Among UR-metabolites, only ribofuranose-1, 2, 3, 5-tetraTMS was specific to SP. Benzoic acid, 2,6-bis(trimethylsiloxy)-; propanoic acid, 2,3-bis[(TMS)oxyl]-,TMS ester and D-glucuronic acid, 2, 3, 4, 5-tetrakis-*O*-(TMS)-, TMS ester were detected in RP, SP and SW but not in RW. The abundance of the propanoic acid was at least seven times higher in pathogen inoculated treatments as compared to water inoculated.

Among the 55 metabolites identified only 15 were significantly different among treatments, including 14 that were up-regulated, after pathogen-inoculation. Among the UR-metabolites nine were significantly up-regulated, PR-metabolites, however, only 5 of these varied significantly among cultivars (Fig. 2). Cinnamic acid, *m*-(trimethylsiloxy)-; myo-Inositol, 1, 2, 3, 4, 5, 6-hexakis-*O*-(TMS); α-D-glucopyranoside, 1, 3, 4, 6-tetrakis-*O*-(TMS)—and D-fructose, 1, 3, 4, 5, 6-pentakis-*O*-(TMS)-, *O*-methyloxime were significant both between cultivars and inoculations (Fig. 2). The difference in abundance of cinnamic acid, *m*-(trimethylsiloxy)-, TMS ester was highly significant ($P < 0.01$) between inoculations (SP > SW) and cultivars (S > R). Myo-Inositol, 1, 2, 3, 4, 5, 6-hexakis-*O*-(TMS) was significantly different between SP and RP (SP > RP) and SW and RW (SW > RW). α-D-glucopyranoside, 1, 3, 4, 6-tetrakis-*O*-(TMS) abundance was not different between RP and RW but was significantly different between SP and SW. The same trend was observed for D-fructose, 1, 3, 4, 5, 6-pentakis-*O*-(TMS)-, *O*-methyloxime (Fig. 2). L-alanine, *N*-(TMS)-, TMS ester and hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester were significantly different between cultivars (Sumai3 > Roblin). 1, 2-ethanediamine, *N*, *N*, *N*′, *N*′-tetramethyl; butanedioic acid, [(TMS)oxy]-, bis(TMS) ester= malic acid (TMS); 1,2,3-propanetricarboxylic acid, 2-[(TMS)oxyl]-, tris(TMS) ester and glucose 2,3,4,5,6-pentakis-*O*-(TMS)-, *O*-methyloxime RT2 were detected in significantly higher abundances in pathogen inoculated spikelets compared to water inoculated ones, irrespective of cultivars.

Several metabolites (Sumai3 = 15 and Roblin = 10) were down regulated following pathogen-inoculation, however, none was significantly different between inoculations, although two were significantly different between cultivars.

3.2.4.1. Factor loadings of metabolites and resistance discrimination. The first and the second factors had positive factor-scores for resistance, and the third factor had positive factor-score for susceptibility. The clustering pattern of treatments, according to three factor-scores, was further used to explain the plausible hidden functions, such as (a) pathogenesis or susceptibility function and (b) defense or resistance functions, by relating the significant positive factor-loadings of metabolites to susceptible or resistant cultivars and to water and pathogen inoculated treatments, and clustering of treatments based on factor-scores.

3.2.4.2. Pathogenesis/susceptibility function. Factor scores for F3 discriminated the levels of pathogenesis, where a higher positive factor-score of F3 was associated with: (i) pathogen-inoculated cluster of treatments (RP, SP) as opposed to water-inoculated (SW, RW), irrespective of cultivars; (ii) susceptible cultivar inoculated with pathogen (RP) as opposed to resistant (SP); (iii) resistant cultivar inoculated with water (SW) as opposed to susceptible (RW), indicating an association of metabolites with significant factor-loading to F3 with pathogenesis or susceptibility of cultivars (Section 3.1). The metabolites with significant factor-loading to F3 (causing higher positive factor-scores) were fatty acids such as 8, 11-octadecadienoic acid, methyl ester, and phenolic compounds such as phenol, 2, 4-bis (1, 1-dimethylethyl-) and benzene related compounds like 2, 4, 6-Tri-*t*-butylbenzenethiol and amino acids like glutamine.

