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

VENKATESH BOLLINA¹, G. KENCHAPPA KUMARASWAMY¹, AJJAMADA C. KUSHALAPPA^{1,*}, THIN MIEW CHOO², YVES DION³, SYLVIE RIOUX⁴, DENIS FAUBERT⁵ AND HABIBALLAH HAMZEZHARGHANI⁶

¹Plant Science Department, McGill University, Sainte-Anne-de-Bellevue, QC, Canada, H9X 3V9

²Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, 960 Carling Ave., Ottawa, ON, Canada, K1A 0C6

³Centre de Recherche sur les Grains Inc., 740 Chemin Trudeau, Saint-Mathieu-de-Beloeil, QC, Canada, J3G 4S5

⁴Centre de Recherche sur les Grains Inc., 2700 Rue Einstein, Ste. Foy, QC, Canada, G1P 3W8

⁵Institut de Recherches Cliniques de Montréal, 110 Av. des Pins Ouest, Montréal, QC, Canada, H2W 1R7

⁶Department of Plant Protection, Shiraz University, Badjgah, Postal Code 71444-65186, Iran

SUMMARY

Quantitative resistance is generally controlled by several genes. More than 100 resistance quantitative trait loci (QTLs) have been identified in wheat and barley against *Fusarium* head blight (FHB), caused by *Gibberella zeae* (anamorph: *Fusarium graminearum*), implying the possible occurrence of several resistance mechanisms. The objective of this study was to apply metabolomics to identify the metabolites in barley that are related to resistance against FHB. Barley genotypes, Chevron and Stander, were inoculated with mock or pathogen during the anthesis stage. The disease severity was assessed as the proportion of spikelets diseased. The genotype Chevron (0.33) was found to have a higher level of quantitative resistance than Stander (0.88). Spikelet samples were harvested at 48 h post-inoculation; metabolites were extracted and analysed using an LC-ESI-LTQ-Orbitrap (Thermo Fisher, Waltham, MA, USA). The output was imported to an XCMS 1.12.1 platform, the peaks were deconvoluted and the adducts were sieved. Of the 1826 peaks retained, a *t*-test identified 496 metabolites with significant treatment effects. Among these, 194 were resistance-related (RR) constitutive metabolites, whose abundance was higher in resistant mock-inoculated than in susceptible mock-inoculated genotypes. Fifty metabolites were assigned putative names on the basis of accurate mass, fragmentation pattern and number of carbons in the formula. The RR metabolites mainly belonged to phenylpropanoid, flavonoid, fatty acid and terpenoid metabolic pathways. Selected RR metabolites were assayed *in vitro* for antifungal activity on the basis of fungal

biomass production. The application of these RR metabolites as potential biomarkers for screening and the potential of mass spectrometry-based metabolomics for the identification of gene functions are discussed.

INTRODUCTION

Quantitative resistance in plants against pathogen stress is generally controlled by several genes. Unlike monogenic traits, polygenic traits are difficult to identify and also to transfer to elite cultivars. Quantitative resistance mechanisms, in addition to structural mechanisms, generally involve both metabolites and proteins (Agrios, 2005). Several metabolites in plants have been identified to have antimicrobial, signalling, cell wall enforcement, etc., properties. In this article, we explore a comprehensive metabolomics approach for the visualization of an array of metabolites and the detection of potential resistance-related (RR) metabolites, using barley and a necrotrophic pathogen *Gibberella zeae*, causal agent of *Fusarium* head blight (FHB), as a model system.

FHB is one of the most destructive and devastating diseases of barley, as well as wheat and Triticale. FHB not only causes a loss in grain yield, but also a deterioration in grain quality, by producing several trichothecene toxins that are detrimental to human and animal health (Bai and Shaner, 2004; Choo, 2006; Sutton, 1982). Breeding for resistance is the most economical and environmentally safe way to manage the disease (Bai and Shaner, 2004). Complete resistance to FHB in barley was not detected in more than 25 000 barley accessions screened (Choo, 2006). The breeding lines are generally screened for two types of

*Correspondence: Email: ajjamada.kushalappa@mcgill.ca

resistance: type I, resistance to infection on spray inoculation of the pathogen; type II, resistance to the spread of disease within the spike on single spikelet inoculation (Schroeder and Christensen, 1963). Barley genotypes, in general, have high type II resistance, unlike wheat genotypes and, accordingly, the screening for resistance in barley against FHB is mainly focused on type I resistance. In addition, the amount of deoxynivalenol (DON), a virulence factor (Ilgen *et al.*, 2009; Jansen *et al.*, 2005), has also been quantified to rank cultivar resistance. However, the genotype ranking based on quantitative resistance (type I) has been highly variable among locations and years. More than 100 quantitative trait loci (QTLs) for FHB resistance have been identified on all seven chromosomes of wheat and barley, but only about 25% are relatively stable (Buerstmayr *et al.*, 2009; Foroud and Eudes, 2009); only the function of the QTL on chromosome 3BS has been partially explained to be caused by the detoxification of DON to DON-3-*O*-glucoside (Lemmens *et al.*, 2005; Poppenberger *et al.*, 2003). However, the latter mechanism is partially associated with type II resistance, as DON-negative mutants are unable to spread within the wheat spike (Ilgen *et al.*, 2009; Jansen *et al.*, 2005). Barley already has high type II resistance, although the mechanisms involved have not been explored. The variation in DON accumulation in spikelets and the occurrence of several FHB resistance QTLs in barley, as in wheat, indicate the existence of several mechanisms of resistance. Thus, it is inadequate to perform resistance evaluation and QTL identification on the basis of the type of resistance and amount of DON only. Molecular breeders have attempted to fine map the QTL locations by further segregation, but have often found no resistance (Lulin *et al.*, 2010). It is possible that a trait, such as quantitative resistance, can be controlled by genes in more than one locus (Keurentjes *et al.*, 2006). In addition, quantitative resistance is strongly influenced by the environment. An uncontrolled environment, under field conditions, leads to large experimental variations, thus resulting in inconsistent results over years and locations. Thus, the evaluation of breeding lines under multiple environments, locations and years is quite expensive and time consuming, and leads to inconsistent genotype ranking. Accordingly, both conventional and molecular breeders are looking for better screening tools that not only discriminate the levels of resistance, but also explain the mechanisms of resistance or have a direct link to resistance genes.

Functional genomics approaches, such as transcriptomics, proteomics and metabolomics, can reveal the biochemical mechanisms of resistance (Fiehn *et al.*, 2000). Several pathogenesis-related (PR) proteins have been identified in wheat and barley against FHB (Geddes *et al.*, 2008; Shin *et al.*, 2008; Zhou *et al.*, 2005). Host enzymes that detoxify the major virulence factor, DON, have been identified (Lemmens *et al.*, 2005; Lulin *et al.*, 2010; Poppenberger *et al.*, 2003). The metabolomics approach has been used in wheat, and several RR metabolites, whose

abundances are higher in resistant than in susceptible genotypes, such as cinnamic acid, myo-inositol, D-fructose, *O*-methyloxime, *p*-coumaric acid, benzoic acid and ferulic acid, have been identified (Hamzehzarghani *et al.*, 2005, 2008a, b; Paranidharan *et al.*, 2008). Metabolic profiling based on NMR identified glutamine, glutamate, alanine and *trans*-aconitate metabolites in wheat resistant to FHB (Browne and Brindle, 2007). Most of the RR metabolites identified in wheat mainly belonged to three major metabolic pathways: phenylpropanoid, fatty acid and polyamine. These studies, however, were based on gas chromatography/mass spectrometry (GC/MS), which can detect only volatile metabolites. Several RR metabolites, such as flavonoids, glucosinolates and terpenoids, are nonvolatiles (Vorst *et al.*, 2005). In this article, liquid chromatography/hybrid mass spectrometry (LC/MS) was explored for widely comprehensive metabolic profiling and to identify RR metabolites. For selected metabolites, the antimicrobial properties were determined. A modelling approach was used to combine the intensities of RR metabolites with their antimicrobial properties to derive resistance equivalence (RE).

