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Quantitative Site-Specific Phosphoproteomics of *Trichoderma reesei* Signaling Pathways upon Induction of Hydrolytic Enzyme Production

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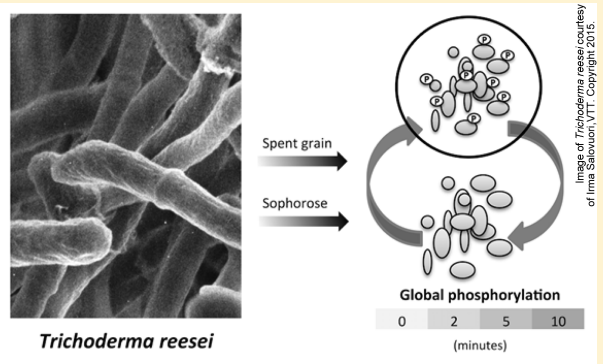
Supporting Information

ABSTRACT: The filamentous fungus *Trichoderma reesei* is used for industrial production of secreted enzymes including carbohydrate active enzymes, such as cellulases and hemicellulases. The production of many of these enzymes by *T. reesei* is influenced by the carbon source it grows on, where the regulation system controlling hydrolase genes involves various signaling pathways. *T. reesei* was cultivated in the presence of sorbitol, a carbon source that does not induce the production of cellulases and hemicellulases, and then exposed to either sophorose or spent-grain extract, which are efficient inducers of the enzyme production. Specific changes at phosphorylation sites were investigated in relation to the production of cellulases and hemicellulases using an MS-based framework. Proteome-wide phosphorylation following carbon source exchange was investigated in the early stages of induction: 0, 2, 5, and 10 min. The workflow involved sequential trypsin digestion, TiO_2 enrichment, and MS analysis using a Q Exactive mass spectrometer. We report on the identification and quantitation of 1721 phosphorylation sites. Investigation of the data revealed a complex signaling network activated upon induction involving components related to light-mediated cellulase induction, osmoregulation, and carbon sensing. Changes in protein phosphorylation were detected in the glycolytic pathway, suggesting an inhibition of glucose catabolism at 10 min after the addition of sophorose and as early as 2 min after the addition of spent-grain extract. Differential phosphorylation of factors related to carbon storage, intracellular trafficking, cytoskeleton, and cellulase gene regulation were also observed.



KEYWORDS: *phosphoproteomics, Trichoderma reesei, mass spectrometry, MS/MS, post-translational modification, phosphoenrichment, TiO_2, cellulase, hemicellulase*

INTRODUCTION

Global need to reduce greenhouse gas emissions and concern for future sustainability has raised interest in biomass-based processes to replace petrochemical processes. Plant biomass offers the most abundant renewable energy source consisting mostly of cellulose, hemicellulose, and lignin. The hydrolytic enzymes produced by fungi of different species play an important role in degradation of biomass to be used in production of bioethanol or other chemicals.[1]

Trichoderma reesei (an anamorph of *Hypocrea jecorina*) is an extremely efficient producer of cellulose- and hemicellulose-degrading enzymes and is widely used as a production host for enzymes in biorefinery and other industrial applications. The large number of carbohydrate active enzymes produced by *T. reesei*[2,3] form a complex system that is regulated by a variety of environmental and physiological factors. The most important determinant for enzyme production is the type of carbon source available for the fungus. Many of the cellulase and hemicellulase genes are repressed by the presence of the easily metabolized carbon sources, such as glucose, and induced by materials derived from the plant cell wall or by specific saccharides. In addition, production of enzymes is regulated by light conditions, growth rate, and secretion stress.[2,4−9] Taking into account the large number of hydrolytic enzymes produced and the multitude of factors affecting their production, a complex signaling cascade and regulatory network is expected.[10]

Despite the fact that carbon-source-dependent regulation mechanisms have been studied in detail over decades, relatively little is known about the molecular mechanisms of sensing different carbon sources and transduction of the signal to induce cellulolytic and hemicellulolytic genes. The studies on signaling

