Title

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Journal

Proceedings of the National Academy of Sciences of the United States of America, 115(1)

ISSN

0027-8424

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Publication Date

2018-01-02

DOI

10.1073/pnas.1717519115

Peer reviewed

Fungal-induced protein hyperacetylation in maize identified by acetylome profiling

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Contributed by Steven P. Briggs, November 16, 2017 (sent for review October 18, 2017; reviewed by Alex Jones and Wenbo Ma)

Lysine acetylation is a key posttranslational modification that regulates diverse proteins involved in a range of biological processes. The role of histone acetylation in plant defense is well established, and it is known that pathogen effector proteins encoding acetyltransferases can directly acetylate host proteins to alter immunity. However, it is unclear whether endogenous plant enzymes can modulate protein acetylation during an immune response. Here, we investigate how the effector molecule HC-toxin (HCT), a histone deacetylase inhibitor produced by the fungal pathogen *Cochliobolus carbonum* race 1, promotes virulence in maize through altering protein acetylation. Using mass spectrometry, we globally quantified the abundance of 3,636 proteins and the levels of acetylation at 2,791 sites in maize plants treated with HCT as well as HCT-deficient or HCT-producing strains of *C. carbonum*. Analyses of these data demonstrate that acetylation is a widespread posttranslational modification impacting proteins encoded by many intensively studied maize genes. Furthermore, the application of exogenous HCT enabled us to show that the activity of plant-encoded enzymes (histone deacetylases) can be modulated to alter acetylation of nonhistone proteins during an immune response. Collectively, these results provide a resource for further mechanistic studies examining the regulation of protein function by reversible acetylation and offer insight into the complex immune response triggered by virulent *C. carbonum*.

acetylome | immunity | maize | proteome

To prevent disease, organisms have evolved elaborate systems to provide immunity against pathogens. To overcome immunity, pathogens employ numerous strategies, including the usage of virulence factors such as toxins to suppress host defense. One common mechanism for suppressing immunity appears to be a result of actually inducing an inappropriate defense response (1–4). In *Arabidopsis*, the bacterial effector coronatine mimics jasmonic acid isoleucine conjugate (JA-Ile) and thereby suppresses salicylic acid (SA)-mediated defense against *Pseudomonas syringae* (2–4). Additionally, *Cochliobolus* species utilize a range of toxins to promote pathogenicity. For example, *Cochliobolus victoriae* secretes the cyclic peptide victorin as its primary effector. Victorin induces susceptibility of oats to *C. victoriae* by activating resistance to the rust fungus *Puccinia coronata* (1). Furthermore, in *Arabidopsis*, victorin elicits a defense response, which enables *C. victoriae* virulence (5).

A related pathogen, *Cochliobolus carbonum*, produces the effector HC-toxin (HCT), which is a cyclic peptide that functions as a histone deacetylase inhibitor (HDACi) (6–8). Specifically, HCT is able to inhibit members of the HD2 and RPD3/HDA1 classes of histone deacetylases (HDACs) in plants, fungi, and animals (6–8). In maize, hyperacetylation of histones H3 and H4 (but not histone H2) was observed following treatment with HCT, but the sites of acetylation were not identified (6, 7). This and subsequent work in a range of species in which the activity of histone acetyltransferases (HATs) or HDAC enzymes was modulated suggests that alterations in histone acetylation status play a central role during host–pathogen interactions (9–13). The changes in HAT or HDAC activity have been interpreted to modulate immunity by directly affecting transcription at specific defense gene promoters. However, recent mass spectrometry-based global acetylation profiling methods have been developed, leading to the realization that lysine acetylation is a major posttranslational modification that impacts a wide range of proteins (14–29). This finding raises the possibly that alterations in HAT or HDAC activity function by means of hyperacetylating proteins, in addition to histones, to promote or modulate plant defense.

Consistently, some pathogens also modulate plant immunity by secreting effector proteins that function as acetyltransferase enzymes and directly acetylate host proteins (12, 30–37). For example, pathogen effectors target host proteins that would otherwise enable immunity, including WRKY transcription factors, RPM1-associated proteins, and microtubule-related proteins. This raises the questions of whether plant HATs or HDACs directly modulate the acetylation status of nonhistone proteins during pathogen infection, and if they do, whether there is selective acetylation of proteins required for immunity.