RESULTS

Disease severity

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

Comparative analysis of metabolic profiles

A total of 1970 peaks, with a signal-to-noise ratio (s/n) of $\geq 5:1$, was detected in this study. Following the sieving of adducts, isotopes and neutral losses, a total of 1826 peaks was retained. The abundances of these were corrected for the variation in extraction by dividing the abundance of each metabolite with that of the internal standard, genistein. The abundances of 1826 peaks were subjected to a *t*-test. A total of 496 peaks showed significant treatment effects, in either of the pairs, and were designated as metabolites: RM $\diamond$ SM = 289; RP $\diamond$ SP = 130; RP $\diamond$ RM = 55; SP $\diamond$ SM = 22, where R = resistant, S = susceptible, P = pathogen-inoculated and M = mock-inoculated. These

Fig. 1 Scatter plot of canonical discriminant analysis based on the abundances of 496 significant metabolites ($P \leq 0.05$) from barley spikelets of resistant (R) and susceptible (S) genotypes, inoculated with mock (M) or pathogen (P). CAN1 separated the genotypes and mainly identified constitutive resistance, whereas CAN2 separated pathogen- from mock-inoculated and mainly explained pathogenesis function.

metabolites may have higher abundance in either of the genotypes, and those with higher abundance in the resistant genotype were designated as RR metabolites. Thus, among these, only 194 were RR metabolites. In addition, 26 were PR metabolites, including five PRr and 21 PRs metabolites (Table S1, see Supporting Information).

Classification of observations and treatments using canonical discriminant analysis

Four hundred and ninety-six metabolites, significant at the $P \leq 0.05$ level from the t -test, were subjected to canonical discriminant analysis and hierarchical cluster analysis to better understand the relationship among treatments. The CAN1 vector explained 70.4% of the variance, discriminating the resistant from the susceptible genotypes, whereas the CAN2 vector explained 22.6% of the variance, discriminating the pathogen from mock inoculation (Figs 1 and 2). A total of 134 metabolites had high positive loading ($L > 0.9$) to CAN1 that explained the constitutive resistance function in Chevron, and two metabolites had high positive loading ($L > 0.9$) to CAN2 that explained the pathogenesis function (the metabolite loadings of these, when are also RRC or PR (217), are presented in Table S1).

PR and RR metabolites

The 496 metabolites with significant treatment effects ($P \leq 0.05$) were further classified on the basis of significance between specific combinations of treatments into different PR and RR metabolite groups. Two hundred and seventeen metabolites were RR or PR (Table S1). Fifty metabolites were assigned putative names on the basis of accurate mass, fragmentation pattern

(Fig. 3) and isotope pattern (Table 1). The median accurate mass error of the internal standard, genistein (m/z 270.0528), was 0.3 p.p.m. for the entire study.

RR constitutive metabolites ($RRC = RM > SM$)

Among the 194 RRC metabolites (Table S1), 47 were assigned putative names of identity (Table 1). These metabolites belonged to different chemical groups: *amino acids*: aspartic acid, arginine, aminoadipic acid and pyroglutamic acid; *fatty acids*: capric acid, methyl dodecanoic acid (fatty acid ester), lauric acid, undecanoic acid and omega-hydroxydodecanoic acid; *alkaloids*: murrainimbin and 3-methylxanthine; *lignans*: dihydrocubebin; *phenolics*: *trans-p*-ferulyl alcohol 4-*O*-[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside, *p*-coumaric acid and sinapate; *flavonoids*: kaempferol 3-*O*-rhamnoside, naringenin 7-glucoside, kaempferol 3-rhamnoside-7-glucoside, kaempferide 3-glucoside-7-rhamnoside and kaempferol 3-sophoroside-7-rhamnoside; *organic acids*: pyruvic acid, malonic acid, D-gluconate and citric acid; *terpenes*: astragaloside III and juanisamin.

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

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

Identification of DON detoxification product

The virulence factor, DON (m/z = 296.1259), and its degradation product, DON-3-*O*-glucoside (m/z = 458.1786) (Fig. 3), were

Fig. 2 Dendrogram based on hierarchical cluster analysis (HCA) of principal components of abundances of 496 metabolites ($P \leq 0.05$). The treatments are: S, susceptible (Stander); R, resistant (Chevron); M, mock inoculation; P, pathogen inoculation. Each line represents one replicate.

Fig. 3 The fragmentation pattern, MS/MS spectra, in negative ionization mode $[M - H]^-$ of deoxynivalenol-3-O-glucoside ($m/z = 458.1786$), a detoxification product of deoxynivalenol (DON) ($m/z = 296.1259$).

Table 1 Resistance-related (RR) metabolites, with putative names of identity, detected in six-row barley genotypes, inoculated with mock or *Gibberella zeae*.