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mechanisms in *T. reesei* have been, to a great extent, focused on signaling in response to light conditions. The major components and the role in nutritional signaling of the light response pathway (BLR1, BLR2, and ENV1) have been identified and studied.[9,11] The light regulation system controlling hydrolase genes has been shown to involve the G proteins and cAMP signaling pathway (cAMP-dependent protein kinase A, PKAC1; adenylate cyclase, ACY1).[12] Furthermore, components of the Ras signaling and mitogen-activated protein kinase (MAPK) pathways have been indicated in regulation of cellulase production.[12−14] On the basis of studies carried out on *T. reesei* grown on a cellulose-containing medium, TrRas2 has been suggested to sense and mediate extracellular signals, via unidentified components, to modulate expression of transcriptional regulators of cellulase genes.[13] Inactivation studies have indicated the MAP kinase TMK3 to have a positive acting role in cellulase production,[14] while TMK2 has a repressing role.[15]

Proteomic methods have been applied to analyze and quantify the proteins secreted by *Trichoderma* under different conditions. The studies have greatly increased the understanding of the vast repertoire of enzyme activities produced in the presence of different substrates, and given an indication of the activities required for utilization, especially in *T. reesei*[16−18] and in *T. harzianum*.[19,20] Total cellular proteomics analysis has also been applied to study the cellular responses to protein production[6] and specifically to the different carbon source substrates.[21] Post-translational modifications, especially phosphorylation, are key determinants in many fundamental cellular functions and are vital in understanding signal transduction in a biological system. Global phosphoproteome profiling has only recently been applied to filamentous fungi;[22] however, no phosphoproteome profiling has yet been performed to analyze cellulase induction in *T. reesei*. In this study, site-specific quantitative phosphoproteomics was used to study signaling and regulation mechanisms of *T. reesei* exposed to either sophorose (2-*O*-*β*-glycopyranosyl D-glucose) or distiller's spent grain.[5] Sophorose is a potent inducer of cellulase and hemicellulase gene expression,[23] and distiller's spent grain is a complex-inducing material. Relatively little is known about the molecular mechanisms of how the fungal cells respond to the presence of different carbon sources inducing cellulase production and how the signal is mediated to induce gene expression. To give further insight into these cellular responses, the overall goal of this study was to identify important phosphorylation sites indicative of regulatory roles in carbon sensing and the induction process.

MATERIALS AND METHODS

Strains and Cultivation Conditions

Trichoderma reesei QM9414 (ATCC 26921, VTT-D-74075) was obtained from VTT Culture Collection. Spore suspensions were prepared by cultivating the fungus on potato-dextrose plates (Difco) for 5 days, after which the spores were harvested, suspended in a buffer containing 0.8% NaCl, 0.025% Tween20 and 20% glycerol, filtered through cotton, and stored at −80 °C.

For the induction experiments, *T. reesei* was first cultivated on minimal medium ($(NH_4)_2SO_4$ 7.6 g L^{-1}, KH_2PO_4 15.0 g L^{-1}, 2.4 mM $MgSO_4 \cdot 7H_2O$, 4.1 mM $CaCl_2 \cdot H_2O$, $CoCl_2$ 3.7 mg L^{-1}, $FeSO_4 \cdot 7H_2O$ 5 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 1.4 mg L^{-1}, $MnSO_4 \cdot 7H_2O$ 1.6 mg L^{-1}, pH adjusted to 4.8 with KOH) supplemented with 2% (w/v) of sorbitol as a carbon source. Precultures for the experiment were started by inoculating the medium with 8×10^7 spores per 200 mL aliquots of the medium and cultivated in shake flasks at 28 °C, with shaking at 250 rpm, until biomass dry weight in the cultures was close to 1.1 g/L (4 days). The preculture aliquots were first mixed together and then divided in 45 mL aliquots in shake flasks to get equal starting material for the inducing conditions. The cultures were let to recover for 30 min at 24 °C with shaking at 250 rpm before the addition of the inducing substrates. Either *α*-sophorose (Serva 35208) or spent-grain extract was added to the cultures to induce cellulase and hemicellulase production. Sophorose was added to a final concentration of 0.72 mM, and spent-grain extract was added to a final concentration of 2% (w/v). The stock solution for spent-grain extract was prepared as follows: 200 g/L of spent grain was suspended to double-distilled deionized water, autoclaved, settled at room temperature overnight, filtered four times through Whatmann 3MM filter paper, and reautoclaved. Five mL of the spent-grain stock solution was added to the 45 mL cultures. For uninduced control cultures, the same amount of water (1/10 of the culture volume) was added. All induction experiments were carried out in triplicate. Mycelial samples for phosphoproteome analysis were collected prior to the addition of inducer (0 min time point) and 2, 5, and 10 min after the addition of sophorose, spent-grain extract, or water. The samples were collected by filtering through Whatmann GF/B and frozen immediately in liquid nitrogen.