To gain insight into how HCT suppresses host defense to promote *C. carbonum* virulence, we used mass spectrometry to quantify global changes in protein abundance and acetylation levels triggered by pathogen infection. Critically, through the application of exogenous HCT and *C. carbonum* strains that do or do not make HCT, we demonstrate that the activity of plant-encoded enzymes (i.e., HDACs) can be modulated to alter both histone and nonhistone protein acetylation. Hyperacetylation was highly selective for proteins involved with transcription,

Significance

How pathogens manipulate host cellular machinery to enable infection is a major question in biology. The ability of *Cochliobolus carbonum* race 1 to infect susceptible corn plants relies on production of HC-toxin (HCT). While it is known that HC-toxin is a histone deacetylase inhibitor, knowledge of how HCT actually promotes virulence has remained elusive. Here, we use mass spectrometry to quantify protein abundance and levels of protein acetylation in HCT-treated or pathogen-infected plants. These analyses revealed that the activity of plant-encoded enzymes can be modulated to alter both histone and nonhistone protein acetylation during a susceptible interaction and suggest that virulent *C. carbonum* utilizes HCT to reprogram the transcriptional response to infection, resulting in an ineffective defense response.

Author contributions: J.W.W. and S.P.B. designed research; J.W.W., Z.S., and E.A.S. performed research; J.W.W., Z.S., M.R.M., and S.P.B. analyzed data; and J.W.W. and S.P.B. wrote the paper.

Reviewers: A.J., University of Warwick; and W.M., University of California, Riverside.

The authors declare no conflict of interest.

Data deposition: The raw spectra for the proteome data have been deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository, https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp (accession ID MSV000079681).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1717519115/-/DCSupplemental.

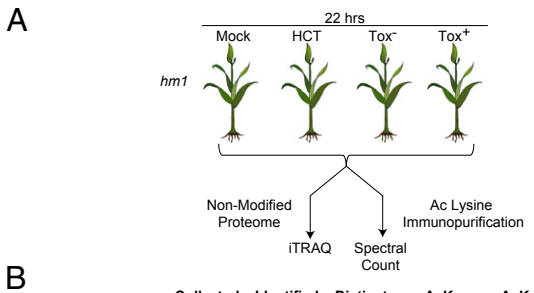
including homologs of known regulators of immunity in *Arabidopsis*. Changes in protein abundance mimicked the JA-mediated induction of the indole biosynthetic pathway in *Arabidopsis*; in maize, this pathway produces benzoxazinoid (Bx) phytoalexins. Additionally, we provide a global acetylome for maize and significantly expand the number of acetylation sites identified in plants. Acetylated proteins carry out a wide range of functions and include numerous well-studied maize proteins. Thus, the data presented here will enable new research approaches to understand the posttranslational regulation and function of both defense- and nondefense-related proteins.

Results

Quantitative Profiling of the Maize Immune Response. We used mass spectrometry-based proteomics to quantify protein abundance and uncover specific acetylation sites altered during pathogen infection. We profiled maize plants carrying the *hm1A* mutation that encodes a defective NADPH-dependent reductase (HCTR), which is not able to inactivate HCT, rendering the plants susceptible to the fungal pathogen *C. carbonum* race 1 (38). Plants were treated with (*i*) mock HCT solution, (*ii*) exogenous HCT, (*iii*) an HCT-deficient (Tox^-) strain of *C. carbonum* (resistant interaction), and (*iv*) an HCT-producing (Tox^+) strain of *C. carbonum* (susceptible interaction) (Fig. 1*A*). To capture early signaling events, we collected tissue at 22 h, which is before visual symptoms develop during a susceptible infection with the Tox^+ strain. These treatments were chosen to globally map protein acetylation sites in maize leaves and to measure changes induced by *C. carbonum* or HCT. Total protein was extracted from the samples, and tryptic peptides were labeled with iTRAQ (SCIEX) reagents to quantify protein abundance (39). In parallel, we enriched for acetylated peptides with a pan–acetyl-lysine antibody and then quantified the immunopurified peptides using spectral counting (14, 40, 41). We compared the levels of 3,636 nonenriched proteins and 2,791 acetylation sites originating from 912 acetylated proteins (Fig. 1*B* and Datasets S1 and S2). The 2,791 sites are more than double the number of acetylation sites previously reported in plant acetylation studies, and therefore significantly expand our knowledge of plant acetylomes (16–21, 27–29).