Exp. mass (M), median	Exp. RT, median	Theor. mass (M)	AME (p.p.m.)	Putative name of identity	Compound group	Molecular formula	RR metabolites	MS/MS fragments	<i>P</i> < 0.05	Fold change	Database
88.01635	2.41	88.01604	3.5	Pyruvic acid	OA	C ₃ H ₄ O ₃	RRC	87.01 , 58.78	0.0116	32.19	A,B,C,D
104.0112	2.53	104.0109	2	Malonic acid	OA	C ₃ H ₄ O ₄	RRC†	103, 59 , 84.99, 74.96	0.007	1.57	A,B,C,D
129.04265	2.42	129.0426	0.39	Pyroglutamic acid	AA	C ₅ H ₇ NO ₃	RRC	128.14, 110.26, 100.11, 84.25	0.0348	2.33	A,B,C,D
133.03765	2.31	133.0375	1.1	Aspartic acid	AA	C ₄ H ₇ NO ₄	RRC	115.19, 114.19, 88.19, 89.1	0.0249	1.38	A,C
161.06865	2.42	161.0688	0.92	2-Aminoadipic acid	AA	C ₆ H ₁₁ NO ₄	RRC		0.0440	2.08	A,B,C,D
164.04735	34.43	164.0473	0.31	<i>p</i> -Coumaric acid	PA	C ₉ H ₈ O ₃	RRC*	119.05, 163, 145.20 , 134.96	0.0076	3.48	A,B,C,D
166.04977	2.35	166.0491	4	3-Methylxanthine	ALK	C ₈ H ₆ N ₄ O ₂	RRC	128.93, 75.00, 96.68 , 105.08	0.0311	1.33	A,B,C,D
172.14575	40.63	172.1463	3.1	Capric acid	FA	C ₁₀ H ₂₀ O ₂	RRC	171.25, 153.22 , 127.26, 148.23	0.0296	1.22	A,B,C,D
174.11165	2.27	174.1116	0.29	L-Arginine	AA	C ₆ H ₁₄ N ₄ O ₂	RRC*	129.05 , 155.04, 142.93, 154.93	0.0095	2.13	A,B,C,D
186.16165	41.94	186.162	1.8	Undecanoic acid	FA	C ₁₁ H ₂₂ O ₂	RRC	141.19, 167.15 , 185.11, 80.17	0.0452	1.31	A,C
192.02715	2.45	192.027	0.78	Citric acid	OA	C ₆ H ₈ O ₇	RRC†	110.99, 172.99, 84.92, 126.96	0.0002	1.74	A,B,C,D
192.06335	2.34	192.0638	2.3	Quinic acid	PP	C ₇ H ₁₂ O ₆	PRs	173.06 , 111.11, 127.19, 85.17	0.0394	1.78	A,B,C,D
196.05825	2.32	196.0583	0.24	D-Glucuronate	OA	C ₆ H ₁₂ O ₇	RRC†		0.0007	1.58	A,B,C,D
200.17755	43.21	200.1776	0.24	Lauric acid	FA	C ₁₂ H ₂₄ O ₂	RRC*, PRs	181.12, 167.13, 155.09, 135.28	0.0139	1.31	A,B,C,D
216.17235	36.04	216.1725	0.68	Omega-Hydroxydodecanoic acid	FA	C ₁₂ H ₂₄ O ₃	RRC		0.0497	1.50	A
224.06865	34.53	224.0684	1.1	Sinapate	PA	C ₁₁ H ₁₂ O ₅	RRC	208.13, 179.11, 164.16	0.0245	1.19	A,C
250.15665	37.82	250.1569	0.99	3β-Hydroxycinnamolide	ST	C ₁₅ H ₂₂ O ₃	PRs	205.17, 231.09, 184.29, 164.09	0.0387	1.26	A
288.22975	39.28	288.2301	1.2	10,16-Dihydroxy-hexadecanoate	FA	C ₁₆ H ₃₂ O ₄	RRC*	243.17, 269.22, 189.19, 259.17, 227.12	0.0021	2.1	A
316.07915	2.49	316.0794	0.78	Quinovic acid	SAP	C ₁₃ H ₁₆ O ₉	RRC†	153.04, 305.64, 165.13, 296.92	0.0007	17.39	A
328.13075	26.02	328.1311	1	Seselinol isovalerate	COU	C ₁₉ H ₂₀ O ₅	RRC	309.19, 291.22, 229.17, 185.09	0.0289	1.74	A
332.07395	2.38	332.0743	1	β-Glucogallin	Tannin	C ₁₃ H ₁₆ O ₁₀	RRC†		0.002	4.0	A,C
340.13065	26.02	340.1311	1.3	6-Prenylarigenin	FLA	C ₂₀ H ₂₀ O ₅	RRC	289.18, 183.04, 307.20, 321.20	0.0279	1.79	A,C
354.10995	24.68	354.1103	0.98	Licoisoflavone A	ISF	C ₂₀ H ₁₈ O ₆	RRC	235.21, 320.31, 255.26	0.0446	1.65	A,C
358.10645	2.27	358.1052	3.4	5-Hydroxy-3,6,7,4'-tetramethoxyflavone	FLA	C ₁₉ H ₁₈ O ₇	RRC*		0.003	2.37	A
358.14095	26.02	358.1416	1.8	(-)-Dihydrocubebin	LIG	C ₂₀ H ₂₂ O ₆	RRC		0.0255	1.9	A
418.08895	27.09	418.0899	2.2	Isoscutellarein 7-xyloside		C ₂₀ H ₁₈ O ₁₀	RRC	180.92, 237.15, 310.47, 313.03	0.0217	1.43	A
428.16775	23.71	428.1682	1	<i>trans-p</i> -Ferulyl alcohol 4- <i>O</i> -[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside	PA	C ₂₀ H ₂₈ O ₁₀	RRC*		0.0077	2.14	A
432.10485	23.54	432.1056	1.7	Kaempferol 3- <i>O</i> -rhamnoside	FLA	C ₂₁ H ₂₆ O ₁₀	RRC*	153.06, 171.03, 399.24, 385.17	0.0091	3.03	A
432.17785	35.41	432.1784	1.2	Juanislamin	ST	C ₂₃ H ₂₈ O ₈	RRC		0.0424	1.64	A
434.12055	31.01	434.1212	1.4	Naringenin 7-glucoside	FLA	C ₂₁ H ₂₂ O ₁₀	RRC	313.19, 253.33, 310.97, 231.08	0.0333	1.62	A
462.11535	24.04	462.1162	1.8	Scoparin	COU	C ₂₂ H ₂₂ O ₁₁	RRC†	299.13, 300.19, 155.09, 307.07	0.0012	3.6	A,C
484.24265	32.43	484.2434	1.5	Segetalin B		C ₂₄ H ₃₂ N ₆ O ₅	RRC		0.0298	2.34	A
510.28125	38.13	510.2828	3	16-Diacetoxy-7α-hydroxy-18-malonyloxy-ent-cleroda-3-ene	DT	C ₂₇ H ₄₂ O ₉	PR*	281.26, 227.12, 153.14	0.017	3.44	A
526.26145	31.07	526.262	1	Murrarimbine	ALK	C ₃₆ H ₃₄ N ₂ O ₂	RRC*		0.0104	2.93	A
536.18865	16.95	536.1893	1.2	7- <i>O</i> -(4-Methoxycinnamoyl) tecomoside	PP	C ₂₆ H ₃₂ O ₁₂	RRC	235.31, 299.30, 192.11, 161.01	0.0233	3.46	A
548.15265	2.44	548.1529	4.5	Hemsleyanoside	ISF	C ₂₆ H ₂₈ O ₁₃	RRC		0.0407	2.57	A
582.20875	28.927	582.2101	2.3	Auriculatin 4'- <i>O</i> -glucoside	ISF	C ₃₁ H ₃₄ O ₁₁	RRC*		0.0193	1.79	A
584.20945	18.45	584.2105	1.7	Sylvestroside III		C ₂₇ H ₃₆ O ₁₄	RRC	195.05, 282.88, 179.43, 165.20	0.0201	1.57	A
594.15755	23.86	594.1584	1.4	Kaempferol 3-rhamnoside-7-glucoside	FLA	C ₂₇ H ₃₀ O ₁₅	RRC†		0.0006	5.45	A
608.17255	25.79	608.1741	2.5	Kaempferide 3-glucoside-7-rhamnoside	FLA	C ₂₈ H ₃₂ O ₁₅	RRC*	299.01, 284.07, 300.49, 269.12	0.004	2.31	A
637.23545	36.90	637.2371	2.5	6'- <i>O</i> -α-D-Xylopyranosylalangiside	TER	C ₃₀ H ₃₈ NO ₁₄	RRC*	310.06, 291.99, 507.23, 618.19, 363.2	0.0168	1.66	A
654.17775	25.77	654.1795	2.6	Syringetin 3-rutinoside	PA	C ₂₉ H ₃₄ O ₁₇	RRC†		0.0012	2.37	A
710.20435	22.80	710.2058	2	Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside	FLA	C ₃₂ H ₃₈ O ₁₈	RRC†	401.08, 311.15, 283.11, 341.12	0.0003	6.48	A
740.21435	21.40	740.2163	2.6	Kaempferol 3-rhamnoside	FLA	C ₃₃ H ₄₀ O ₁₉	RRC†		0.0009	8.94	A
740.21465	24.93	740.2163	2.2	Kaempferol 3-rhamnoside-7-glucosyl-(1->2)-rhamnoside	FLA	C ₃₃ H ₄₀ O ₁₉	RRC*		0.0152	1.61	A
756.20965	19.57	756.2112	2	Kaempferol 3-gentiobioside-7-rhamnoside	FLA	C ₃₃ H ₄₀ O ₂₀	RRC†		0.0004	5.15	A
756.20965	22.81	756.2112	2	Kaempferol 3-sophoroside-7-rhamnoside	FLA	C ₃₃ H ₄₀ O ₂₀	RRC†		0.0003	6.12	A
770.22485	21.72	770.2269	2	Rhamnetin 3-rhamnoside	FLA	C ₃₄ H ₄₂ O ₂₀	RRC†		0.0004	7.67	A
784.45935	42.53	784.4609	1.9	Astragaloside III	TT	C ₄₁ H ₆₈ O ₁₄	RRC		0.0473	3.62	A,C
786.22015	21.41	786.2218	2	Isorhamnetin 3-rutinoside-7-glucoside	FLA	C ₃₄ H ₄₂ O ₂₁	RRC†		0.0003	9.94	A

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

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

*Significant at *P* ≤ 0.01.