Samples for determining biomass dry weight were withdrawn separately from the precultures and uninduced control cultures during induction. Biomass dry weight was determined by drying the mycelium samples, collected as previously described, to constant weight. pH of the cultures was measured throughout the cultivation.

Phosphoroteomic Sample Preparation

Cell extracts for the proteome analysis were prepared as follows: Samples of mycelium were ground under liquid nitrogen using a mortar and pestle, resuspended in 20 mM *N*-ethylmaleimide (NEM), 10 mM NaN_3, 10% trichloroacetic acid (TCA) supplemented with Halt Protease, and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific), and kept on ice for 30 min. Two volumes of cold acetone (−20 °C) was added, and after 30 min of incubation on ice the samples were centrifuged 14 000*g* at 4 °C for 15 min. The pellets were washed with −20 °C acetone, air-dried, and resuspended in the sample buffer (7.5 M urea, 2 M thiourea, 15 mM Tris base), and the pH was adjusted to 8 by the addition of NaOH. RapiGest SF (Waters) was added to a concentration of 0.1% (w/v). Insoluble material was removed by centrifugation. Triplicate biological replicates were prepared. Total protein was measured using 2D Quant kit (GE Healthcare).

Ten micrograms of *α*-casein (Sigma) was added to 1 mg of the *T. reesei* lysates. The samples were diluted to 200 *μ*L with 8 M urea in 50 mM Tris-HCl (pH8.5), reduced with 10 mM dithiothreitol (DTT) at 37 °C for 1 h, and alkylated with 50 mM iodoacetamide in the dark for 30 min. Alkylation was stopped by the addition of 50 mM DTT. The samples were digested with 20 *μ*g of modified trypsin (Promega) in a total volume of 920 *μ*L of 2 M urea, 50 mM Tris-HCl (pH8.5) at 37 °C for 18 h. Tryptic digests were slightly acidified to pH 1 to 2 with 80 *μ*L of 10% trifluoroacetic acid (TFA) and incubated at RT for 1 h, followed by centrifugation at 14 000 rpm for 15 min to remove RapiGest. Supernatant was desalted with an Empore C18-SD 10 mm/6 mL cartridge (3M), and eluted with 1 mL of 6% TFA, 80% acetonitrile (ACN). Phosphopeptides were enriched on 5 mg of TiO_2 (Sachtopore-NP, 20 *μ*m, 300 Å; ZirChrom) placed in

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a 200 μL pipet tip and eluted with 200ul of 5% NH_4OH.[24] The enriched phosphopeptides were desalted immediately with a Empore C18 column prepared in house, eluted manually with 50 μL of 0.1% formic acid (FA), 80% ACN, and evaporated to dryness in a SpeedVac. The dried peptides were reconstituted with 0.5% FA in 2% ACN.

LC–MS/MS Analysis

Each sample was analyzed using a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The Q Exactive was coupled to an EASY-nLC 1000 nanoflow LC instrument (Thermo Fisher Scientific). A 100 μm × 2 cm trap column and a 75 μm × 15 cm analytical column were in-house packed with Magic C18AQ resin (5 μm, 200 Å pore size; Michrom Bioresources). After injecting ∼45% (v/v) of the sample onto the column, phosphopeptides were eluted using a 3-step linear gradient at a flow rate of 300 nL/min using solvent A (0.2% FA in 2% ACN) and solvent B (0.2% FA in 95% ACN). The initial linear gradient was 2 to 20% B for 30 min, then 40% B until 40 min, and finally 100% B for 45–50 min. Data-dependent acquisition was performed in positive ion mode. MS spectra were acquired from 300 to 2000 m/z in the Orbitrap with resolution of 70 000, an AGC target value of 1×10^6 ions, and a maximal injection time of 120 ms. The 10 most abundant ions of charge states 2+ or higher were selected for subsequent fragmentation by HCD with normalized collision energy of 30. The MS/MS spectra were acquired in the Orbitrap with a resolution of 17 500, isolation window of 2.0 m/z, an AGC target value of 5×10^4 ions, maximal injection time of 250 ms, lowest mass fixed at 100 m/z, and dynamic exclusion duration set to 20 s. The lock-mass option was used (m/z 445.12003).