3.2.4.3. Defense/resistance function. Factor-scores for F1 and F2 discriminated the resistant and susceptible cultivars (Section 3.1). A higher positive factor-scores of F2 were associated with: (i) resistant cultivar cluster of two treatments (SP, SW) as opposed to susceptible cultivar (RP, RW), irrespective of inoculation agents; (ii) resistant pathogen-inoculated cultivar (SP) as opposed to susceptible pathogen-inoculated cultivar (RP); (iii) resistant water inoculated cultivar (SW) as opposed to susceptible water inoculated cultivar (RW). The metabolites with high positive factor-loadings for F2 that partly (also confounding effects of cultivar-related metabolites) explaining defense functions were: cinnamic acid, *m*-(trimethylsiloxy)-, TMS ester (also Cinnamic acid, *p*-(trimethylsiloxy)-, methyl ester); myo-inositol, 1, 2, 3,4,5,6 hexakis; fructose; galactose and glucose; 1,2,3-propanetricarboxylic acid, 2-[(TMS)oxyl]-, tris(TMS) ester RT2; α-D-glucopyranoside, 1,3,4,6-tetrakis-*O*-(TMS)-beta-*d*-fructofuranosyl 2,3,4,6-tetrakis-*O*-(TMS); Tris-TMS malonic acid and an unidentified compound (retention time: 23.39–23.43 min mass spectrum: 217, 73, 218, 147, 219, 45, 305).

The highest positive factor-score of F1 was associated with: (i) pathogen-inoculated resistant cultivar (SP) as opposed to others (RW, RP, SW), thus, a higher positive F1 score means a higher level of resistance (note: a moderately high positive F1 scores for RP and RW and a negative score for SW). The metabolites with high positive factor-loadings for F1 that partly (also confounding with cultivar-related metabolites) explained defense functions were: benzene, (1-Butylopentyl)-; tetradecanoic acid, TMS ester; pentadecnoic acid, 14-methyl-, methyl ester; heptadecanoic acid, methyl ester; hexadecanoic acid, TMS ester; octadecanoic acid, TMS ester; myristic acid, 2,3-bis(trimethylsiloxy) propyl ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; octadecanoic acid, 2,3-bis[(TMS)oxyl]propyl ester and 2-monostearin TMS ether.

There is negative association between factor-score with metabolite(s) with negative factor-loading(s), which signifies negative correlation of *F*-score with abundance of the metabolite. The smaller factor-scores were associated with down-regulation of defense/resistance and up-regulation of susceptible/pathogenesis related metabolites. 9,12-octadecadienoic acid (*Z*, *Z*)-, TMS ester; α-linolenic acid, TMS ester; benzene, 1, 3-bis (1, 1-dimethylethyl)-; L-valine, *N*-(TMS)-, TMS ester; *N*, *N*-bis [2-trimethylsiloxylethyl] ethaneamine and an unidentified peak at retention time 24.89–24.93 (mass spectrum 95, 67, 123, 81, 82, 69, 55) had negative loadings to F1. SP and SW had the highest and lowest F1 scores, respectively (down-regulated following pathogen-inoculation). Benzoic acid, 2,4-bis(trimethylsiloxy)- with the highest abundances in SW. Glycine, *N*, *N*-bis(TMS)-, TMS ester, L-proline, 1-(TMS)-,TMS ester, L-proline, 5-oxo-1-(trimethysilyl)-, TMS ester and 8,11-octadecadienoic acid, methyl ester had significant negative loadings to F3. Pathogen-inoculated spikelets of both cultivars (SP and RP) with the highest F3 scores as opposed to the water-inoculated ones (SW and RW) had the lowest abundances of these metabolites contributed to the higher F3 score, the susceptible/pathogenesis factor.