Tryptophan Biosynthetic Proteins and Bx Phytoalexin Levels Are Induced by HCT. To uncover specific biological processes that may be targeted by HCT to promote *C. carbonum* virulence, we examined our protein abundance data (Dataset S1). Globally, we determined that 171 and 116 proteins increased and decreased, respectively, in abundance following treatment with HCT or infection by *C. carbonum* strains (Fig. 2*A* and Dataset S1). Using these data, we identified gene ontology (GO) enrichment for a number of biosynthetic processes that are specific to HCT and Tox^+ treatments (Fig. S1). We were intrigued by enrichment of GO terms related to indole/tryptophan biosynthesis in HCT- and Tox^+-treated plants and examined these genes further. This analysis revealed that proteins required for every step in the biosynthesis of indole and tryptophan from chorismate were increased in HCT and/or Tox^+ treatments (Fig. 2*B*). Indole is the precursor for the class of phytoalexins known as Bx phytoalexins (42). To determine whether the increased enzyme levels caused accumulation of Bx, we measured total Bx 44 h after treatment (Fig. S2) and found that both HCT and Tox^+ treatments increased the levels of Bx. While further research is necessary to verify that HCT transcriptionally activates expression of indole/tryptophan genes, these findings suggest that *C. carbonum* alters indole/tryptophan biosynthesis to enable infection.

Pathogen Infection Alters Histone and Nonhistone Protein Acetylation. To determine if HCT targets proteins in addition to histone H3 and H4, we quantified acetylation levels after infection with



B

	Collected Spectra	Identified Spectra	Distinct Peptides	AcK Spectrum	AcK Peptides	AcK Sites	Proteins
Non-Modified Proteome (FDR)	4,499,409	353,491 (0.02%)	15,145 (0.09%)	NA	NA	NA	3,636
Acetylome (FDR)	15,636,094	441,336 (0.01%)	13,258 (0.08%)	69,277 (0.03%)	2,292 (0.1%)	2,791	912

Fig. 1. Overview of treatments and proteome profiling. (*A*) Susceptible *hm1A* plants were exposed to a mock HCT solution or 100 μM HCT, HCT-deficient (Tox^-), or HCT-producing (Tox^+) strain of *C. carbonum* race 1. For each condition, four biological replicates were collected 22 h posttreatment to quantify protein abundance (iTRAQ) and acetylation levels (spectral counting). (*B*) Summary of sampled spectra, peptides, acetylated peptides, and identified proteins. Ac, acetylated; AcK, acetylated lysine; FDR, false discovery rate; NA, not applicable.

Tox^- and Tox^+ *C. carbonum* or treatment with HCT (Dataset S2). We first verified our acetylation measurements using a commercial antibody that recognizes tetra-acetylated histone H4 (H4K5/8/12/16). Using mass spectrometry, we confirmed that the histone H4 tetra-acetylation pattern quantified matched the pattern determined using Western blotting (Fig. 3 *A* and *B* and Dataset S2). While it has previously been shown that histone H3 and H4 acetylation increases following HCT treatment and by infection with Tox^+, the specific sites of acetylation were not previously identified (6, 7). Both the mass spectrometry and the independent Western blot data demonstrate that histone H4 tetra-acetylation induced by HCT or Tox^+ infection occurs on histone H4 lysine residues 5, 8, 12, and 16. In addition, we observed increased lysine acetylation on specific residues of H2A.W, H3.1, H3.3, and a linker histone-like protein following HCT or Tox^+ treatment (Dataset S2).

Globally, we found that 62 acetylated peptides (155 sites) increased following treatment, while only nine acetylated peptides (12 sites) decreased (Fig. 3 *C* and *D* and Dataset S2). Furthermore, the majority of hyperacetylation events occurred with either the HCT or Tox^+ treatment but not in response to Tox^- infection (Fig. 3 *C* and *D*). This observation is consistent with HCT promoting protein hyperacetylation by inhibiting HDACs (6).

