Effects of Atmospheric CO₂ Level on the Metabolic Response of Resistant and Susceptible Wheat to *Fusarium graminearum* Infection

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Rising atmospheric CO₂ concentrations and associated climate changes are thought to have contributed to the steady increase of *Fusarium* head blight (FHB) on wheat. However, our understanding of precisely how elevated CO₂ influences the defense response of wheat against *Fusarium graminearum* remains limited. In this study, we evaluated the metabolic profiles of susceptible (Norm) and moderately resistant (Alsen) spring wheat in response to whole-head inoculation with two deoxynivalenol (DON)-producing *F. graminearum* isolates (DON⁺), isolates 9F1 and Gz3639, and a DON-deficient (DON⁻) isolate (Gzt40) at ambient (400 ppm) and elevated (800 ppm) CO₂ concentrations. The effects of elevated CO₂ were dependent on both the *Fusarium* strain and the wheat variety, but metabolic differences in the host can explain the observed changes in *F. graminearum* biomass and DON accumulation. The complexity of abiotic and biotic stress interactions makes it difficult to determine if the observed metabolic changes in wheat are a result of CO₂-induced changes in the host, the pathogen, or a combination of both. However, the effects of elevated CO₂ were not dependent on DON production. Finally, we identified several metabolic biomarkers for wheat that can reliably predict FHB resistance or susceptibility, even as atmospheric CO₂ levels rise.

The global average atmospheric carbon dioxide (CO₂) concentration surpassed 400 ppm in 2016 and is rising at an unprecedented pace (NOAA 2017). By 2100, the CO₂ concentration is predicted to reach 800 ppm, contributing to significant climate changes (Stocker et al. 2013). The Average global temperature is expected to rise 2 to 4°C and seasonal

and regional climate conditions will very likely become more variable and extreme (Stocker et al. 2013). In the Great Plains, where most of the United States wheat is produced, the frequency and intensity of heat waves and drought are predicted to increase in the south, while the northern states are expected to have increased precipitation and heavy downpours (Barton and Clark 2014).

Climate directly impacts the growth, development, and productivity of agricultural crops as well as the frequency and severity of disease epidemics. Elevated CO₂ has been shown to increase wheat growth, flowering time, maturation, and yield but also reduces grain nutritional quality and alters plant primary and secondary metabolism (Högy et al. 2009). All these changes have the potential to influence plant susceptibility pathogens. This is concerning because future climate conditions are also projected to increase the geographic distribution, growth rate, and virulence of major agricultural pathogens. Crop-destroying fungal pathogens, in particular, are expected to be among the greatest threat to future global food security (Bebber 2015; Bebbber and Gurr 2015; Fisher et al. 2012).

Fusarium head blight (FHB) is one of the most devastating fungal diseases of wheat. FHB can be caused by many closely related fungal species, but, in many regions of the world, *Fusarium graminearum* is the primary etiological agent. FHB epidemics substantially reduce grain yield and quality and contaminate grain with harmful trichothecene mycotoxins that can render the grain unsafe for human or animal consumption. Trichothecenes are a serious food safety concern because they inhibit protein synthesis and cause neurologic, gastrointestinal, and immune function disorders (Pestka 2010; Wu et al. 2014). However, trichothecene production, especially the production of deoxynivalenol (DON), is an important virulence factor allowing the pathogen to overcome the plant defenses and spread throughout the host (Bai et al. 2002; Mesterhazy et al. 1999). Over the past few decades, *F. graminearum* has caused widespread outbreaks of FHB resulting in significant economic losses to the wheat industry (Dweba et al. 2017). The prevalence of FHB and highly DON-contaminated grain has increased in recent years, due to changes in climate and agricultural practices (Edwards 2004; Fisher et al. 2012; Kane et al. 2013). FHB epidemics and DON contamination of grain are strongly associated with climate and occur when wheat anthesis coincides with warm wet conditions. The frequency of such favorable conditions for disease is projected to increase in the future, with climate change intensifying the risk of FHB and mycotoxin food safety issues (Chakraborty and Newton 2011; Madgwick et al. 2011; Vaughan et al. 2016; Zhang et al. 2014).

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Two main types of wheat resistance to FHB are: type I, resistance to initial infection, which is typically evaluated by the incidence of infected florets following whole-head inoculation and type II, resistance to spread within the head, also termed rachis resistance, which is assessed as the percentage of infected florets over time following the inoculation of a single floret (Schroeder and Christensen 1963; Zhang et al. 2018). Quantitative trait loci (QTL), such as *Fhb1* and *Fhb2*, which provide type II resistance, and *Fhb5*, which confers type I resistance, derived from the Chinese spring wheat cultivar Sumai 3, are the major sources of FHB resistance but only provide partial protection (Buerstmayr et al. 2009; Zhang et al. 2018). Genetic sources of complete FHB resistance have not yet been identified in wheat. Currently, the most effective FHB management strategies integrate chemical fungicide treatment, agronomic practices that discourage the development of pathogen inoculum (e.g., crop rotation), and the selection of moderately resistant wheat varieties (that have *Fhb* loci) (Dweba et al. 2017). Still, under favorable environmental conditions, even this integrated approach frequently fails to consistently minimize disease and keep mycotoxin contamination below regulatory levels (Dweba et al. 2017).

Recent studies suggest that a rising atmospheric CO₂ concentration also has the potential to compromise wheat resistance to FHB and associated DON contamination (Bencze et al. 2017; Kane et al. 2013; Váry et al. 2015). Transcriptomic analyses comparing winter wheat grown under ambient and elevated CO₂ conditions revealed that elevated CO₂ markedly suppressed the expression of important plant defense-response genes (Kane et al. 2013). Additionally, single-floret inoculations (evaluation of type II resistance) of winter wheat varieties with *Fusarium culmorum* usually resulted in higher grain DON content at elevated CO₂ than at ambient CO₂, even in the absence of visual disease differences (Bencze et al. 2017). However, with whole-head inoculations (evaluation of type I resistance), disease and DON content of grain grown at elevated CO₂ either did not change, decreased, or increased, depending on the host variety (Bencze et al. 2017). Furthermore, acclimation of *F. graminearum* and spring wheat to elevated CO₂ resulted in greater disease development and reduced effectiveness of innate plant defenses in a moderately resistant variety originating from Sumai 3 (Váry et al. 2015).

These findings amplify concerns that the occurrence of FHB and highly DON-contaminated grain will rise under projected future climatic conditions. In particular, rising CO₂ will be a global and consistent abiotic pressure that can influence wheat susceptibility to FHB and, ultimately, grain productivity and food safety. However, in order to develop more climate-resilient FHB-resistant wheat varieties and agricultural management strategies, additional research is needed to understand the underlying processes involved, such as determining changes that occur in the wheat defense response to *F. graminearum* at elevated CO₂ leading to altered resistance.