4. Discussion

With progress in genomic initiatives to profile genome and gene expression of plant-pathogen interaction, the need for metabolome profiling is increasing to better understand plant defense against various environmental stress, including pathogen stress. Wheat breeders on the other hand are looking for fast, easy and precise tools for screening resistance against FHB, in addition to an understanding of functions of FHB-resistance genes to help them pyramid suitable QTL alleles into elite cultivars. In the present investigation, GC/MS metabolic profiling of wheat spikelets at 24 hai has enabled identification of several plant-pathogen interaction metabolites and a putative relation of UR-metabolites to wheat cultivars varying in resistance to FHB. Many of these are known to play significant roles in the metabolism of plants leading to the production of defense related compounds [9,13,37,38]. The technology 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 have been able to discriminate resistance in wheat against FHB considering several metabolic profiling criteria: (a) UR/PR-metabolites (water-methanol and chloroform fractions) unique/specific to a cultivar resistant or susceptible to FHB; (b) UR/PR-metabolites common to both the susceptible and resistant cultivars but in higher abundance in one of these cultivars; (c) metabolites (present in all treatments) with significant factor-loadings to factor-scores or treatments, allowing us to suggest possible explanations for differences among-cultivars in resistance against FHB.

Both the susceptible and resistant cultivars produced some novel compounds or mostly up-regulation of compounds following pathogen inoculation. These metabolites were designated here as UR-metabolites, or when the abundance was significantly up-regulated as PR-metabolites, in a context similar to PR-proteins and PR-genes [29,34]. In this study, 49 UR-metabolites were identified, including 12 UR-metabolites that were unique to Roblin, 13 unique to Sumai3 and 24 common to both cultivars. Out of 24 common UR-metabolites 11 and 13 were in higher abundance in R and S, respectively. There were in total 23 pathogen-induced metabolites from Roblin (R/R > S-UR) and 26 from Sumai3 (S/S > R-UR) that could be used to discriminate FHB-responses between the two cultivars that varied in their resistance against FHB. However, out of 55 metabolites detected here only 15 were significant among treatments, including nine that were PR-metabolites, of which only 5 varied significantly among treatments (Table1, Fig. 2). Even though cinnamic acid, *m*-(trimethylsiloxy)- was detected in all the treatments its, abundance in SP was about six times higher than that in SW, while the abundance was low in RW which changed slightly following pathogen inoculation. Higher abundances of sugars such as myo-Inositol (significantly in higher abundances in Sumai3) an important signal molecule and glucose a precursor of shikmic acid and monomer of cellulose and hemicelluloses can also account for resistance of Sumai3 to FHB [5]. Benzoic acid (BA) was up-regulated in the resistant cv. Sumai3 pathogen-inoculated and while it was not detected in RW but detected in lower abundances in RP. BA can easily be converted to cinnamic acid a key compound in phenylpropanoid pathway. Decarboxylation of trans-cinnamic acid to BA and further 2-hydroxylation of BA to salicylic acid (SA) has also been reported [23]. Some aromatic compounds such as BA and SA besides their role in signal transduction are directly antimicrobial [18]. Higher abundance of glutamine which helps the plant cell recycle liberated ammonia ions from phenylalanine can also be considered as another evidence for a more active PAL pathway in Sumai3 and discriminating resistance. Glutamine was detected with increased abundances in pathogen-inoculated plants of both cultivars but the increase was higher in SP.

Several metabolites detected in this study were down-regulated-metabolites, yet these can be involved in plant defense [9,29]. Prolin and glycine had negative factor-loading to F3 which means less abundance of these amino acids in pathogen inoculated spikelets of both cultivars (with high F3 scores). This indicates that the genes coding for these proteins are induced, and consequently prolin and glycine are utilized by cell of attacked spikelets to synthesize these proteins [24]. Fatty acids such as linolenic acid and 9,12-octadecadienoic acid (*Z*,*Z*)-, TMS ester had negative facto-loading to F1, more resistance at

lower/reduced abundances of these fatty acids in SP. 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 [5,8,19]. F1 score was maximum for SP and minimum for SW, which indicates 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.