To gain insight into how HCT-induced hyperacetylation promotes *C. carbonum* virulence, we performed GO overrepresentation analyses. First, we examined GO categories overrepresented among the acetylated peptides that were altered in abundance following HCT treatment or infection with *C. carbonum* strains (Dataset S3). Strikingly, there are 31 and eight GO categories related to transcriptional regulation overrepresented in the HCT and Tox^+ treatments, respectively (Fig. 3*E*). These hyperacetylated transcriptional regulatory proteins include gene-specific transcription factors, subunits of the general transcription factor TFIID (TAF5 and TAF6), transcriptional corepressors, chromatin remodeling enzymes, and HAT enzymes (Datasets S2 and S3). Examination of the hyperacetylated transcriptional regulatory proteins reveals numerous proteins that have homologs in *Arabidopsis* with known roles in plant immunity. For example, the hyperacetylated maize protein GRMZM2G406712 is a homolog of RNA pol II C-terminal domain phosphatase-like 3 (*At*CPL3), which is a negative regulator of immune gene expression (43). Several chromatin remodeling enzymes are also hyperacetylated. These include GRMZM2G387890, a homolog of *At*SPLAYED (SYD), which is a negative regulator of SNC1-mediated immunity and is required for defense against

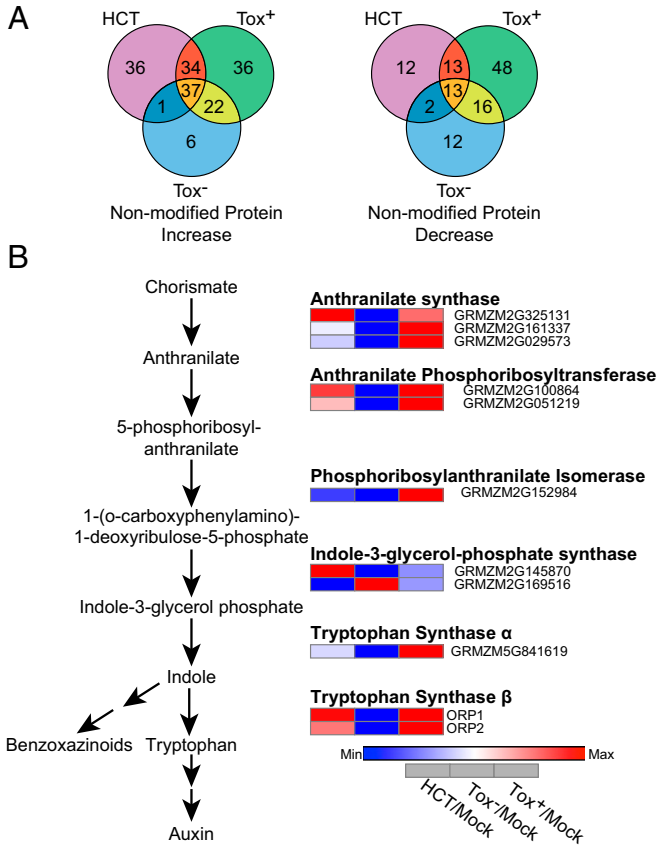


Fig. 2. HCT increases the levels of tryptophan biosynthetic proteins. (*A*) Overlap of protein abundance changes in response to treatments. (*B*) All detected proteins related to each step in tryptophan biosynthesis are shown. Heat maps represent the relative abundance of each protein following treatment by HCT, Tox$^-$, or Tox$^+$ relative to mock treatment.