Wheat resistance to FHB is dependent on a highly complex and integrated network of chemical defense metabolites that counteract *F. graminearum* infection (Gauthier et al. 2015). Metabolomics, the high-throughput analysis of metabolites in biological systems, have aided in the identification of many wheat resistance-related metabolites and metabolic biomarkers that can be used in breeding for enhanced resistance (Cuperlovic-Culf et al. 2016; Dhokane et al. 2016; Gunnaiah and Kushalappa 2014; Gunnaiah et al. 2012; Hamzehzarghani 2007). Metabolomics measurements can be performed on a variety of samples from cell extracts and cell media or tissue extracts and body fluids, with preferred analytical methods currently being nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry preceded by chromatography

separation. Mass spectrometry provides information about larger numbers of compounds and is particularly appropriate for analysis of small samples and qualitative analysis. Metabolomics analysis in current work was performed for a large number of samples collected over time, and NMR was chosen as a more appropriate method, due to its high reproducibility, minimal sample preparation, possibility to identify compounds that are difficult to ionize or require derivatization, and possibility for metabolite quantification over a wide dynamic range. Analysis of NMR data can be performed in an untargeted or qualitative sense, in which all measured signals are analyzed and compared, or in a targeted or quantitative sense, in which accurate identification and quantification of defined set of metabolites is performed initially and these quantitative data are further analyzed (Bingol 2018). In this study, we have used both approaches, in which overall comparison of samples is performed in an untargeted sense and, then, quantitative metabolite data are used for more detailed analysis and biological interpretation.

In the present study, we evaluated the impact of elevated CO₂ on the metabolic response of spring wheat to *F. graminearum* inoculation and assessed how changes in the defense response influenced susceptibility to FHB and DON contamination. In order to determine the potential underlying causes for changes in host resistance level, we used targeted and nontargeted metabolomics to compare the biochemical response of whole-head *F. graminearum*-inoculated wheat under ambient and elevated CO₂ conditions. To determine if elevated CO₂ impacted the response of wheat varieties containing *Fhb* loci differently, we evaluated the metabolic response of both a susceptible (Norm) and a moderately resistant variety of wheat (Alsen, which contains the *Fhb1* and *Fhb5* loci [Bokore et al. 2017]). Furthermore, we assessed for *F. graminearum* isolate-specific effects and the potential influence of DON production on the host defense responses by using two distinct wild-type *F. graminearum* isolates (9F1 and Gz3639), which have consistently produced DON and spread in previous disease assays, and a mutant form of Gz3639 (Gzt40), which lacks a functional biosynthetic (*Tri5*) gene and is thereby incapable of producing DON (Desjardins et al. 1996). Since wheat variety was a major contributor to metabolic differences, we also examined specific metabolic biomarkers for resistance that are not affected by CO₂ level.

RESULTS

Preliminary experiments determined that the published method of pathogen acclimation (Váry et al. 2015), i.e., performing multiple consecutive subcultures at corresponding CO₂ concentrations, results in virulence variability between strains that is not associated with CO₂ concentration. Recent reports similarly suggest that subculturing fungal isolates can result in altered virulence (Jirakkakul et al. 2018). Additionally, since our research objective was focused on the host responses, the *F. graminearum* isolates were not acclimated to the different CO₂ concentrations and inoculums were all treated identically prior to inoculations.

The effect of elevated CO₂ on wheat-*F. graminearum* interactions following whole-head inoculation was dependent on the host variety as well as the infecting *F. graminearum* strain (Fig. 1). At 2×CO₂, Alsen inoculated with *F. graminearum* 9F1 displayed significantly more disease symptoms at both 3 and 7 dpi and accumulated 23% more *F. graminearum* biomass (measured as relative *F. graminearum* DNA to wheat DNA) and 38% more DON (Fig. 1 A to C) than respective plants inoculated at 1×CO₂. In contrast, Norm grown at 2×CO₂ and inoculated with 9F1 displayed significantly fewer disease

symptoms at 3 days postinoculation (dpi) (Fig. 1A). However, at 7 dpi, no significant difference in incidence of spikelet infection was detected. It is worth noting that, while our scoring method indicated that diseased florets were approaching 100% at both CO₂ concentrations, at 7 days after whole-head inoculation, many of these florets were only partially symptomatic. Nevertheless, the amount of *F. graminearum* biomass in the tissue collected 7 dpi was 30% lower in Norm plants at 2×CO₂ in comparison with respective plants at 1×CO₂ (Fig. 1A and B). Despite lower *F. graminearum* biomass at 2×CO₂, the amount of DON that accumulated in Norm was not significantly affected by CO₂ concentration, which suggests that the amount of DON produced per unit of pathogen biomass was greater at 2×CO₂. Whole-head inoculation with Gz3639 resulted in significantly reduced pathogen biomass at 2×CO₂ in both Alsen and Norm, even though only Alsen appeared to be less diseased at 7 dpi (Fig. 1D and E). Despite the reduction in *F. graminearum* biomass, DON contamination was not significantly affected, suggesting that, at 2×CO₂, Gz3639 produced more DON per unit of pathogen biomass in both wheat varieties (Fig. 1E and F). As expected, the mutant *F. graminearum* strain Gzt40, which lacks a functional *Tri5* gene and is thereby incapable of producing DON, caused less disease overall (Fig. 1G and H). No significant difference was visually observed between the Gzt40-inoculated

plants at 2×CO₂ in comparison with 1×CO₂. However, on average, the amount of Gzt40 biomass was reduced in plants grown at 2×CO₂, although this reduction was only significant in susceptible variety Norm (Fig. 1G and H).

To elucidate the underlying molecular processes involved in the observed differences in FHB and DON under 1×CO₂ and 2×CO₂ concentrations, we conducted metabolomic analyses of the host plant in response to different *F. graminearum* treatments. One- and two-dimensional (1D and 2D, respectively) NMR measurements, including 1D proton, HSQC (heteronuclear single quantum coherence) and TOCSY (total correlated spectroscopy), were performed for hydrophilic metabolites in all samples. 1D proton spectra for all samples, grouped by treatment and colored by sample type following spectral processing and alignments using the *icoshift* method (Savorani et al. 2010), are shown in Figure 2A. Signals from a mixture of water-soluble compounds were detected with high signal quality and consistency across all measurements. Residual methanol solvent peaks were observed in the sample spectra (shown with yellow overlay in Figure 2) but were removed from the analyses. No other impurities were detected. Minor differences in baseline and signal phasing were corrected as described below, resulting in the high-quality spectra used in the analyses (Fig. 2A).