Factor analysis of the metabolites, common to all treatments whose role in resistance was not as clear as those metabolites that were specific or not common to all the four treatments as discussed above, enabled explanation of hidden functions underneath resistance in wheat cultivars tested here against FHB, namely (i) pathogenesis and (ii) defense. The large difference in F1 scores between SP and SW indicated up-regulation of defense-related metabolites following pathogen inoculation (UR/PR-metabolites), while such a difference was not as dramatic in Roblin (Fig. 1). Also, the highest F2 scores for Sumai3 pathogen inoculated can partly explain higher level of resistance due to increase in the abundances of metabolites with positive and significant factor-loading to F2 (Table 1), even though some of the effect could be due to cultivar differences. Thus, metabolites with positive and significant factor-loading to F1 and F2 can be used to discriminate resistance in Sumai3 against FHB. On the other hand the F3 explained mainly the pathogenesis, as the pathogen inoculated had high positive scores with highest positive factor score for RP.

Following pathogen attack plants normally switch their metabolic pathways from primary metabolite production to produce more defense-related compounds [9,13]. It appears that the phenyl ammonia lyase (PAL) is more active in the resistant cv. Sumai3 than in the susceptible cv. Roblin. Following pathogen inoculation, the abundance of *m*-hydroxycinnamic acid (also *p*-hydroxycinnamic acid) in Sumai3 increased in higher proportions than in the Roblin. PAL converts phenylalanine to trans-cinnamic acid which is also a precursor for salicylic acid. Cinnamate 4-hydroxylase enzyme hydrolyses t-cinnamic acid into 4-coumaric acid, which finally produces coumaric acid, a key compound which directly or indirectly serves as precursors of other phenolic compounds and monomers of cell wall [3–5,9]. 4-hydroxycinnamic acid is a precursor for the production of a group of phytoalexines in oat (*Avena sativa* L.) leaves infected with *Puccinia coronata* f. sp. *avenae* [29,31]. 4-Hydroxy-3-methoxycinnamic acid (ferulic acid) has a clear role in polymer cross-linking within the plant cell walls [39].

Higher abundances of 4-hydroxycinnamic acid (*p*-coumaric acid) as well as m-coumaric acid observed in the resistant cultivar (Sumai3) as the downstream outcome of resistant gene expression may be due to several hypothetical causes. There is evidence that PAL gene is on chromosome 3B which carries the QTL for resistance to FHB [24]. Thus, the reason for higher abundances of *m*-hydroxycinnamic acid in Sumai3 may be the PAL factor. 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 also been observed in vitro [43]. Accumulation of phenylpropanoid metabolites after infection by plant pathogens has been reported [43,45]. Higher concentrations of free phenolic compounds have been found in resistant wheat cv. Frontana as compared to susceptible cv. Argent inoculated with *F. graminearum*, especially *p*-coumaric acid in glumes, lemmas and paleas [42].

Abundance of malonic acid was higher in pathogen inoculated than in water inoculated, in both the cultivars, implying following pathogen inoculation both the cultivars activate malonate pathway, in addition to PAL pathway. The former is known to produce phenolics like isoflavonoids, though it is not as efficient as PAL pathway for the production of phenolics. Isoflavonoids are important both as toxic substances to fungi and as signal molecules in plant-microbe communication [5,9,29].