†Significant at *P* ≤ 0.001.

Fig. 4 Antimicrobial properties of resistance-related (RR) metabolites assessed *in vitro* against *Gibberella zeae*. The metabolite concentrations in mM for 50% inhibition (LD₅₀ values) of the mycelial biomass by 10 RR metabolites; the letters A–D indicate the Duncan rankings of RR metabolites at $P \leq 0.01$.

RR metabolites	Relative abundance of RR metabolites	LD ₅₀ value of RR metabolites (mM)	Resistance equivalence*
Pyroglutamic acid	2.33	3.172	0.73
p-Coumaric acid	3.48	1.154	3.01
Capric acid	1.22	0.1003	12.16
Quinic acid	1.78	3.155	0.56
Ferulic acid	1.97	1.766	1.11
D-Gluconate	1.58	2.65	0.59
Lauric acid	1.31	2.142	0.61
Sinapate	1.19	1.747	0.68
Naringenin	1.43	1.58	0.9
Kaempferol	1.24	4.768	0.25

*Resistance equivalence (RE) = $[(AR/AS)/LD_{50}]$, where AR is the abundance of the metabolite in the resistant genotype, AS is the abundance of the metabolite in the susceptible genotype and LD₅₀ is the concentration (mM) of metabolite that inhibited 50% of the biomass of *Gibberella zeae*. The higher the RE value, the higher the potential resistance.

Table 2 Relative abundance, LD₅₀ value and resistance equivalence of resistance-related (RR) metabolites identified in barley genotype Chevron.

detected in the resistant but not susceptible genotype. This is the first report of DON-3-*O*-glucoside in barley.

Relative antifungal activity and RE of RR metabolites

Seven RR metabolites (pyroglutamic acid, *p*-coumaric acid, capric acid, quinic acid, D-gluconate, lauric acid, sinapate), naringenin (parent compound of naringenin-7-glucoside which was detected) and kaempferol (parent compound of kaempferol glucoside which was detected) were used for fungal biomass inhibition studies. Ferulic acid, a phenolic compound, was used as a positive check as it has been reported to reduce the biomass of *G. zeae* (Boutigny *et al.*, 2009). Except for kaempferol, all other compounds reduced significantly ($P \leq 0.01$) *G. zeae* biomass production (Fig. 4). When kaempferol was added to the liquid culture medium, it precipitated and

failed to dissolve completely. Capric acid inhibited biomass completely at a dose of ≥ 0.5 mM, and thus lower concentrations were evaluated; significant biomass was observed at 0.1 mM. In addition, both *p*-coumaric acid and naringenin showed greater biomass inhibition than that of ferulic acid, which was equal to that of sinapic acid.

RE for the RR metabolites detected here in the resistant genotype Chevron was derived as $RE = [(AR/AS)/LD_{50}]$, where AR is the abundance of the metabolite in the resistant genotype, AS is the abundance of the metabolite in the susceptible genotype and LD₅₀ is the concentration (mM) of metabolite that inhibited 50% of the biomass of *G. zeae*. RE ranged from zero for ferulic acid, as we did not detect this metabolite in our study, to 12.16 for capric acid and 3.01 for *p*-coumaric acid (Table 2). The higher the RE value, the higher is the potential resistance. The lowest RE for capric acid was mainly a result of its lowest LD₅₀.

DISCUSSION

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

In the present study, 1970 peaks were detected by the LC-ESI-LTQ-Orbitrap (Thermo Fisher, Waltham, MA, USA). Of these, 496 metabolites had significant treatment effects ($P \leq 0.05$). A canonical discriminant analysis of these metabolites identified constitutive resistance, but failed to identify induced resistance; however, it explained PR function. One hundred and thirty-four metabolites had high positive loading to CAN1, which mainly explained constitutive resistance, and two metabolites had high positive loading to CAN2, which explained pathogenesis function. More specific plant–pathogen interaction was further explored using univariate analysis. A *t*-test identified 194 RR metabolites, where all were RRC metabolites and none were RRI metabolites, as observed by canonical discriminant analysis. Of these, 50 were assigned putative names, and these metabolites belonged mainly to four chemical groups: phenylpropanoids, flavonoids, fatty acids and terpenoids. However, our previous studies using GC/MS detected only phenylpropanoids and fatty acids (Hamzehzarghani *et al.*, 2005, 2008a, b; Paranidharan *et al.*, 2008), but not flavonoids and terpenoids, making LC/MS a more comprehensive MS-based metabolomics tool to study biotic stress (Vorst *et al.*, 2005). Furthermore, we report here, for the first time, the occurrence of the DON degradation product, DON-3-*O*-glucoside, in barley (Fig. 3). However, the fragmentation pattern library for metabolites based on LC/MS is rather limited, when compared with GC/MS; accordingly, not many compounds were assigned putative names. The RR metabolites that were not assigned putative names are still useful markers, and may be identified in future with progress in metabolomics databases (Tohge and Fernie, 2009). This is the first study to report RR metabolites in barley against FHB following a metabolomics approach, which enabled the visualization of several metabolites of the plant–pathogen interaction. The RR metabolites reported here have several known mechanisms of resistance, and, in addition, the relative antimicrobial properties of some of these were also demonstrated.

We detected a resistance indicator metabolite, DON-3-*O*-glucoside, the degradation product of DON to the less toxic glucoside, through enzymatic activity in the resistant genotype (Lemmens *et al.*, 2005; Poppenberger *et al.*, 2003). Although we detected DON and its degradation product, they were not detected in all replicates. This is because, in this study, we used a nonpolar column; the use of a polar column should better detect trichothecenes (Berthiller *et al.*, 2007). Interestingly, the

accumulation of a small amount of DON in the Stander allele at chromosome 3 has been reported, even though it was used as a susceptible parent in the production of a recombinant inbred line population (Smith *et al.*, 2004). Although both genotypes used in this study accumulate a small amount of DON, the mechanisms may be different, either by inhibition of synthesis through antioxidants (Boutigny *et al.*, 2009) or by degradation of already produced DON through enzymatic action (Lemmens *et al.*, 2005; Poppenberger *et al.*, 2003).

The metabolites identified here were interlinked in a satellite metabolic pathway (Fig. 5) to better understand the role of metabolites in plant defence. The RR metabolites identified here belong to different metabolic pathways, in particular flavonoid, phenylpropanoid, fatty acid and terpenoid. The putative mechanisms of resistance of RR metabolites are discussed below.