Botrytis cinerea (44, 45). A homolog of SEUSS-like 2 (SLK2; GRMZM2G071491), which is a transcriptional corepressor involved in abiotic stress responses and distribution of auxin (46, 47), is hyperacetylated (HCT: $P = 0.06$, Tox$^+$: $P = 0.08$). Additionally, a homolog of the basic helix–loop–helix (bHLH) transcription factor *At*MYC2 (GRMZM2G001930) is hyperacetylated in response to HCT ($P = 0.07$) and Tox$^+$ ($P = 0.06$) treatments. In *Arabidopsis*, MYC2, along with homologs MYC3 and MYC4, regulates JA-mediated immunity and biosynthesis of tryptophan-derived indole-glucosinolates (48–51). Finally, we only observe acetylation of ramosa1 enhancer locus 2 (REL2), a transcriptional corepressor (52), following HCT treatment. In *Arabidopsis*, the REL2 homolog TOPLESS (TPL) functions in the *At*MYC2 complex (53).

There were no GO categories associated with transcriptional regulation among the acetylated peptides that respond to Tox$^-$ infection (Fig. 3*E* and Dataset S3). Furthermore, defense-related GO terms were only present among the Tox$^+$-responding acetylated peptides. Taken together, these data suggest that virulent *C. carbonum* utilizes HCT to reprogram the transcriptional response to infection, resulting in a defense response that is inappropriate for arresting *C. carbonum* infection. Another nonmutually exclusive possibility is that acetylation of defense proteins by Tox$^+$ infection results in inactivation of their function and suppresses host defense.

Acetylation Is a Global Protein Modification. To gain insight into the composition of the overall maize acetylome, we examined the functional category distribution of acetylated proteins using MapMan classification, which is an ontology system developed for plants to classify gene function (54–56). Proteins in 32 of the 35 major MapMan bins (i.e., functional categories) contained acetylated proteins (Fig. 4 and Dataset S2). The only bins that did not contain acetylated proteins were the sparsely populated bins "fermentation," "polyamine metabolism," and "microRNA, natural antisense" comprising 59, 41, and 3 proteins, respectively, out of a total of 63,542 MapMan-annotated proteins. Therefore, it is possible that sampling additional tissues and/or deeper acetylome profiling may reveal that proteins in these functional categories are also acetylated.

We found that the third largest MapMan bin of acetylated proteins is "RNA," which contains proteins annotated to be involved in RNA splicing and transcriptional regulation (Fig. 4). Specifically, we detected 108 acetylated proteins from the RNA bin, which is greater than expected by chance ($P = 2.9E^{-7}$). Furthermore, we observe acetylation of 17 GRASSIUS annotated transcription factors (57). While earlier studies have described acetylated plant transcription factors, the prevalence of this functional category is greater than previously reported in *Arabidopsis* (16). Potentially, the increased identification of transcriptional regulatory proteins is due to increased acetylome depth.

We observed acetylation of 61 of the 468 "classical" maize proteins (Dataset S2), which are a set of maize genes that have been the subject of a disproportionate share of publications recorded at MaizeGDB (58). For example, photosynthetic proteins, including light harvesting proteins (LHCB3, LHCB7, and