Fig. 1. Effect of elevated CO₂ on *Fusarium* head blight development in susceptible (Norm) and moderately resistant (Alsen) wheat hosts caused by two wild-type *Fusarium graminearum* strains (9F1 and Gz3639) and a mutant strain (Gzt40) disrupted in deoxynivalenol (DON) production. **A, D, and G,** Average percentage of symptomatic spikelets per head observed on days 3 and 7 after whole-head inoculation with 9F1, Gz3639, or Gzt40 on Alsen or Norm grown at 1×CO₂ (400 ppm) and 2×CO₂ (800 ppm). **B, E, and H,** Average pathogen biomass measured as *F. graminearum* DNA (9F1, Gz3639, or Gzt40) relative to wheat DNA (Alsen or Norm) at day 7 postinoculation under 1×CO₂ or 2×CO₂. **C and F,** Average DON contamination in Alsen and Norm wheat heads 7 days postinoculation with 9F1 or Gz3639 under 1×CO₂ or 2×CO₂. Bars indicate standard error of the mean and colored asterisks designate significant differences (means from Alsen and Norm were analyzed separately, using *t* tests to compare differences between 1×CO₂ and 2×CO₂, *P* < 0.05).

Principal component analysis (PCA) of complete 1D spectra illustrated variances in the metabolic profiles across all samples based on complete metabolic profile in an untargeted, i.e., qualitative, sense (Fig. 2B). In the PCA of all samples, principal component 2 (PC2) revealed major differences between metabolic profiles of Norm heads treated with DON-producing *F. graminearum* isolates (DON⁺, 9F1 and Gz3639) and treatments lacking DON (DON⁻, control and Gzt40). Although less-pronounced, PC2 also showed separation between the metabolomes of DON⁺-treated samples of Norm and Alsen. Furthermore, principal component 1 (PC1) illustrated some separation between the metabolomes of DON⁻ treatments and Alsen inoculated with DON⁺ isolates. These results suggest that wheat variety and treatment were the most significant contributing factors to metabolic profile differences.

To further evaluate metabolic differences between wheat varieties and treatments, additional PCA was conducted on subgroups of samples. Separate PCA of Norm and Alsen wheat samples under different treatments (Fig. 3A) revealed that, in Norm, the metabolic profiles of control and Gzt40-treated heads clustered but distinctly separated from 9F1- and Gz3639-treated (DON⁺) heads. However, the metabolic profiles of Alsen treated with Gzt40, noticeably separated from both the controls and the DON⁺ fungal treatments. This would suggest that Alsen metabolically responds to *F. graminearum* even in

the absence of DON and this response is different when the *F. graminearum* strains produce DON. Differences between the metabolic responses of Norm and Alsen became apparent when PCA was performed separately for each treatment of Norm and Alsen wheat (Fig. 3B). Greatest separation between Alsen and Norm metabolomes was observed in Gz3639- and Gzt40-treated heads. However, separation for the two varieties were illustrated by PC1 for Gz3639 and PC2 for Gzt40. The effect of CO₂ level was difficult to discern in the presence of the dominant source of variation (host variety), so a supervised analysis was performed via orthogonal projections to latent structures (O-PLS). This analysis determines combinations of components in the spectral data that separate 1×CO₂ and 2×CO₂ samples for all treatments (Fig. 3C). Control and Gz3639-treated samples displayed the most distinct separation between 1×CO₂ and 2×CO₂ as illustrated by PC1.

Targeted metabolites were used to also quantitatively analyze the data. Assignment and determination of relative metabolite concentrations was performed as described below with the list of analyzed metabolites and their 1D spectra shown in Supplementary Figure S1.

The resulting relative concentrations for the 49 identified metabolites following scaling to a mean of 0 and standard deviation of 1 across metabolites and samples are graphically represented in a heat plot (Fig. 4). This schematic illustrates the

Fig. 2. A, Average one-dimensional ¹H spectra grouped by treatment. Blue lines represent spectra for Norm and red lines represent spectra for Alsen. Yellow overlay highlights the methanol peak. **B,** Principal component analysis of spectra for all samples, labeled by wheat variety (outline color), *F. graminearum* treatment type (fill color), and 800 ppm CO₂ (2×CO₂) level marked with an X. Metabolite profiles clustered into three distinct groups indicated by different color circles: Blue circle surrounds Norm inoculated with DON-producing (DON⁺) *Fusarium graminearum* strains (9F1 and Gz3639); red circle groups, Alsen inoculated with DON⁺ *Fusarium graminearum* strains (9F1 and Gz3639); green circle clusters, the mock- (control) and Gzt40-inoculated samples from both Norm and Alsen. Percentage of total variation in the spectral data that is accounted for in principal components PC1 and PC2 is provided.

variability between metabolic profiles across wheat varieties and treatments but, also, among biological replicates. For example, the concentration of glutamine in control Norm samples appears to be more abundant in half of the samples but less abundant in the other half at both CO₂ concentrations. It is worth noting that, although the concentration of shikimate was relatively low (Supplementary Fig. S3) and varied between samples, the average concentration in Alsen was significantly higher than Norm in DON⁺ *F. graminearum* treatments and displayed a slight reduction at 2xCO₂. Comparison of PCA for spectral and quantitative metabolic data are shown in Supplementary Figure S2. PCA of quantified metabolic data shows an overall agreement with the PCA of spectra, suggesting that major variances in the data have been preserved in the quantification. Consistent with spectral analyses, metabolite concentrations were primarily affected by wheat variety and treatment (as shown by hierarchical cluster analysis in Supplementary Figure S4).

Significant differences between Alsen and Norm metabolite concentration for the treatments were determined using significance analysis of microarrays (SAM) (Tusher et al. 2001). The metabolites with significant concentration differences between Alsen and Norm for each treatment, independent of CO₂ concentration, were graphically represented in a heat plot for each treatment (Fig. 5). Significant differences in metabolite concentrations between Norm and Alsen were found even without any *F. graminearum* treatment (control). Relative to Norm, control Alsen samples had higher concentrations of glutamate, L-(−)-glutathione, phosphocholine, and L-alanine and lower concentrations of beta-alanine, succinate, trehalose, raffinose, oxoglutarate, methionine, leucine, asparagine, and phenylalanine (Fig. 5). However, more metabolites displayed significant differences with *F. graminearum*

inoculation. Based on the SAM, the metabolites that were significantly less abundant in Alsen than in Norm, regardless of treatment, included beta-alanine, succinic acid, oxoglutarate, trehalose, raffinose, methionine, and leucine (Fig. 5, blue dots). The concentrations of asparagine and phenylalanine in control Alsen samples were lower than in control Norm samples but significantly higher in Alsen than in Norm with *F. graminearum* treatment, including Gzt40, suggesting that the induction of these metabolites was not DON-dependent. Metabolic differences between Alsen and Norm treated with 9F1 and Gz3639, which both produce DON (DON⁺), overlapped significantly and are indicated by red lines in Figure 5. Six of these metabolites also displayed differences with Gzt40 treatment (Fig. 5, red dots). However, while the concentration of tryptophan was greater in DON⁺-inoculated Alsen than respective Norm plants, the amount of tryptophan was lower in Gzt40-inoculated Alsen.