Higher abundances of sugars such as myo-Inositol and glucose can also account for resistance in Sumai3 to FHB. Myo-Inositol abundance in Sumai3 increased following pathogen inoculation in higher proportions than in Roblin. Higher factor-loading of myo-inositol on F2, which had high factor-score for treatment SP, indicates an important contribution of this compound in resistance of Sumai3 to the pathogen. Myo-Inositol is involved in cell signaling in animals and plants [10,30,33] and plants with higher levels of resistance to diseases have over expression of inositol [2,34,43]. A richer pool of inositol-derived metabolites in resistant cultivar (Sumai3) can supply a higher signal transduction capacity and rapid response to the attacking pathogen. Galactose and glucose had higher factor-loadings for F2 and this hidden factor may run the production of some enzymes involved in their synthesis or an enzymatic hydrolysis of their parent glycosides. Sugars such as glucose, galactose and xylose are all used in the synthesis of hemicelluloses with xylose as side chains [5].

Fatty acid production by the two cultivars appears to be complex and appears to be controlled by functions hidden mainly in F1 for Sumai3 and in F3 for Roblin. Highest F1 scores for SP and lowest scores for SW suggest more active PAL pathway in Sumai3-FHB system, as opposed to Roblin which appears to suppress PAL, as the factor-scores for RP were lower than for RW.

Following pathogen inoculation certain fatty acids appear to increase in both the cultivars, but more in Roblin, as indicated by higher F3 scores for RP (Table 1). Highest increase in F3 values for Roblin as compared to Sumai3, following pathogen inoculation, implies that Roblin plausibly takes more advantage of JA signal transduction system than Sumai3. Octadecanoic acid pathway produces signal molecules with vital roles in regulating secondary pathways [3]. Despite the fast increase in the production of JA pathway fatty acids the pathogen invasion advanced in the cv. Roblin.

Some increase in F3 score for both SP and RP can be assigned to glutamine which plays an important role in recycling ammonia ions and guarantees quick and apt functioning of PAL [5]. Higher abundance of glutamine implies higher activity of PAL and phenylpropanoid metabolism in resistant cv. Sumai3.

Metabolic 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 susceptible cultivar, Roblin. There is potential to develop this technology for high throughput cultivar screening, once the defense metabolites are characterized. This technology can also be used to better understand the mode of action of pathotoxin, DON, in pathogenesis, and five different types of resistance mechanisms [18]. The knowledge base 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 [29] and this knowledge base could be used to pyramid genes into an elite cultivar.

Plants are known to produce thousands of metabolites [13,46], while we have detected only a few metabolites in wheat-FHB system. However, we have increased the chance of detecting PR-metabolites by extracting and profiling metabolites following pathogen inoculation. In spite of complexity of resistance phenomenon in wheat-FHB system we were able to identify groups of compounds that discriminated resistance, and additionally, able to explain the plausible functions of metabolites in wheat plant defense against *F. graminearum*. However, various steps involved in metabolic profiling such as metabolite extraction, metabolite identification and use of suitable wheat and pathogen genotypes to prove certain metabolic functions, etc. have to be improved to achieve an in depth understanding of wheat-FHB interactions. Following pathogen inoculation, plants use one or more metabolic pathways to synthesize novel compounds to defend against the attacking pathogen [9,13,35]. The metabolite synthesis and plant defense are dynamic processes, however, we have profiled metabolites only at 24 h following pathogen inoculation. Further studies involving temporal assessment of metabolites, different wheat and *F. graminearum* genotypes, environmental variables, etc., are needed to better understand metabolite function in plant defense.

In this study, compounds were identified based on NIST library match and manual comparison of spectra. Since there was no prior knowledge of metabolite identity GC/MS technology appears to be the best. However, the identity of the compounds reported here are tentative and further studies involving spiking with pure compounds or use of other instruments to identify the compound structure are required [46]. The GC/MS system detects only relatively low molecular weight compounds [44]. For more complex molecules the use of other hyphenated instrumentations such as LC/MS/MS, LC/NMR, etc. should be explored [36,46], which are also relatively cost effective and fast methods. Likewise, the solvents and extraction methods used in this study were selected to compromise the goal of extracting as many compounds as possible. More than one extraction method and analytical instrument is needed to detect sufficient number of compounds to better explain the nature of resistance in plants against diseases. Metabolic profiling, thus, 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, transcripts and genes.