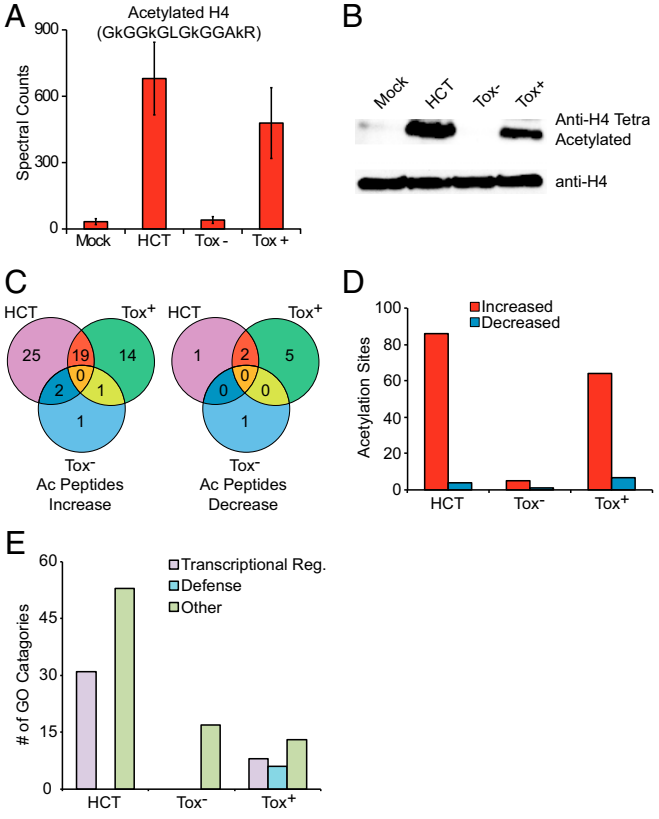


Fig. 3. Dynamics of protein abundance and acetylation. (*A*) Spectral counts of the peptide corresponding to tetra-acetylation of H4 lysines 5, 8, 12, and 16. Data are means of four independent biological replicates ± SEM. (*B*) Western blot confirmation of H4 tetra-acetylation quantified by proteomics. (*C*) Overlap of acetylated peptides that change in response to treatments. (*D*) Number of acetylation sites that are altered, relative to mock treatment, by HCT, Tox$^-$, or Tox$^+$ treatment. (*E*) Number of GO categories related to transcriptional response, defense, or any other process (Other) that are overrepresented in HCT, Tox$^-$, or Tox$^+$ treatment. Reg., regulation.

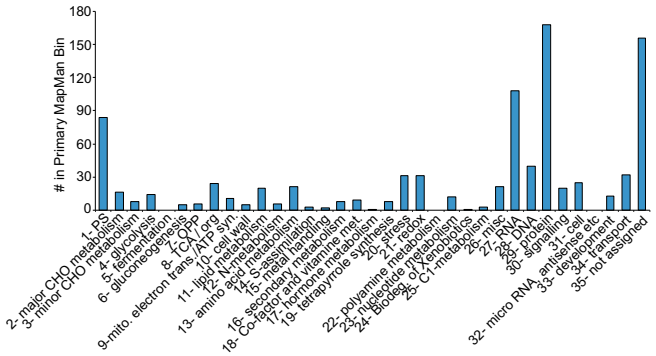


Fig. 4. Diverse maize proteins are acetylated. Proteins from 32 of 35 major MapMan bins are acetylated.

LHCB9) as well as the small and large subunits of Rubisco (RBCL and SSU3), are acetylated, which is consistent with the acetylation of homologous proteins in *Arabidopsis* (16, 17). We observed acetylation of phosphoenolpyruvate carboxylase (PEP1 and PEP4) proteins. Additionally, the oxylipin signaling proteins lipoxygenase (LOX6) and 12-oxo-phytodienoic acid reductase (OPR1) were detected as acetylated proteins (59, 60). We also identified acetylation of starch biosynthetic enzymes, including sucrose synthase1 (SUS1), UDP-glucose pyrophosphorylase1 (UGP1), ADP glucose pyrophosphorylase small subunit Leaf1 (AGPSLZM), and starch branching enzyme 3 (SBE3) (61–63). Finally, genes involved in transcriptional regulation such as REL2 are acetylated.

Discussion

In this study, we used global mass spectrometry-based acetylome profiling in maize to identify over 2,700 acetylation sites arising from 912 proteins in the maize leaf. This represents an ∼twofold increase in acetylome depth relative to previous plant acetylome reports, which have reported several hundred to ∼1,300 sites of acetylation (16–21, 27–29). The acetylated proteins we identified represent a wide range of functional categories, suggesting that this posttranslational modification can regulate diverse biological processes (Fig. 4). Notably, we discovered that many well-characterized maize proteins are acetylated, including proteins responsible for major commercial traits such as starch and oil biosynthesis. These descriptions of acetylation sites enable new approaches to study the potential regulation of well-characterized proteins and agronomically important traits.

In addition to the well-characterized alteration in histone acetylation levels that occurs during an immune response (6, 7, 9–13, 64), pathogen infection has been shown to induce nonhistone protein acetylation and to alter host immunity (12, 30–33, 65). However, all of the induced nonhistone protein acetylation events previously identified are a result of pathogen effector molecules functioning as acetyltransferase enzymes that act on host proteins. Here, through the application of the exogenous HDACi HCT and *C. carbonum* strains, we demonstrate that the activity of plant-encoded enzymes (i.e., HDACs) can be modulated to alter both histone and nonhistone protein hyperacetylation in response to pathogen infection.