Metabolite markers that can distinguish between susceptible and resistant FHB wheat varieties are in high demand (Cuperlovic-Culf et al. 2016), with particular interest in the effect of rising CO₂ on the concentrations of these markers and their potential diagnostic accuracy with climate change. Therefore, features providing the highest classification power for Norm versus Alsen wheat in control, mock-treated samples were further selected, using logistic regression and analysis of variance (ANOVA). According to the analysis, the metabolites with the highest classification power were L-alanine, isoleucine, 3-hydroxybutyrate, and myoinositol (Fig. 5, red Xs). The concentrations of these four metabolites also provide high classification accuracy between Alsen and Norm wheat, regardless of the atmospheric CO₂ level, as shown in the PCA graph in Supplementary Figure S5. Also, naïve Bayesian classifier shows high classification accuracy with obtained area

Fig. 3. Analysis of one-dimensional (1D) ¹H nuclear magnetic resonance (NMR) spectra. **A**, Principal component analysis (PCA) of all, complete 1D ¹H NMR spectra comparing the metabolic profiles of Norm (top) and Alsen (bottom) wheat in response to treatments (control, 9F1, Gz3639, and Gzt40) at both CO₂ concentrations. Percentage of total variation in the spectral data that is accounted for in principal components PC1 and PC2 is provided. **B**, PCA of 1D ¹H NMR spectra showing differences between the metabolic responses of Norm and Alsen to different treatments. Percentage of total variation in the spectral data that is accounted for in principal components PC1 and PC2 is provided for each plot. **C**, Orthogonal projections to latent structures analysis illustrating the effect of CO₂ level on metabolic responses for each treatment.

under receiver operating curve (AUC) and the classification precision of 95.5% for these four markers. Although concentrations of these four markers varied at $2\times\text{CO}_2$ in both Alsen and Norm (Fig. 5), their relative concentration remained different, suggesting that the power of these resistance markers will be preserved even as CO_2 concentrations rise.

Features providing the highest classification accuracy for Norm and Alsen wheat in all samples, with all treatments, and at both CO_2 levels according to naïve Bayes analysis (AUC of 94.4%) are sucrose, methionine, asparagine, oxoglutarate, tyrosine, phenylalanine, raffinose, L-alanine, and succinate (not shown in a separate figure). These metabolites have been selected as significantly different in individual SAM analysis for some or all treatments.

To identify the metabolic differences between $1\times\text{CO}_2$ and $2\times\text{CO}_2$ samples, we have used a logistic regression and ANOVA significance test, followed by PCA of samples showing the level of separation. Metabolites with the greatest significant differences between $1\times\text{CO}_2$ and $2\times\text{CO}_2$ with respect to treatments are shown in Figure 6.

An analysis of all samples, distinguished only by CO_2 concentration, revealed that, on average, malate, betaine, phosphocholine, oxoglutarate, succinate, glutamine, and phenylalanine concentrations were higher in plants at $1\times\text{CO}_2$, while uridine and sucrose concentrations tended to be higher in plants at $2\times\text{CO}_2$ (Fig. 6A). The major effect CO_2 level on these metabolites may reflect the changes that occur in primary metabolism at higher atmospheric CO_2 .

As above, the data were further analyzed for each treatment independent of the others (Fig. 6B). There was little overlap between the most significantly different metabolites of the treatments. However, within the individual treatments, some of

the same metabolites differed in both Norm and Alsen (Fig. 6B, highlighted in blue). Only the concentration of cysteine was consistently different between the CO_2 concentrations in all the *F. graminearum*-treated Norm plants. Furthermore, the amount of cysteine was more abundant in Gzt40-inoculated Norm and Alsen plants at $2\times\text{CO}_2$. Choline and myoinositol were significantly different at $1\times\text{CO}_2$ in comparison with $2\times\text{CO}_2$ in all *F. graminearum*-treated Alsen plants (Fig. 6B, black Xs). However, the concentration of myoinositol in Alsen treated with Gz3639 or Gzt40 was greater in plants at $2\times\text{CO}_2$ in comparison with $1\times\text{CO}_2$ but not in 9F1-treated Alsen. Furthermore, the concentration of asparagine was different in $1\times\text{CO}_2$ versus $2\times\text{CO}_2$ Alsen plants treated with DON⁺ *F. graminearum* and control (Fig. 6B, red Xs), however the direction of the change was opposite in 9F1 and Gz3639 treatments. Additional analyses elucidating the major metabolic differences in *F. graminearum*-treated Alsen relative to the control treatment at both CO_2 levels are shown in Supplementary Figure S6.

DISCUSSION

To the best of our knowledge, this work represents the first report on differences in the metabolic profiles of *F. graminearum*-inoculated wheat at ambient and elevated CO_2 . Our results demonstrate that the effects of elevated atmospheric CO_2 on FHB and DON contamination are dependent on both the *F. graminearum* strain and the wheat variety. Furthermore, changes in host metabolites can partly explain the observed changes in FHB and DON levels. Previous studies observed variable effects of elevated CO_2 on head blight between wheat varieties and have suggested that CO_2 has the

Fig. 4. Heat map of targeted metabolite concentrations relative to the reference trimethylsilylpropionate peak and scaled to calculated mean of 0 and standard deviation of 1 across all samples and metabolites, with red indicating samples with significantly higher metabolite concentrations than the overall metabolite mean and green indicating lower metabolite concentrations than mean concentration. Metabolites are ordered based on their concentration similarities across all samples determined with hierarchical clustering.

potential to affect both the fungal pathogen and the host plant (Bencze et al. 2017; Kane et al. 2013; Váry et al. 2015). Bencze et al. (2017), further demonstrated that inoculation method, single floret versus whole head (evaluating type I versus type II resistance, respectively), can produce variable results at elevated CO₂. However, our data provides additional insight into the effects of elevated CO₂ on the metabolome of wheat varieties with (Alsen) and without (Norm) *Fhb* loci in response to whole-head inoculation with two DON-producing (DON⁺) *F. graminearum* isolates and a mutant DON-deficient (DON⁻) *F. graminearum* isolate.

Our results also indicate that visual disease severity and *F. graminearum* biomass do not always correspond with DON contamination levels (Fig. 1). Climate can skew the classic disease triangle and the inlaid mycotoxin triangle in different ways (Medina et al. 2017). Our results are consistent with other reports evaluating *Fusarium* diseases under abiotic stress conditions (Bencze et al. 2017; Paterson and Lima 2010; Vaughan et al. 2014). When pathogen proliferation was constrained at 2×CO₂, DON production per unit of *F. graminearum* biomass was higher (Fig. 1B, C, D, and F), leading to the observed reduction in pathogen biomass but no significant difference in DON for Norm inoculated with 9F1 and both Norm and Alsen inoculated with Gz3639 (Fig. 1B, C, D, and F). Furthermore,

the increased resistance of Norm at 2×CO₂ was independent of DON production (Fig. 1H), suggesting that 2×CO₂ may compromise other pathogen effectors, enhance host defenses, or a combination of both. Nevertheless, an increase in DON production per unit of *F. graminearum* biomass is concerning. While the susceptible variety Norm appears to have enhanced resistance to *F. graminearum* colonization at 2×CO₂, DON contamination remained the same. Therefore, current mycotoxin food safety issues will persist. Additionally, while current FHB control strategies and breeding efforts rely heavily on parent lines containing *Fhb* loci for resistance, it is disturbing that these moderately resistant lines may become significantly more susceptible to mycotoxin accumulation when infected by certain *F. graminearum* strains at elevated CO₂ (Fig. 1B and C).