Phenylpropanoid pathway

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

Significant amounts of *p*-coumaroyl-hydroxyagmatine in barley near-isogenic lines were observed following *Erysiphe graminis hordei* inoculation, and this was corroborated with *in vitro* and *in vivo* antifungal activity (von Ropenack *et al.*, 1998). In our study, both *p*-coumaric acid and sinapate had low LD₅₀ values, and the former had the second highest RE. These metabolites are also known for cell wall lignification (Jansen *et al.*, 2005), but their conversion to lignomonomers (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) was not significant in the study. Quinic acid was detected here as a PRs not PR metabolite, and this also had a high LD₅₀ value. It is possible that quinic acid is used in resistance to enhance the cell wall. The incorporation of radiolabelled quinic acid into a resistant tomato genotype against *Fusarium oxysporum* degraded most of the quinic acid, and converted it to lignin, whereas it was accumulated in the susceptible plant (Dixon and Paiva, 1995; Fuchs and Vries, 1969). Flux analysis of this pathway can reveal the role of quinic acid against FHB.

Flavonoid pathway

This is a downstream phenylpropanoid pathway. In our study, 16 flavonoids and isoflavonoids were identified as RR metabolites. The flavonol kaempferol and its glucosylated forms, identified

Satellite metabolic pathway of barley

Fig. 5 Satellite metabolic pathway of barley. The resistance-related (RR) metabolites detected in barley inoculated with mock or *Gibberella zeae*: bold, significant (at $P \leq 0.05$); italic, not significant, but identified; regular font, not identified in this study.

here as RR metabolites, were linked to the flavonol biosynthesis pathway (Fig. 6). The flavonoids identified in this study have been reported previously from plants, and the naturally occurring flavonoids and flavonoid coumarins inhibited the biosynthesis of trichothecene in *F. sporotrichioides* (Desjardins *et al.*, 1988). The flavonoids, kaempferol-*O*-rutinoside and kaempferol-3-*O*- β -D-glucopyranosyl, isolated from *Dianthus caryophyllus*, showed significant inhibition of *F. oxysporum* growth (Galeotti *et al.*, 2008). Flavonoid glucosides have excellent antioxidant activities (Ko *et al.*, 2005), and thus it is quite possible that they also inhibit DON synthesis, as in ferulic acid (Boutigny *et al.*, 2009). Some flavonoid monomers from barley testa were potent inhibitors of *Fusarium* spp. (Skadhauge *et al.*, 1997).

Fatty acid pathway

In this study, five fatty acids were detected. Capric acid and lauric acid, identified here as RR metabolites, inhibited *G. zeae* mycelial

biomass significantly (Table 2). These also have antifungal activity against *Pseudomonas aeruginosa* and *Candida albicans* (Kabara, 1984) and *Fusarium* spp. (Liu *et al.*, 2008). Among the RR metabolites tested here, capric acid had the highest mycelial inhibition and also had the highest RE.

Other RR metabolites

In the present study, we identified six amino acids, four of which were RR metabolites (pyroglutamic acid, aspartate, 2-aminoadipic acid and arginine), and four organic acids (pyruvic acid, melonic acid, citrate and gluconate). Pyroglutamic acid, a nonprotein amino acid, showed antimicrobial activity against *Bacillus subtilis* and *Pseudomonas putida* (Huttunen *et al.*, 1995). Arginine, identified here as an RR metabolite ($P \leq 0.01$), acts as a precursor for the biosynthesis of a polyamine, putrescine (Nakada and Itoh, 2003). Polyamines have been reported from wheat (Paranidharan *et al.*, 2008), and these are

Fig. 6 Schematic diagram of part of the flavonoid biosynthesis pathway leading to kaempferol production in barley, inoculated with mock or *Gibberella zeae*: bold, significant (at $P \leq 0.05$); italic, not significant at $P \leq 0.05$, but identified with AME < 5 p.p.m.; K, kaempferol.

involved in a variety of stress responses (Bajaj *et al.*, 1999). Gluconic acid, an organic acid, identified here as an RRC metabolite, had an LD₅₀ value of 2.8 mM for mycelial inhibition. Enhanced disease resistance was observed following the over-expression of glucose oxidase, an enzyme that converts glucose to gluconic acid, in cabbage and tobacco against *Xanthomonas campestris* pv. *campestris* (Lee *et al.*, 2002), and in rice against *Magnaporthe grisea* and *X. oryzae* pv. *oryzae* (Kachroo *et al.*, 2003). This indicates that many such diverse compounds are involved in a complex network of disease suppression and plant resistance to biotic and abiotic stresses.

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

The goal of this study was to explore the potential of comprehensive metabolomics to identify RR metabolites. Accordingly, we used the cultivar Chevron, as it has been proposed to possess several mechanisms and QTLs for resistance. Such an exploratory step at the outset is not possible using near-isogenic lines as they are expected to possess a specific mechanism. However, metabolites that are significantly different between genotypes may also be a result of genotype background effects. Accordingly, for selected RR metabolites, we established their antifungal effects and also derived their RE to obtain a combined parameter to better discriminate resistance. In addition, we used information in the literature to obtain the defensive role of RR metabolites.

Metabolic fluxes are also important, as resistant genotypes can use a given RR metabolite as a precursor to produce a metabolite with greater RE, whereas a susceptible genotype will accumulate the metabolite (Dixon and Paiva, 1995; Fuchs and Vries, 1969). Our study indicates that the resistance in barley to FHB, as in wheat, is controlled by several RR metabolites. Various combinations of these RR metabolites can be accumulated through breeding in a cultivar to achieve higher levels of quantitative resistance (Hamzehzarghani *et al.*, 2008a). In addition, the knowledge base on the occurrence of several RR metabolites in different metabolic pathways can be used to overexpress certain important metabolites through metabolic engineering. Alternatively, individuals or mixtures of these RR metabolites can be exploited as biofungicides, in particular capric acid, which had the lowest LD₅₀, applied to spikes to manage FHB. The RR metabolites identified in this study have shed some light onto the different resistance mechanisms against FHB involved in barley. However, we have not identified all possible RR metabolites in barley, and improvement of the metabolomics protocol for the detection of more polar metabolites and the analysis of other genotypes, including cultivars, recombinant inbred and near-isogenic lines, may reveal other important metabolites that might explain more mechanisms of resistance.

EXPERIMENTAL PROCEDURES

Plant and fungus production

Six-row barley genotypes, Chevron (R = resistant) and Stander (S = susceptible), varying in quantitative resistance to FHB, were used in this study. Plants were produced under glasshouse conditions. Seeds were sown in pots containing pasteurized soil and pro-mix (50:50). Plants were fertilized once every 2 weeks with 200 mL of a 0.3% solution of Plant-Prod (20–20–20 NPK + trace elements; Plant Products Co Ltd., Brampton, ON, Canada).

(Hamzehzarghani *et al.*, 2005). The glasshouse conditions were maintained at 22 ± 3 °C, $70 \pm 10\%$ relative humidity and 16 h photoperiod throughout the growing period. At each 2-week interval, plants were thinned to retain one tiller in addition to the main stem. *Gibberella zeae* Schwein (Petch) (anamorph: *Fusarium graminearum* Schwabe; isolate 15–35) was obtained from the Centre de Recherche sur les Grains Inc. (CEROM, Saint-Mathieu-de-Beloeil, QC, Canada) and maintained on potato dextrose agar (PDA). Fresh cultures were produced using spezieiller nutrient agar (SNA) medium (Nirenberg, 1981). Seven-day-old cultures were flooded with sterile water, the surface of the medium was gently scraped with a sterile glass slide to dislodge macroconidia, and these were filtered through four layers of cheesecloth. A conidial suspension of 1.5×10^5 macroconidia/mL in an aqueous solution of 0.02% Tween 80 was produced using a haemocytometer. Fresh inoculum was prepared for each inoculation.