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References

[1] Bai G, Shaner G. Scab of wheat: prospects for control. Plant Dis 1994; 78:760–6.
[2] Berridge MJ, Irvine RF. Inositol phosphates and cell signaling. Nature 1989;341:197–205.
[3] Blechert S, Brodschelm H, Holder S, Kammerer L, Kutchan TM, Mueller MJ, et al. The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci 1995;92: 4099–105.
[4] Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA. Altering expression of cinnamic acid 4-Hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol 2000;122:107–16.
[5] Buchanan BB, Gruissem W, Jones RL. Biochemistry and molecular biology of plants.: APS Press; 2000.
[6] Buerstmayr H, Lemmens M, Fedak G, Ruckenbauer P. Backcross reciprocal analysis of Fusarium head blight resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 1999;98:76–85.
[7] Comeau A. Fusarium head blight—emerging issues, an overview. In: Canadian Worskshop on Fusarium Head Blight Proceedings, Winnipeg, Manitoba, November 28–30; 1999. p. 26–7.
[8] Digiacomo E, Frugis G, Mariotti D. Characterization of a novel *Arabidopsis thaliana* protein that interacts with methyl-jasmonate inducible NAC transcription factor Proceedings of the XLVI Italian Society of Agricultural Genetics—SIGA Annual Congress, 18–21 September 2002.
[9] Dixon RA, Achnine L, Kota P, Liu C, Reddy SMS, Wang L. The phenylpropanoid pathway and plant defense—a genomic perspective. Mol Plant Pathol 2002;3:371–90.
[10] Exton JH. Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu Rev Pharmacol Toxicol 1996;36:481–509.
[11] Fedak G, Gilbert A, Comeau A, Voldeng H, Savard M, Butler G. Sources of Fusarium head blight resistance in spring wheat; 2002 http://res2.agr.gc.ca/ecorc/fusarium01/session1h_e.htm.