The metabolic profiles of *F. graminearum*-inoculated Alsen and Norm were quite distinct. Multiple metabolites with significant concentration differences were identified between the varieties and treatments. All the 49 quantified metabolites can be produced by *F. graminearum* and wheat, thus, without conducting separate analyses of *F. graminearum* alone or carbon labeling experiments, we cannot be certain which organism is responsible for these metabolic changes during the interaction. Trehalose has been shown to play an important role in *F. graminearum* virulence and mycotoxin production (Song

Fig. 5. Metabolites that were determined by significance analysis of microarrays (SAM) analysis as having significantly different concentrations between Norm and Alsen for each treatment (control, 9F1, Gz3639, and Gzt40), regardless of CO₂ level, are depicted as separate heat maps. Metabolites that have a significant concentration difference between Norm and Alsen for all treatments, including control, are indicated with blue dots. Metabolites that are common between the DON⁺ treatments (9F1 and Gz3639) are indicated with a red line. Red dots indicate metabolites that are in both DON⁻ (Gzt40) and DON⁺ treatments but not in the control. Metabolites that provide the most accurate classification for control Norm and Alsen samples are designated with a red X. In each group metabolites have been hierarchically clustered, indicating similarities in behavior. Data are normalized across samples and metabolites for each group prior to SAM.

et al. 2014), thus, lower levels in Alsen are consistent with enhanced FHB resistance. On the other hand, asparagine has been described as a constitutive defense metabolite marker and phenylalanine has been reported as a DON-induced resistance metabolite in wheat (Paranidharan et al. 2008). However, asparagine and phenylalanine levels were higher in control Norm as compared with Alsen but their concentrations were significantly higher in Alsen inoculated with 9F1, Gz3639, or Gzt40, suggesting that these metabolites would best be considered as inducible markers that are not always dependent on DON production. The production of asparagine may deprive the pathogen of a necessary nitrogen source (Seifi et al. 2013), which is in agreement with increased asparagine concentration in Alsen plants treated with *F. graminearum*. The lower concentration of succinate, oxoglutarate, methionine, and leucine, together with the higher levels of phenylalanine and asparagine in *F. graminearum*-treated Alsen, suggests an increased flux in the shikimate pathway. Indeed, although quite variable and at relatively low concentration overall, the average concentration of shikimate was significantly higher in Alsen in comparison with Norm for all treatments (Fig. 4), which is consistent with previous reports on the metabolomes of other moderately resistant varieties, including Sumai 3 (Cuperlovic-Culf et al. 2016). Phenylalanine is an end product of the shikimate pathway and the starting substrate of the phenylpropanoid pathway; these pathways provide essential precursors for a wide range

of important FHB resistance metabolites. For example, both salicylic acid, a key defense-related hormone, and flavonoids, which typically possess antioxidant activity, are phenylpropanoids (Gunnaiah and Kushalappa 2014; Makandar et al. 2012). Chemical resistance mechanisms of wheat varieties containing the *Fhb* loci rely on induction of phenylpropanoids and thickening of cell walls that reduce pathogen advancement and synthesis of antifungal and antioxidant metabolites that reduce pathogen proliferation and DON production (Gunnaiah and Kushalappa 2014). Therefore, the metabolic response of Alsen to *F. graminearum* inoculation appears to be consistent with reports from other *Fhb*-containing varieties. Additionally, and perhaps most imperative, the metabolic pathways that contribute to the primary source of FHB resistance appear to be upregulated in *F. graminearum*-inoculated Alsen relative to Norm at both CO₂ concentrations. Therefore, *Fhb*-containing varieties will likely remain more resistant to FHB than non-*Fhb* containing varieties even at elevated CO₂. This is consistent with our disease assessment results (Fig. 1) and previously published data (Váry et al. 2015). Even in the case in which the 9F1 biomass and DON concentration were significantly higher in Alsen at 2×CO₂ in comparison with 1×CO₂, these levels were still less than the amounts in corresponding Norm plants. Metabolic differences were also found between 1×CO₂ and 2×CO₂ samples (Figs. 3C and 6). Metabolic differences between control plants at the two CO₂ concentrations were

Fig. 6. Metabolites with significant concentration differences at 1×CO₂ versus 2×CO₂. **A**, Relative concentration of metabolites that are significantly different at 1×CO₂ (blue line) and 2×CO₂ (red line) based on analysis of variance (ANOVA) of all samples for all treatment groups. The metabolites are ordered by statistical significance based on ANOVA F-values, (provided in the table). Bars indicate standard variation across all samples in all treatment groups. **B**, Metabolites with significantly different concentrations between samples grown under 1×CO₂ and 2×CO₂ with respect to different treatments (control, 9F1, Gz3639, or Gzt40). Principal component analysis subplots show major variances (PC1 and PC2) for samples when analyzing concentrations of only the select metabolites, with red showing samples grown at 2×CO₂ and blue indicating samples grown at 1×CO₂. Metabolites that are more abundant in plants at 2×CO₂ relative to 1×CO₂ are highlighted in red. Metabolites that are significantly affected by CO₂ in both Norm and Alsen wheat for a given treatment are highlighted in blue. Metabolites discussed in the text are marked with an X.

expected, given that elevated CO₂ can increase wheat photosynthesis and alter primary metabolism (Habash et al. 1995; Pal et al. 2005). A higher concentration of sucrose at elevated CO₂ is in agreement with previously observed increases in the expression of genes associated with sucrose synthesis (Fukayama et al. 2011; Gray and Brady 2016). Several tricarboxylic acid cycle intermediates, including malate, oxoglutarate, and succinate, were at lower concentrations at elevated CO₂, possibly due to increased flux through this pathway, another aspect that is supported by previous gene expression data (Gray and Brady 2016; Fukayama et al. 2011).