Inoculation and incubation

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

Disease severity assessment

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

mental units consisted of 10–12 spikes for the spray inoculation, to assess type I resistance, and five spikes for the individual spikelet inoculation of two spikelets, to assess type II resistance.

Sampling and metabolite extraction

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

Metabolites were extracted from samples 1–5 days ahead of their analysis. The samples were crushed in liquid nitrogen using a mortar and pestle which was cleaned with methanol and precooled with liquid nitrogen. The metabolites were extracted according to de Vos *et al.* (2007) with some modifications. One hundred milligrams of the powdered sample were placed in a 2.2-mL microcentrifuge tube that was washed with methanol and precooled with liquid nitrogen; 400 µL of 100% cold methanol was added and, finally, the methanol concentration in the sample was adjusted to 65% with high-performance liquid chromatography (HPLC)-grade water. To this an internal standard, genistein (210 pg/µL), was added for abundance correction and the mixture was stirred using a vortex stirrer. Each sample was sonicated for 15 min at 40 kHz in a water bath at room temperature. Sample extracts were centrifuged for 10 min at 20 000 *g* at room temperature. The supernatant was filtered through a 0.22-µm poly(vinylidene difluoride) (PVDF) membrane filter (Millipore Corporation, Bedford, MA, USA) and centrifuged at 2520 *g* for 10 min. The filtrate was placed in labelled sampling glass vials and stored at -20 °C.

Metabolite analysis

The metabolites were analysed using LC/MS, with electrospray ionization, a quadrupole linear ion trap capable of MS^n and an Orbitrap electrostatic Fourier transform mass spectrometer capable of high mass accuracy and resolution (LC-ESI-LTQ-Orbitrap). The Orbitrap was externally calibrated every day. A 5-µL sample extract was injected automatically using a 96-well autosampler maintained at 20 °C. For chromatographic separation of the compounds, a capillary C-18 reversed-phase column, with an internal diameter of 500 µm, length of 10 cm and packed with a Jupiter stationary phase of 5-µm particle, 300-Å pore, reversed-phase material (Phenomenex, Torrance, CA, USA), was used. This column was installed on the LC-2D system (Eksigent, Dublin, CA, USA) and coupled to the LTQ-Orbitrap. The column was maintained at 25 °C and the mobile phase was

adjusted to a flow rate of 800 nL/min and eluted with 2.5 mM ammonium acetate (buffer A) and 100% methanol (buffer B). During the first 10 min, a 5- μ L sample was loaded onto the column with a flow rate of 8 μ L/min and, subsequently, the gradient was shifted from 10% to 90% buffer B in 30 min and then back to 10% buffer B for 10 min. Electrospray, capillary and tube lens voltages were set to -3.5 kV, -37 V and -110 V, respectively. The capillary temperature was set to 275 °C. The MS and MS/MS data acquisitions were accomplished using a four-scan event cycle comprising a full-scan MS for scan event one acquired in the Orbitrap, which enabled high resolution and high mass accuracy analysis. The mass resolution for MS was set at 60 000 (at m/z 400) and used to trigger the three additional MS/MS events acquired in parallel in the linear ion trap for the top three most intense ions. The mass over charge ratio range was 70–1000 for MS scanning, with a target value of 500 000 charges, and from approximately one-third of the parent m/z ratio to 2000 for MS/MS scanning, with a target value of 20 000 charges. Data were recorded in centroid mode. For all scan events, the maximum ion fill time was set to 100 ms and the number of microscans to unity. For the MS/MS mode, the normalized collision energy was maintained at 35 eV, the activation q was set to 0.25 and the activation time to 30 ms. Target ions already selected for MS/MS were dynamically excluded for 15 s.

Peak deconvolution

The raw output files from the LTQ-Orbitrap were converted into mzData format using Bioworks (Thermo Fisher Scientific, San Jose, CA, USA), keeping only MS1, the parent ion. The mzData files were later imported to the XCMS 1.12.1 platform (Smith *et al.*, 2006). Baseline was corrected and the peaks were deconvoluted and aligned across samples (treatments and replicates) using default program settings, except for the s/n threshold of 5:1 and bandwidth (bw) of 10 s. A frame width of $m/z = 0.001$ and a retention time $RT = 10$ s were used for peak alignment. The aligned output for each sample consisted of accurate masses (70–1000 m/z), retention times ($RT = 1$ –3600 s) and abundances (ion current counts) of each peak. CAMERA (<http://www.bioconductor.org/packages/release/bioc/html/CAMERA.html>) a bioinformatics tool based on the R platform and XCMS, was used to identify adducts, isotopes and neutral losses, which were multiple peaks of the same compound found at a given retention time. The output from XCMS was imported to MS-EXCEL. Multiple peaks with adducts, isotopes and neutral losses were excluded from the total peak list. The retained peak abundances were subjected to statistical analyses.

Experimental design and statistical analysis

The experiment on metabolic profiling was a randomized complete block design with two genotypes, Chevron resistant (R) and

Stander susceptible (S) to FHB, and two inoculations, mock (M) and pathogen (P), with five blocks, conducted over a time interval of 3–5 days. Each experimental unit consisted of 60 spikelets harvested from six spikes produced by three plants in one pot. The data on the accurate masses of peaks and their abundances (ion current count) for all samples were subjected to a t -test using SAS version 9.2 (Johnson, 1998), and those with significant treatment effects at $P \leq 0.05$ were retained, and designated as metabolites. Four different treatment combinations were compared (RM vs. SM, RP vs. RM, SP vs. SM and RP vs. SP, where R = resistant, S = susceptible, P = pathogen-inoculated and M = mock-inoculated) to assess treatment effects.

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

Identification of PR and RR metabolites

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

Assignment of putative names of identity to metabolites

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

1. Accurate mass match: the accurate masses were automatically searched, using XCMS linked to METLIN (http://metlin.scripps.edu/metabo_search.php) and other libraries (Tohge and Fernie, 2009), including PubChem (<http://pubchem>).

- ncbi.nlm.nih.gov/), KNApSACk (<http://kanaya.naist.jp/KNApSACk/>), HMDB (<http://hmdb.ca/>) and MoTo (<http://appliedbioinformatics.wur.nl/>). For all the metabolites, the accurate mass error [AME = (observed – exact mass)/(exact mass)] was calculated and, if AME > 5 p.p.m., the compound was considered to be unidentified.
- Mass fragmentation pattern: the mass fragments were obtained using IntelliXtract version 12 (ACDLabs, Toronto, ON, Canada) and the fragmentation patterns were searched in the above databases, if available. In addition, the chemical structure was manually verified for a given fragment using the ChemSketch function of IntelliXtract. A few RR metabolite standards were spiked under similar LC/MS conditions and fragmentation patterns were compared to identify a given metabolite.
 - Number of carbon atoms in the molecule: IntelliXtract was also used to calculate the number of carbon atoms in the peak if isotope abundances were available. The isotope abundances that passed the criteria of containing only ^{13}C based on IntelliXtract were further used to calculate the possible number of carbon atoms from the relative intensity of the ^{12}C and ^{13}C peaks [(Intensities of $^{13}\text{C}/^{12}\text{C} \times 100\%)/1.1\%$, where 1.1% is the natural abundance of ^{13}C]. The predicted number of carbon atoms in the putatively identified metabolite was used to reduce false annotations.