We observed that 52% of the proteins hyperacetylated in response to HCT or Tox$^+$ are associated with transcriptional regulation (Dataset S2). These transcriptional regulatory proteins are not limited to histones and include gene-specific transcription factors, subunits of the general transcription factor TFIID (TAF5 and TAF6), chromatin remodeling enzymes, and HAT enzymes. Of particular interest are homologs of *Arabidopsis* MYC2, CPL3, SYD, and SLK2 proteins, which play key roles in *Arabidopsis* stress responses (43–48, 50, 66). The role of transcription factors in plant defense is well documented, and the importance of both chromatin remodeling and modifying enzymes is now recognized (9, 44, 67). Furthermore, acetylation of these types of transcriptional regulatory proteins is able to both reduce and enhance function, depending on the protein and/or specific site of acetylation (68–71). This suggests that the observed HCT-induced hyperacetylation will result in alteration of the transcriptional response during pathogen infection, thereby promoting pathogen virulence either through induction of an inappropriate immune response or by induction of a suppressor(s) of defense (2–4).

Consistent with this notion, suppressing resistance by eliciting an inappropriate defense response is a common mechanism employed by pathogens (1–4). *Cochliobolus* species utilize a range of toxins to promote pathogenicity. For example, *C. victoriae* secretes victorin as its primary effector, which induces susceptibility of oats by activating resistance to the rust fungus, *P. coronata* (1). Furthermore, in *Arabidopsis*, victorin elicits an inappropriate defense response, which enables *C. victoriae* virulence (5). In *Arabidopsis*, the bacterial effector coronatine mimics JA-Ile and thereby suppresses SA-mediated defense against *P. syringae* (2–4).

By measuring protein abundance levels, we determined that treatment with either HCT or Tox$^+$ *C. carbonum* results in an increase of metabolism-related proteins. We are particularly intrigued by the observed increase in proteins in the indole/tryptophan biosynthesis pathway, which may promote susceptibility through several mechanisms. For example, up-regulation of the indole/tryptophan biosynthesis pathway may result in increased auxin biosynthesis (Fig. 2), thereby altering plant resistance. Auxin is known to promote either susceptibility or resistance, depending on the pathogen and host (72–76). In maize, *Ustilago maydis* infection induces auxin biosynthesis and auxin-responsive gene expression, which are hypothesized to promote susceptibility (77, 78).

Finally, in line with the increased accumulation of indole/tryptophan biosynthetic enzymes, we observed an increase in the level of indole-derived Bx phytoalexins, which are a class of defensive secondary metabolites found in grass species (42, 79–81), following treatment with HCT or Tox$^+$ *C. carbonum* (Fig. S1). Intriguingly, we also observed that a maize homolog (GRMZM2G001930) of the *Arabidopsis* bHLH transcription factors *At*MYC2, *At*MYC3, and *At*MYC4 is hyperacetylated. In *Arabidopsis*, transcript levels of tryptophan biosynthesis genes are induced in a dominant gain-of-function *myc3*(*atr2D*) mutant (82). Additionally, *At*MYC2 directly binds to the promoters and is required, along with MYC3 and MYC4, for the expression of genes responsible for production of the class of tryptophan-derived defensive secondary metabolites termed indole glucosinolates (48). Thus, the hyperacetylated *Zm*MYC2 protein may function to directly activate indole and Bx production. In this scenario, Bx would represent inappropriate defensive metabolites to *C. carbonum* and their induction would prevent an effective defense response. Future work will need to address whether auxin and/or Bx directly functions in promoting *C. carbonum* virulence.

Materials and Methods

Plant Material. *Zea mays* plants in which the *hm1A* (83) allele was introgressed into the B73 inbred were used for all experiments. Plants were grown in a growth chamber in a 16-h light/8-h dark photoperiod at temperatures of 28 °C (day) and 24 °C (night). Leaves 2, 3, and 4 of 15-d-old plants were sprayed midday until runoff with mock HCT solution (0.1% Tween-20), 100 μM HCT (Sigma), or 400,000 spores per milliliter of an HCT-deficient (Tox$^-$) or HCT-producing (Tox$^+$) strain of *C. carbonum* race 1 (Guri Johal, Purdue University, West Lafayette, IN). Following treatment, plants were bagged to increase humidity and placed back in the growth chamber for either 22 h for proteomics or 44 h for Bx analysis, at which point tissue was collected and flash-frozen. Four independent biological replicates were used for each treatment. Each biological replicate is composed of leaf tissue pooled from multiple plants.