Significant differences were also observed between the *F. graminearum*-treated samples, suggesting that *F. graminearum* treatment was also a significant factor contributing to metabolic differences at the two CO₂ levels (Fig. 3C and 6B). At 2×CO₂, not as much asparagine accumulated in Alsen treated with 9F1, which could explain the observed increase in susceptibility to 9F1 biomass and DON contamination. Only the concentration of glycerol significantly increased at 2×CO₂ in Alsen treated with 9F1 (Fig. 6B). In barley, glycerol was identified as a putative resistance-related metabolite (Bollina et al. 2011), but, despite its induction here at 2×CO₂ in Alsen treated with 9F1, the plants were relatively more susceptible. Therefore, increased concentration of glycerol alone is not sufficient to enhance resistance. In Alsen treated with Gz3639 at 2×CO₂, only the concentration of phosphocholine was significantly reduced, while all other metabolites with significant differences, including asparagine and phenylalanine, were increased in concentration. Additionally, the concentration of asparagine was slightly reduced at 2×CO₂ in Alsen treated with Gzt40, indicating that this response in the host was not entirely dependent on DON and is, therefore, unlikely a response to changes in fungal mycotoxin production at 2×CO₂. Nevertheless, in moderately resistant wheat varieties carrying the *Fhb* loci, asparagine concentration may represent a useful biomarker for enhanced wheat susceptibility at elevated CO₂. It has been proposed that moderately resistant wheat varieties hinder DON production and fungal growth by sequestering nitrogen in asparagine, making it unavailable to the invading pathogen (Fagard et al. 2014; Gaufichon, et al. 2016). The difference between asparagine levels in Alsen compared with Norm supports this premise. However, in comparison with corresponding plants at 1×CO₂, the concentration of asparagine was lower in Alsen treated with 9F1 at 2×CO₂ but greater in Alsen treated with Gz3639 at 2×CO₂. This difference in asparagine is therefore dependent on the infecting *F. graminearum* strain and not only an inherent trait of the host. It is possible that the aggressiveness of *F. graminearum* strains on a moderately resistant host variety is determined by its ability to inhibit the host from sequestering nitrogen in asparagine and elevated CO₂ interferes with this process in a strain-specific manner, leading to the observed strain-dependent results.

Another important resistance metabolite that significantly differed between 9F1- and Gz3639-treated Alsen at 2×CO₂ was myoinositol (Fig. 6). Although myoinositol is listed among the metabolites with the greatest significant concentration difference between 1×CO₂ and 2×CO₂ for all three *F. graminearum* treatments, at 2×CO₂ the concentration of myoinositol was significantly reduced in Alsen treated with 9F1, while it was increased in Alsen treated with Gz3639 or Gzt40. Myoinositol functions as a building block for a variety of molecules that are involved in signal transduction, including regulation of plant hormone receptors, mediation of abiotic and biotic defense responses, and fungal pathogen recognition (Gillaspy 2011). Furthermore, a myoinositol is a precursor for D-glucuronic acid, which is an essential component of cell-wall biosynthesis,

and ascorbic acid, which has strong antioxidant activity (Gauthier et al. 2015). Alsen treated with Gz3639 or Gzt40 displayed a similar induction in myoinositol, choline, and acetate concentration at 2×CO₂. As Gzt40 is a mutant form of Gz3639, it cannot be excluded that these metabolic differences are a result of 2×CO₂-induced changes in the fungus rather than the host plant or a combination of changes in both the fungus and the host plant. The induction of these metabolites was not observed in Norm inoculated with Gz3639 or Gzt40 at 2×CO₂, but the inherent genotypic differences between the two varieties does not allow for such comparisons. Therefore, the potential direct effects of elevated CO₂ on *F. graminearum* effectors or virulence factors other than DON require further investigation.

In Norm treated with either 9F1 or Gz3639 all significantly different metabolites were less abundant at 2×CO₂, which is perplexing considering that, in both cases, *F. graminearum* biomass was reduced but DON levels were unaltered. These two *F. graminearum* treatments share the reduction of betaine and cysteine (Fig. 6). Betaine, a major component of wheat anthers and germ, has been shown to stimulate *F. graminearum* growth (Strange et al. 1974) and cysteine has been implicated in DON resistance (Gardiner et al. 2010). Therefore, a reduction in the concentration of these two metabolites could potentially explain the observed results. However, Norm treated with Gzt40 at 2×CO₂ also displayed less fungal biomass, suggesting that the increase in resistance was independent of DON production. There are no common metabolites between the three *F. graminearum* treatments in Norm. Thus, further experiments are needed to elucidate the underlying mechanisms involved in constraining *F. graminearum* biomass in Norm at 2×CO₂.

Additional metabolic profiling that includes the quantification of metabolites derived from the phenylpropanoid pathway, which make up the largest (more than 50%) chemical group of potential resistance metabolites against DON-producing *Fusarium* species (Gauthier et al. 2015), will likely provide additional insight into the underlying mechanism involved in CO₂-induced changes in wheat resistance to FHB. Although, we were able to make inferences based on changes in the concentration of essential precursors, these inferences and hypotheses need to be empirically validated.

Our findings suggest that individual *F. graminearum* strains can respond differently to elevated CO₂. Váry et al. (2015) previously demonstrated that pathogen virulence can be influenced by the CO₂ concentration, but here, we further show that this influence is strain-dependent. Given this variability among strains, rising CO₂ concentration has the potential to further shape *F. graminearum* population dynamics by giving an advantage to strains that have increased pathogenicity under conditions of elevated CO₂. Nevertheless, since the effects of elevated CO₂ are dependent on both the pathogen strain and the host variety, it is essential to evaluate multiple combinations of diverse strains and varieties when conducting such analyses.

Finally, our analysis has also identified several promising, constitutive, FHB resistance markers, L-alanine, isoleucine, 3-hydroxybutyrate, and myoinositol, which have significant concentration differences between susceptible Norm and moderately resistant Alsen spring wheat at both ambient and elevated CO₂. Metabolomic analyses of other wheat varieties have similarly identified L-alanine, 3-hydroxybutyrate, and myoinositol as wheat FHB resistance-related metabolite markers (Gunnaiah, et al. 2012; Hamzehzarghani 2007; Kushalappa and Gunnaiah 2013). However, 3-hydroxybutyrate was previously reported as a constitutive resistance-related metabolite marker (Gunnaiah, et al. 2012; Hamzehzarghani 2007). In our analysis, 3-hydroxybutyrate was less abundant in control Alsen as compared with Norm, but the concentration of 3-hydroxybutyrate was significantly higher in Alsen treated

with *F. graminearum*, suggesting that the constitutive concentration of this particular metabolite may not reflect the resistance level in all varieties. Furthermore, isoleucine has been reported as a metabolite related to mechanisms of resistance in barley against *F. graminearum* (Bollina et al. 2011). Nevertheless, the metabolic panel identified here is unique in that it provides breeders with markers that will reliably predict resistance or susceptibility, even as atmospheric CO₂ levels rise.

MATERIALS AND METHODS

Experimental design.