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

Antifungal activity and RE for RR metabolites

Selected RR metabolites were used to evaluate the antimicrobial properties: pyroglutamic acid, *p*-coumaric acid, capric acid, quinic acid, D-gluconate, lauric acid, sinapate and ferulic acid (the latter was used as a positive check). In this study, naringenin and kaempferol were used instead of their detected glucosidal forms, as they were unavailable. Antifungal studies were performed in a liquid culture medium containing 5 mL of potato dextrose broth. *Gibberella zeae* macroconidial suspension was inoculated to medium to contain 10^4 spores/mL. The macroconidia were harvested from SNA medium and washed twice with sterile water by centrifugation. The RR metabolites at mock final concentrations of 0, 1, 2 and 4 mM were individually inoculated to the culture tubes containing medium and spores. For capric acid, mock final concentrations of 0, 0.01, 0.05 and 0.1 mM were used, as no growth was observed at higher concentrations. As many of the compounds are hydrophobic, all were dissolved in methanol. The pH of the medium was adjusted

using NaOH and, for the entire study, the pH was in the range 6.35–6.45. Liquid cultures were incubated at 25 °C in the dark on an orbital shaker at 120 r.p.m. After 5 days of incubation, mycelia were separated by centrifugation, lyophilized and the biomass was quantified. The amount of fungal biomass was expressed as the proportion of the control. The data for different concentrations were subjected to probit analysis to derive LD₅₀ values for each compound using SAS. The LD₅₀ values were subjected to analysis of variance (ANOVA) and Duncan's multiple range test using SAS. RE for a metabolite was calculated using $\text{RE} = (\text{AR}/\text{AS})/\text{LD}_{50}$, where AR is the abundance in the resistant genotype, AS is the abundance in the susceptible genotype and LD₅₀ is the millimolar concentration. A higher RE value of a metabolite indicates a higher level of resistance, which may be caused by the higher abundance of the compound in the genotype or a lower LD₅₀ value.

ACKNOWLEDGEMENTS

This project was funded by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ), Centre de Recherche sur les Grains Inc. (CEROM) and the Fédération des Producteurs de Porc du Québec (FPPQ), QC, Canada.

REFERENCES

- Agrios, G. (2005) *Plant Pathology*. San Diego, CA: Academic Press.
- Bai, G. and Shaner, G. (2004) Management and resistance in wheat and barley to fusarium head blight. *Annu. Rev. Phytopathol.* **42**, 135–161.
- Bajaj, S., Targolli, J., Liu, L.F., Ho, T.H.D. and Wu, R. (1999) Transgenic approaches to increase dehydration-stress tolerance in plants. *Mol. Breed.* **5**, 493–503.
- Berthiller, F., Sulyok, M., Krska, R. and Schuhmacher, R. (2007) Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *Int. J. Food Microbiol.* **119**, 33–37.
- Bily, A.C., Reid, L.M., Taylor, J.H., Johnston, D., Malouin, C., Burt, A.J., Bakan, B., Regnault-Roger, C., Pauls, K.P. and Arnason, J.T. (2003) Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: resistance factors to *Fusarium graminearum*. *Phytopathology*, **93**, 712–719.
- Boutigny, A.L., Richard-Forget, F. and Barreau, C. (2008) Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. *Eur. J. Plant Pathol.* **121**, 411–423.
- Boutigny, A.L., Barreau, C., Atanasova-Penichon, V., Verdal-Bonnin, M.N., Pinson-Gadais, L. and Richard-Forget, F. (2009) Ferulic acid, an efficient inhibitor of type B trichothecene biosynthesis and Tri gene expression in *Fusarium* liquid cultures. *Mycol. Res.* **113**, 746–753.
- Browne, R. and Brindle, K. (2007) 1H NMR-based metabolite profiling as a potential selection tool for breeding passive resistance against *Fusarium* head blight (FHB) in wheat. *Mol. Plant Pathol.* **8**, 401–410.
- Buerstmayr, H., Ban, T. and Anderson, J.A. (2009) QTL mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. *Plant Breed. Rev.* **128**, 1–26.