[12] Fernando D. Overview of the fusarium situation in Canada. In: Canadian Workshop on Fusarium Head Blight Proceedings, Winnipeg, Manitoba, November 28–30; 1999. p. 12–5.
[13] Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L. Metabolic profiling for plant functional genomics. Nat Biotechnol 2000;18:1157–61.
[14] Fiehn O, Kopka J, Trethewey RN, Willimitzer L. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. Anal Chem 2000;72:3573–80.
[15] Franc LJ, Nether DA. Exercises in plant disease epidemiology.: APS Press; 1997.
[16] Gary CP, Muehlbauer J, Bushnell WR, Somers DA, Vance CP. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. Mol Plant Microbe Interact 2000;13:159–69.
[17] Gilbert J, Tekauz A. Recent developments in research on fusarium head blight of wheat in Canada. Can J Plant Pathol 2000;22:1–8.
[18] Hammond-Kosack KE, Jones JDG. Resistance gene-dependent plant defense responses. Plant Cell 2000;8:1773–91.
[19] Johnson DE. Applied multivariate methods for data analysts. North Scituate, MA: Duxbury Press; 1998.
[20] Johnson RA, Wichern W. Applied multivariate statistical analysis. 3rd ed. Englewood Cliffs, NJ: Prentice Hall; 1996.
[21] Khattree R, Naik DN. Multivariate data reduction and discrimination with SAS software. New York: SAS institute/Wiley; 2000.
[22] Kolb FL, Bai G-H, Muehlbauer GJ, Anderson JA, Smith KP, Fedak G. Host plant resistance genes for fusarium head blight: mapping and manipulation with molecular markers. Crop Sci 2001;41:611–9.
[23] Leon J, Shulaev V, Yalpani N, Lawton MA, Raskin I. Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis. Proc Natl Acad Sci USA 1995;92:10413–7.
[24] Li WL, Faris JD, Chittoor JM, Leach JE, Hulbert SH, Liu DJ, et al. Genomic mapping of defense response genes in wheat. Theor Appl Genet 1999;98:226–33.
[25] Liu ZZ, Wang ZY. Improved scab resistance in China: sources of resistance and problems. In: Saunders DA, editor. Wheat for non-traditional warm areas. Mexico, DF: CIMMYT; 1991. p. 178–88.
[26] McMullen M. Chemical control of FHB. In: Canadian Workshop on Fusarium Head Blight Proceedings, Winnipeg, MN, November 28–30; 1999. p. 73–8.
[27] McMullen M, Jones R, Gallenberg D. Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis 1997;81: 1340–8.
[28] Mesterházy Á. Types and components of resistance to Fusarium head blight of wheat. Plant Breed 1995;114:377–86.
[29] Muthukrishnan S, Liang GH, Trick HN, Gill BS. Pathogenesis-related proteins and their genes in cereals. Plant Cell Tissue Organ Cult 2001;64: 93–114.
[30] Nelson DE, Rammesmayer G, Bohnert HJ. Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. Plant Cell 1998;10:753–64.
[31] Okazaki Y, Isobe T, Iwata Y, Matsukawa T, Matsuda F, Miyagawa H, et al. Metabolism of avenanthramide phytoalexins in oats. Plant J 2004;39:560–72.
[32] Parry DW, Jenkinson P, McLeod L. Fusarium ear blight (scab) in small grain cereals—a review. Plant Pathol 1995;44:207–38.
[33] Poovaiah BW, Reddy AS. Calcium and signal transduction in plants. CRC Crit Rev Plant Sci 1993;12:185–211.
[34] Pritsch C, Muehlbauer GJ, Bushnell WR, Somers DA, Vance CP. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. Mol Plant Microbe Interact 2000;13:159–69.
[35] Kofalvi SA, Nassuth A. Influence of wheat streak mosaic virus infection on phenylpropanoid metabolism and the accumulation of phenolics and lignin in wheat. Physiol Mol Plant Pathol 1995;47: 365–77.
[36] Roepenack-Lahaye E, Degenkolb T, Zerjeski M, Franz M, Roth U, Wessjohann L, et al. Profiling of *Arabiopsis* secondary metabolites by capillary liquid chromatography coupled to electro spray ionization quadrupole time-of-flight mass spectrometry. Plant Physiol 2004;134: 548–59.
[37] Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L. Simultaneous analysis of metabolites in tuber by gas chromatography-mass spectrometry. Plant J 2000;23:131–42.
[38] Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willimitzer L, et al. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell 2001;13:11–29.
[39] Russell WR, Burkitt MJ, Provan GJ, Chesson A. Structure-specific functionality of plant cell wall hydroxycinnamates. J Sci Food Agric 1999;79:408–10.
[40] Schroeder HW, Christensen JJ. Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. Phytopathology 1963;53:831–8.
[41] Shaner G, Finney RE. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 1977;70:1183–6.
[42] Siranidou E, Kang Z, Buchenauer H. Studies on symptom development, phenolic compounds and morphological defense responses in wheat cultivars differing in resistance to Fusarium head blight. J Phytopathol 2002;150:200–8.
[43] Smart CC, Flores S. Over-expression of D-myo-inositol-3-phosphate synthase leads to elevated levels of inositol in Arabidopsis. Plant Mol Biol 1997;33:811–20.
[44] Sneh X, Zhou M, Lu W, Ohm H. Detection of Fusarium head blight resistance QTL in a wheat population using bulked segregant analysis. Theor Appl Genet 2003;106:1041–7.
[45] Southerton SG, Deverall BJ. Changes in phenolic acid levels in wheat leaves expressing resistance to *Puccinia recondita* f. sp. *tritici*. Physiol Mol Plant Pathol 1990;37:231–43.
[46] Sumner LW, Mendes P, Dixon A. Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 2003; 62:817–36.
[47] Zadoks JC, Chang TT, Konzak CF. A decimal code for growth stages of cereals. Weed Res 1974;14:415–21.