To evaluate the effect of elevated atmospheric CO₂ concentrations on the metabolomic response of wheat following *F. graminearum* inoculation, we conducted FHB assays in growth chambers programmed to ambient CO₂ (400 ppm, 1×CO₂) and elevated CO₂ (800 ppm, 2×CO₂). Second-generation 1×CO₂- and 2×CO₂-acclimated (Váry et al. 2015) hard red spring wheat varieties Norm and Alsen were used for all the experiments. Norm (pedigree = MN73167/MN 81070, released by the Minnesota Agricultural Experiment Station and the United States Department of Agriculture-Agricultural Research Service in 1992) is highly susceptible to head blight, while Alsen (pedigree = ND 674/ND 2710/ND 688, released by the North Dakota Agricultural Experiment Station in 2000) is derived from the resistant Sumai 3 and contains the *Fhb1* and *Fhb5* loci (Bokore et al. 2017). Two wild-type 15-acetyldeoxynivalenol-producing strains, 9F1 (isolated from the Netherlands [McCormick et al. 2010]) and Gz3639 (isolated from Kansas in the United States [Desjardins et al. 1996]) as well as a trichothecene-nonproducing *tri5* deletion mutant (Gzt40) of Gz3639 (Desjardins et al. 1996) were used. Whole-head inoculations were conducted to test the combined resistance to the penetration and spread of the pathogen by dipping the entire flowering wheat head in 50 ml of inoculum and then evaluating initial disease symptoms at 3 days postinfection and spread at 7 days postinfection (type I and II resistance). As a negative control, heads were mock-inoculated with sterile 0.04% Tween solution. At each CO₂ concentration, 30 heads from at least six independent pots were inoculated with one of the three *F. graminearum* strains or the control 0.04% Tween solution. Following the disease assays, three heads were combined into one tube as a single biological pool (resulting in $n = 10$ per treatment). Due to growth-chamber space limitations and differences in flowering time, experiments with Norm and Alsen were conducted separately.

Wheat growth.

Five seeds were germinated in each 20 × 15-cm plastic pot filled with approximately 4 liters of SunGrow Horticulture potting mix (Agawam, MA, U.S.A.). Plants were grown in two separate climate-controlled growth chambers, both programmed at 25°C day and 23°C night, 550 μmol m⁻² s⁻¹ photosynthetic photon flux density with a 14 h photoperiod, with 50 to 60% relative humidity. The only difference in growing conditions was that one chamber was set at 400 ppm CO₂ (1×CO₂) and the other at 800 ppm CO₂ (2×CO₂). Plants were watered daily and received biweekly nutrient supplement with soluble Peters 20-20-20 (The Scotts Company, Marysville, OH, U.S.A.) until anthesis, at which point the flowering heads were inoculated.

F. graminearum inoculation and disease assessment.

Inoculum was prepared by recovering isolates from glycerol stocks, allowing them to grow for 7 days on V8 plates and then transferring an agar plug to 100 ml of mung bean broth. Cultures were then grown in a New Brunswick Innova 44 incubator

shaker (Eppendorf, Hauppauge, NY, U.S.A.) at 28°C and 200 rpm for 48 h. The conidial suspension of each strain was spun down and resuspended in 0.04% Tween 20 (Fisher Scientific, Waltham, MA, U.S.A.) solution at a concentration of 10⁵ microconidia per milliliter, based on cell counts obtained using a Scepter automated cell counter (Millipore, Billerica, MA, U.S.A.). Fresh inoculum was prepared on the day of each experiment.

To minimize metabolic differences due to circadian rhythm, all experiment inoculations were performed between 1:00 and 2:00 p.m. Inoculated heads were covered with plastic bags to ensure high humidity during the early stages of pathogenesis. At 3 dpi, the bags were removed and the number of visibly infected florets were scored. The heads were again scored at 7 dpi, were then collected and frozen in liquid nitrogen, and were kept at -80°C until processing. All collections were done between 1:00 and 2:00 p.m. Collected tissues were lyophilized in a VirTis lyophilizer (Model 24DX48GPF 25LXL70; SP Scientific, Warminster, PA, U.S.A.) and were then pulverized, using a SPEXsamplePrep Geno/Grinder 2010 (Metuchen, NJ, U.S.A.). Samples were separated into three aliquots for DNA quantification of pathogen and plant biomass, mycotoxin quantification, and metabolite analyses.

Percent disease was calculated by dividing the number of visually symptomatic florets by the total number of florets on the head. Florets displaying any sign (even if minor) of premature bleaching or spots of necrosis were deemed symptomatic. Student's *t* tests were then performed to compare the average percentage of disease on plants grown at 1×CO₂ with those at 2×CO₂. Comparisons for Alsen and Norm varieties were conducted independently.

Estimation of pathogen and plant biomass.

In order to assess the amount of fungal biomass in the inoculated tissues, the ratio of *F. graminearum* DNA to wheat DNA was estimated using quantitative polymerase chain reaction (qPCR) (Vaughan et al. 2014). Genomic DNA was extracted from 80 mg of tissue ground using the ZR Fungal/Bacterial DNA MiniPrep and the Genomic DNA Clean and Concentrate kits (Zymo Research, Boston), following the manufacturer protocol. The quantity of 9F1 DNA was estimated using gene-specific primers for *Tri5* (forward TCTATGGCC CAAGGACCTGT and reverse ACGTCATCGTCGAATTCCT; efficiency = 116, R² = 0.996) and the quantity of Gz3639 and Gzt40 was estimated using gene-specific primers for *Tri6* (forward TAACCACATCGTCGGGACTG and reverse GCCGACTT CTTGCAGGTCTT; efficiency = 116, R² = 0.996). The quantity of *Triticum aestivum* DNA was estimated using primers for *PR1* (forward CGTCTTCATCACCTGCAACTA and reverse CAAA CATAAACACACGCACGTA; efficiency = 115; R² = 0.997). For DNA quantifications, a tenfold dilution series spanning 6 logs from 50 ng of pure fungal or plant DNA was used to generate standard curves by plotting the quantification cycle values obtained by qPCR against the log of DNA concentration. Statistical differences between the *F. graminearum*/wheat DNA biomass ratio at 1×CO₂ and 2×CO₂ were determined by conducting pairwise Student's *t* tests.

Mycotoxin analyses.

Mycotoxins were extracted from 0.5 g of pulverized tissue from the whole head with 10 ml of extraction solvent (acetonitrile/water, 86:14 vol/vol) in a 50-ml polypropylene screw-cap centrifuge tube with shaking for 15 min. After centrifugation, a portion of each extract was purified by forcing 5-ml extract through a MycoSep 225 Trich cartridge (Romer Labs, Union, MO, U.S.A.). A 2-ml aliquot of the purified extract was then transferred to a 1-dram vial and was dried under a nitrogen stream. Trimethylsilyl (TMS) derivatives were

prepared by adding 100 μ l of a 100:1 freshly prepared mixture of N-trimethylsilylimadazole/trimethylchlorosilane (Sigma-Aldrich, St. Louis) to the dried extract. After 30 min, 900 μ l of isooctane was added to the reaction mixture, followed by 1 ml of water. The mixture was mixed gently, until the organic (top) layer became translucent. The organic layer was then transferred to 2-ml autosampler vials for gas chromatography mass spectrometry (GC-MS) analysis. TMS derivatives of purified DON (0.3125 to 80 μ g) were also prepared to construct a standard curve for quantification. Significant differences between the amount of DON in Alsen or Norm at 1 $\times$ CO₂ and 2 $\times$ CO₂ were evaluated, using pair-wise Student's *t* test.