- Capettini, F., Rasmusson, D.C., Dill-Macky, R., Schiefelbein, E. and Elakkad, A. (2003) Inheritance of resistance to *Fusarium* head blight in four populations of barley. *Crop Sci.* **43**, 1960–1966.
- Chen, Z.Y., Brown, R.L., Rajasekaran, K., Damann, K.E. and Cleveland, T.E. (2006) Identification of a maize kernel pathogenesis-related protein and evidence for its involvement in resistance to *Aspergillus flavus* infection and aflatoxin production. *Phytopathology*, **96**, 87–95.
- Choo, T.M. (2006) Breeding barley for resistance to fusarium head blight and mycotoxin accumulation. *Plant Breed. Rev.* **26**, 125–169.
- Choo, T.M., Martin, R.A., Ho, K.M., Shen, Q., Fedak, G., Savard, M., Voldeng, H., Falk, D.E., Etienne, M. and Sperry, E. (2004) *Fusarium* head blight and deoxynivalenol accumulation of barley in eastern Canada: cultivar response and correlation analysis. *Plant Dis.* **88**, 837–844.
- Desjardins, A.E., Plaitner, R.D. and Spencer, G.F. (1988) Inhibition of trichothecene toxin biosynthesis by naturally occurring shikimate aromatics. *Phytochemistry*, **28**, 761–771.
- Dixon, R. and Paiva, N. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell*, **7**, 1085–1097.
- Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R.N. and Willmitzer, L. (2000) Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* **18**, 1157–1161.
- Foroud, N. and Eudes, F. (2009) Trichothecenes in cereal grains. *Int. J. Mol. Sci.* **10**, 147.
- Fuchs, A. and Vries, F.W. (1969) Metabolism of radioactively labeled quinic acid and shikimic acid in healthy and *Fusarium*-infected tomato plants. *Eur. J. Plant Pathol.* **75**, 186–192.
- Galeotti, F., Barile, E., Curir, P., Dolci, M. and Lanzotti, V. (2008) Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity. *Phytochem. Lett.* **1**, 44–48.
- Geddes, J., Eudes, F., Laroche, A. and Selinger, L.B. (2008) Differential expression of proteins in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare*. *Proteomics*, **8**, 545–554.
- Hamzehzarghani, H., Kushalappa, A.C., Dion, Y., Rioux, S., Comeau, A., Yaylayan, V., Marshall, W.D. and Mather, D.E. (2005) Metabolic profiling and factor analysis to discriminate quantitative resistance in wheat cultivars against fusarium head blight. *Physiol. Mol. Plant Pathol.* **66**, 119–133.
- Hamzehzarghani, H., Paranidharan, V., Abu-Nada, Y., Kushalappa, A., Dion, Y., Rioux, S., Comeau, A., Yaylayan, V. and Marshall, W.D. (2008a) Metabolite profiling coupled with statistical analyses for potential high-throughput screening of quantitative resistance to fusarium head blight in wheat. *Can. J. Plant Pathol.* **30**, 24–36.
- Hamzehzarghani, H., Paranidharan, V., Abu-Nada, Y., Kushalappa, A.C., Mamer, O. and Somers, D. (2008b) Metabolic profiling to discriminate wheat near isogenic lines, with quantitative trait loci at chromosome 2DL, varying in resistance to fusarium head blight. *Can. J. Plant Sci.* **88**, 789–797.
- Humphreys, J.M. and Chapple, C. (2002) Rewriting the lignin roadmap. *Curr. Opin. Plant Biol.* **5**, 224–229.
- Huttunen, E., Noro, K. and Yang, Z. (1995) Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains. *Int. Dairy J.* **5**, 503–513.
- Ilgen, P., Hader, B., Maier, F.J. and Schafer, W. (2009) Developing kernel and rachis node induce the trichothecene pathway of *Fusarium graminearum* during wheat head infection. *Mol. Plant–Microbe Interact.* **22**, 899–908.
- Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K.H., Felk, A. and Maier, F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl. Acad. Sci. USA*, **102**, 16892–16897.
- Johnson, D.E. (1998) *Applied Multivariate Methods for Data Analysts*. North Scituate, MA: Duxbury Press.
- Kabara, J. (1984) Antimicrobial agents derived from fatty acids. *J. Am. Oil Chem. Soc.* **61**, 397–403.
- Kachroo, A., He, Z., Patkar, R., Zhu, Q., Zhong, J., Li, D., Ronald, P., Lamb, C. and Chattop, B.B. (2003) Induction of H₂O₂ in transgenic rice leads to cell death and enhanced resistance to both bacterial and fungal pathogens. *Transgenic Res.* **12**, 577–586.
- Keurentjes, J.J.B., Fu, J., De vos, C.H.R., Lommen, A., Hall, R.D., Bino, R.J., Van der plas, L.H.W., Jansen, R.C., Vreugdenhil, D. and Koornneef, M. (2006) The genetics of plant metabolism. *Nat. Genet.* **38**, 842–849.
- Ko, C.H., Shen, S.C., Hsu, C.S. and Chen, Y.C. (2005) Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome c, and caspase cascade. *Biochem. Pharmacol.* **69**, 913–927.
- Lee, Y., Yoon, I., Suh, S. and Kim, H. (2002) Enhanced disease resistance in transgenic cabbage and tobacco expressing a glucose oxidase gene from *Aspergillus niger*. *Plant Cell Rep.* **20**, 857–863.
- Lemmens, M., Scholz, U., Berthiller, F., Dall'Asta, C., Koutnik, A., Schuhmacher, R., Adam, G., Buerstmayr, H., Mesterházy, Á., Krska, R. and Ruckebauer, P. (2005) The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus. *Mol. Plant–Microbe Interact.* **18**, 1318–1324.
- Liu, S., Ruan, W., Li, J., Xu, H., Wang, J. and Gao, Y. (2008) Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia*, **166**, 93–102.
- Lulin, M., Yi, S., Aizhong, C., Zengjun, Q., Liping, X., Peidu, C., Dajun, L. and Xiu, E.W. (2010) Molecular cloning and characterization of an up-regulated UDP-glucosyltransferase gene induced by DON from *Triticum aestivum* L. cv. Wangshuibai. *Mol. Biol. Rep.* **37**, 785–795.
- Ma, Z., Steffenson, B.J., Prom, L.K. and Lapitan, N.L.V. (2000) Mapping of quantitative trait loci for *Fusarium* head blight resistance in barley. *Phytopathology*, **90**, 1079–1088.
- Mesfin, A., Smith, K.P., Dill-Macky, R., Evans, C.K., Waugh, R., Gustus, C.D. and Muehlbauer, G.J. (2003) Quantitative trait loci for *Fusarium* head blight resistance in barley detected in a two-rowed by six-rowed population. *Crop Sci.* **43**, 307–318.
- Nakada, Y. and Itoh, Y. (2003) Identification of the putrescine biosynthetic genes in *Pseudomonas aeruginosa* and characterization of agmatine deiminase and N-carbamoylputrescine amidohydrolase of the arginine decarboxylase pathway. *Microbiology*, **149**, 707–714.
- Nirenberg, H.I. (1981) A simplified method for identifying *Fusarium* ssp. occurring on wheat. *Can. J. Bot.* **59**, 1599–1609.
- Paranidharan, V., Abu-Nada, Y., Hamzehzarghani, H., Kushalappa, A.C., Mamer, O., Dion, Y., Rioux, S., Comeau, A. and Choiniere, L. (2008) Resistance-related metabolites in wheat against *Fusarium graminearum* and the virulence factor deoxynivalenol (DON). *Botany*, **86**, 1168–1179.
- de la Pena, R.C., Smith, K.P., Capettini, F., Muehlbauer, G.J., Gallo-Meagher, M., Dill-Macky, R., Somers, D.A. and Rasmusson, D.C.

- (1999) Quantitative trait loci associated with resistance to *Fusarium* head blight and kernel discoloration in barley. *Theor. Appl. Genet.* **99**, 561–569.
- Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glossl, J., Luschnig, C. and Adam, G. (2003) Detoxification of the fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *J. Biol. Chem.* **278**, 47905–47914.
- von Ropenack, E., Parr, A. and Schulze-Lefert, P. (1998) Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *J. Biol. Chem.* **273**, 9013–9022.
- Schroeder, H.W. and Christensen, J.J. (1963) Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. *Phytopathology*, **53**, 831–838.
- Shin, S., Mackintosh, C.A., Lewis, J., Heinen, S.J., Radmer, L., Baldridge, G.D., Zeyen, R.J. and Muehlbauer, G.J. (2008) Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. *J. Exp. Bot.* **59**, 2371–2378.
- Skadhauge, B., Thomsen, K.K. and Wettstein, D. (1997) The role of the barley testa layer and its flavonoid content in resistance to *Fusarium* infections. *Hereditas*, **126**, 147–160.
- Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R. and Siuzdak, G. (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **78**, 779–787.
- Smith, K.P., Evans, C.K., Dill-Macky, R., Gustus, C., Xie, W. and Dong, Y. (2004) Host genetic effect on deoxynivalenol accumulation in fusarium head blight of barley. *Phytopathology*, **94**, 766–771.
- Sutton, J.C. (1982) Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Can. J. Plant Pathol.* **4**, 195–209.
- Tohge, T. and Fernie, A.R. (2009) Web-based resources for mass-spectrometry-based metabolomics: a user's guide. *Phytochemistry*, **70**, 450–456.
- Urrea, C.A., Horsley, R.D., Steffenson, B.J. and Schwarz, P.B. (2002) Heritability of *Fusarium* head blight resistance and deoxynivalenol accumulation from barley accession Cih0 4196. *Crop Sci.* **42**, 1404–1408.
- Vorst, O., Vos, C.H.R., Lommen, A., Staps, R.V., Visser, R.G.F., Bino, R.J. and Hall, R.D. (2005) A non-directed approach to the differential analysis of multiple LC–MS-derived metabolic profiles. *Metabolomics*, **1**, 169–180.
- de Vos, R., Moco, S., Lommen, A., Keurentjes, J.J.B., Bino, R.J. and Hall, R.D. (2007) Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat. Protocols*, **2**, 778–791.
- Zadoks, J.C., Chang, T.T. and Konzak, C.F. (1974) A decimal code for the growth stages of cereals. *Weed Res.* **14**, 415–421.
- Zhou, W., Kolb, F.L. and Riechers, D.E. (2005) Identification of proteins induced or upregulated by *Fusarium* head blight infection in the spikes of hexaploid wheat (*Triticum aestivum*). *Genome*, **48**, 770–780.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Experimental median accurate masses (m/z), retention times (RT) and respective CAN1 loadings of resistance-related (RR) and pathogenesis-related (PR) metabolites detected in mock- or *Gibberella zeae*-inoculated barley genotypes in a negative mode of ionization based on an LC-ESI-LTQ-Orbitrap (total of 217 metabolites).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.