Proteomics. Peptide preparation and protein abundance profiling by mass spectrometry were based on previously described methods (41, 63, 84, 85). Quantification of total nonenriched protein abundance was conducted by labeling peptides before acetyl enrichment with iTRAQ reagent, which labels primary amines and lysine side chains (39, 86). For acetylated peptide enrichment, 2 mg of anti–acetyl-lysine antibody immobilized on agarose beads (ImmuneChem-ICP0388) was added to ∼10 mg of maize peptides, which were not iTRAQ-labeled, in 50 mM Tris·HCl pH 7.4. Samples for each biological replicate series (e.g., Mock_Rep1, HCT_Rep1, Tox^{-}_Rep1, Tox^{+}_Rep1) were processed in parallel. The antibody-peptide mixture was incubated for 1 h with rotation at 4 °C on a 0.2 μM centrifugal device (Microsep MCPM02C68). Following incubation, the beads were washed three times with 50 mM Tris·HCl pH 7.4, and acetyl peptides were then eluted using 1.5 mL of 0.1% trifluoracetic acid. The antibody-conjugated beads were washed twice with 50 mM Tris·HCl pH 7.4 and then used for a second round of immunoprecipitation of the same sample (i.e., the original flow-through). The two enrichments for a given sample were then pooled and desalted using a Sep-Pak C18 column (Waters WAT054960) before liquid chromatography-tandem mass spectrometry. Acetylome quantification was conducted using spectral counting of the acetyl-enriched peptides (40). Full proteomic methods are detailed in *SI Materials and Methods*.

Western Blotting. Proteins for Western blot analysis were extracted using trichloroacetic acid/acetone from 250 mg of the same maize leaf tissue used for proteomics. Extracted proteins were quantified using a Pierce BCA Protein Assay Kit with bovine serum albumin as a standard. Fifteen micrograms of protein was loaded per sample onto a NuPAGE Novex 4–12% Bis-Tris protein gel (Thermo Fisher Scientific). Proteins were transferred to a nitrocellulose membrane using a Bolt Western transfer kit (Thermo Fisher Scientific), blocked at room temperature (RT) in 5% nonfat milk for 2 h, and then incubated overnight at 4 °C with commercial antibodies specific for H4Kac5/8/12/16 (06-866; Millipore) or nonmodified H4 (05-858; Millipore) at 1:1,000 and 1:2,000 dilutions, respectively. After washes with Tris-buffered saline containing 0.1% Tween-20, a 1:3,000 dilution of secondary goat anti-rabbit HRP-conjugated antibody was added, and after 1 h at RT, the immunoreactive bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) imaged using a ChemiDoc XRS System with ImageLab software (Bio-Rad).

Functional Annotations and Enrichment. The MapMan functional annotation file "Zm_B73_5b_FGS_cds_2012 download" was downloaded from mapman.gabipd.org (54). MapMan bin enrichment was determined with a hypergeometric test carried out in R using the dhyper command. GO term enrichment was carried out using VirtualPlant 1.3 (virtualplant.bio.nyu.edu/cgi-bin/vpweb/virtualplant.cgi) (87). Enrichment was calculated using the Fisher exact test with false discovery rate correction. GO terms with a *P* value less than 0.05 were considered enriched.

Bx Measurement. Total Bx was measured as previously described (79).

ACKNOWLEDGMENTS. We thank Dior Kelley for critical and thoughtful comments regarding this manuscript and Gurmukh Johal for seeds of B73 with introgressed Hm1A. This work was supported by National Science Foundation Grant 1546899 (to S.P.B.), a new faculty start-up grant (to J.W.W.) from Iowa State University, NIH National Research Service Award Grant F32GM096707 (to J.W.W.), and The San Diego Center for Systems Biology (J.W.W./S.P.B.).

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