GC-MS analyses were performed on an Agilent 7890 gas chromatograph fitted with a HP-5MS column (30 m, 0.25 mm, 0.25 μ m) and a 5977 mass detector. The injection temperature was kept at 250°C and the column flow rate was 1 ml min⁻¹. A temperature program was used, with an initial column temperature of 150°C for 1 min which was then increased to 280°C at 30°C min⁻¹ and held for 3.5 min. Selective ion monitoring was applied to detect the characteristic ions of triTMS-DON with fragment ion (*m/z* value) of 235.1 as the quantifier ion and 259.1, 295.1, 392.2, 422.2, and 512.2 as qualifier ions.

Metabolite extraction for NMR analysis.

Hydrophilic metabolites were extracted from an approximately 100-mg of tissue (the precise amount for each sample was recorded) with 1 ml of methanol/water solution (40:60 vol/vol) by homogenizing the sample for 30 s at 6,000 rpm, using a Precellys 24 homogenizer (Bertin Technologies, Atkinson, NH, U.S.A.), followed by centrifugation at 3500 $\times$ *g* for 20 min. The supernatant was then collected into a 15-ml tube. The extraction process was repeated and the supernatants from both extractions were combined. The tubes were covered with Breath-Easier film (Diversified Biotech, Boston), were frozen by placing the bottom of the tube in liquid nitrogen, and were then lyophilized for 24 to 48 h. The dry samples were covered and were then stored at -80°C. For NMR analysis, the samples were resuspended in 700 μ l of deuterated phosphate buffer (0.0912 g of monobasic potassium phosphate in 10 ml of deuterium oxide was mixed with 0.1167 g of dibasic potassium phosphate in 10 ml of deuterium oxide to create a 0.067 M phosphate solution, to which 20 mg of sodium azide and 20 mg of deuterated sodium trimethylsilylpropionate were added), were vortexed, and were transferred to NMR sample tubes.

NMR experimentation, data processing, and quantification.

All ¹H NMR measurements were performed on a Bruker 500 MHz spectrometer at 298 K. 1D ¹H (proton) NMR was measured for all samples using 1D ¹H with water suppression sequence. The 2D TOCSY (total correlated spectroscopy) and HSQC (heteronuclear single quantum coherence) spectra were used for metabolite identification. All 1D and 2D spectra were processed using MestReNova 9.1.0 software (Mestrelab Research Solutions, Santiago de Compostela, Spain). Preprocessing for 1D spectra included exponential apodization, global phase correction, and normalization using the reference peak. Spectral regions from 0.5 to 9.5 ppm were included in the normalization and analysis. The 2D spectra were processed using a standard procedure recommended in the MestReNova documentation.

Metabolite assignment was performed using 1D ¹H, HSQC, and TOCSY data. Assignment of peaks in the TOCSY spectra was performed using Madison Metabolomics Consortium Database and tools (Markley et al. 2007). Assignment of individual peaks was performed using searches provided in metabolomics databases HMDB (Human Metabolome Database)

(Wishart et al. 2013) and BMRB (Biological Magnetic Resonance Databank) (Markley et al. 2007) as well as NMR spectral peaks search tool MetaboHunter (Tulpan et al. 2011) and literature assignments for metabolites previously observed in related samples. A total of 49 metabolites were included in the analyses, comprising molecules that were present in both TOCSY and HSQC spectra. Spectra for metabolites that were used in quantification of 1D ¹H spectra were obtained from the HMDB or BMRB and were processed using MestReNova 9.1.0 software. Spectral preprocessing for standards spectra included exponential apodization, global phase correction, and normalization using the total spectral area. Spectral regions from 0.5 to 9.5 ppm were included in the normalization and analysis. Prior to quantification analysis, the standard spectra were aligned to the reference peak (trimethylsilylpropionate) using peak alignment by fast Fourier transform cross-correlation (Wong et al. 2005). The set of quantified metabolites can be divided into carbohydrate pathway metabolites, amino acids, and derivatives, organic acids, and others. Metabolites from these chemical groups have previously been linked to FHB resistance and also provide insight into potential fluxes in the biosynthetic pathways of other chemical groups involved in host resistance (Gauthier et al. 2015), because many of the metabolites from the groups analyzed serve as precursors for others that have not been directly evaluated in this study.

An automated method for quantification using multivariable linear regression that finds the best fit of spectra for individual metabolites from a database to the measured 1D sample spectra was developed previously (Cuperlovic-Culf et al. 2012) and was utilized, in this study, to determine relative metabolite concentrations. The partial least square regression analysis result was used as the starting point and the model was constrained to concentrations greater than or equal to zero. The deconvolution of spectra of mixtures, such as in metabolomics, with many strongly overlapping lines, possibly with an unknown number of lines and atomic groups, each with a different line width is extremely difficult, and thus, it is important to determine an optimal solver for this problem. The best result, i.e., the model with a minimal error was obtained with Levenberg-Marquardt curve fitting, and this method was used for quantification of metabolic data used in further analysis. Multivariate linear regression analysis was performed using lsqcurvefit running under Matlab. Metabolite concentrations across samples were determined using the same standard spectra that were normalized to the total intensity equal to one and sample spectra normalized to the same reference concentration. The resulting concentrations, therefore, provide relative metabolite measures in different samples using the same standard scale that allows comparison between samples without having to determine absolute metabolite concentrations.

Data analysis.

Preprocessing including data organization, removal of undesired areas, normalization, as well as data presentation was performed with Matlab vR2010 and vR2017a (Mathworks). Minor adjustments in peak positions (alignment) between different samples were performed using icoshift (Savorani et al. 2010). PCA was performed in Matlab, using routine ppca for probabilistic principal component analysis (Tipping and Bishop 1999), and was performed on sample spectra as well as relative metabolite concentration data. Supervised classification analysis O-PLS method running in Matlab was used to separate spectral data for the two CO₂ conditions. Selection of metabolic panels with statistically significant difference between groups was done using the SAM method (Tusher et al 2001), as provided in TMeV, selecting panels with zero false significant genes and Δ (delta) value of over 1 representing distance from

the expected line based on the permutation analysis performed in SAM. Machine learning methods running under Matlab and Orange, a component-based data mining software running under the Anaconda Python data science platform, were used for feature selection in different CO₂ concentrations. Specifically, feature selection was done using Logistic Ridge Regression performing L2 regularization as well as ANOVA ranking as presented in Orange, giving the same set of significant metabolites.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Anaconda Python data science platform: <https://anaconda.org>
 Biological Magnetic Resonance Databank: www.bmrb.wisc.edu
 Human Metabolome Database: www.hmdb.ca
 Orange data mining software: <https://orange.biolab.si>