Phosphopeptide Identification

MS/MS data were searched using Mascot (v2.4.0; Matrix Science, London, U.K.) against the UniProt database (v2013-01, *Trichoderma*) supplemented with common contaminants and reverse sequences for FDR determination (total 46 414 sequences) via Proteome Discoverer (v1.3.0.339, Thermo Fischer Scientific). Search parameters allowed modified residues (fixed cysteine carbamidomethylation and variable methionine oxidation, phosphorylation of serine/threonine/tyrosine, and acetylation on protein N-terminus) and two missed cleavage sites. Mass tolerance was set to 5 ppm and 0.02 Da in MS and MS/MS mode, respectively. Expectation value of ≤0.05 indicated peptide sequence identifications. PhosphoRS was used to validate phosphorylation sites.

Phosphopeptide Quantification

Nonlabeled quantification was performed using Progenesis LC–MS (v4.1, Nonlinear). Here extracted peptide intensity (MS1) features were generated for the evaluation of differential peptide regulations. The best reference run was selected and an automatic alignment was performed for all data based on RP_HPLC retention times of each analysis. Detected features represent peptide ions detected. Each sample was normalized to the abundance of phosphopeptides detected from the α-casein standard. All features that had no associated MS/MS spectra were deleted. After alignment, the samples were divided into their respective time point and compared. Phosphopeptide abundance was calculated by averaging the total cumulative abundance of an individual phosphopeptide across the triplicate samples. Differentiation of phosphopeptides was determined using analysis of variance (ANOVA) across the four time points. Phosphopeptides with a p value ≤0.05 were considered significant with a power ≥0.8. In addition, the data were clustered using the R package Mfuzz[25] and hierarchical clustering was performed using the Multi-Experiment Viewer analysis tool.[26]

RESULTS

Experimental Setup

To understand how phosphorylation profiles change with time upon stimulation of *T. reesei* with either sophorose or distiller's spent grain, we applied a label-free quantitative phosphoproteomic approach. For the analysis, *T. reesei* was first cultivated on medium containing an noninducing/nonrepressing carbon source, sorbitol, and induction of cellulase and hemicellulase production was initiated in the actively growing cultures by the addition of either sophorose or distiller's spent grain. Samples collected at early time points during induction (0, 2, 5, and 10 min after addition of the substrate) were subjected to the analysis to identify phosphorylation events relevant in sensing of the inducing carbon source and the onset of the induction process.

Global Phosphoproteomic Analysis

As a result of the global phosphoproteome analysis, a total of 9225 peptide features matching to MS/MS identifications were detected, and 2594 of the features were mapped to phosphorylated peptides. The median CV of phosphopeptide features after normalization was 0.4. A total of 1721 unique phosphorylation sites were identified from the phosphopeptide features (FDR = 0.22%). Singly, doubly, and higher phosphorylated peptides represented 87, 12, and 1% of the total phosphopeptides, respectively (Figure 1A). The distribution of pS, pT, and pY sites was 82.9, 16.6, and 0.5%, respectively (Figure 1B), in agreement with data on protein phosphorylation in the yeast *Saccharomyces cerevisiae*.[27] 20% of phosphorylation occurred within 1/10 of the amino acid sequence length from the N-terminus of the proteins (Figure 1C). Altogether, the phosphoproteome data matched 823 *T. reesei* proteins in the Uniprot database. Details of the quantitative results of phosphopeptides are presented in Supporting Information Table S-1. The majority of protein identifications from the UniProt database mapped to predicted proteins, encompassing over 50% of all identifications. The annotation information was supplemented by the data provided by JGI (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Furthermore, the PANNZER tool[28] (freely available at http://ekhidna.biocenter.helsinki.fi/pannzer) was used for more concise functional prediction of the proteins. Details of the PANNZER, KOG, JGI, and Interpro annotation of the proteins are presented in Supporting Information Table S-2. Functional classification of the phosphoprotein identifications is presented in Figure 1D.

Phosphoproteome Dynamics in Cultures Treated with Sophorose or Distiller's Spent Grain

Phosphoproteome analysis was carried out during a time course of induction of cellulose and hemicellulose production. To measure the overall dynamic phosphorylation, we further investigated patterns in the time profiles of the regulated phosphopeptides. Significantly regulated phosphopeptides for each time point were plotted (Figure 2; see also Supporting Information Tables S3 and S4). The highest amount of regulated phosphopeptides during the induction experiment was detected after 10 min of treatment with sophorose (366 upregulated phosphopeptides as compared with the uninduced control cultures). In cultures treated with distiller's spent grain, the highest amount of upregulated phosphopeptides as compared with the control

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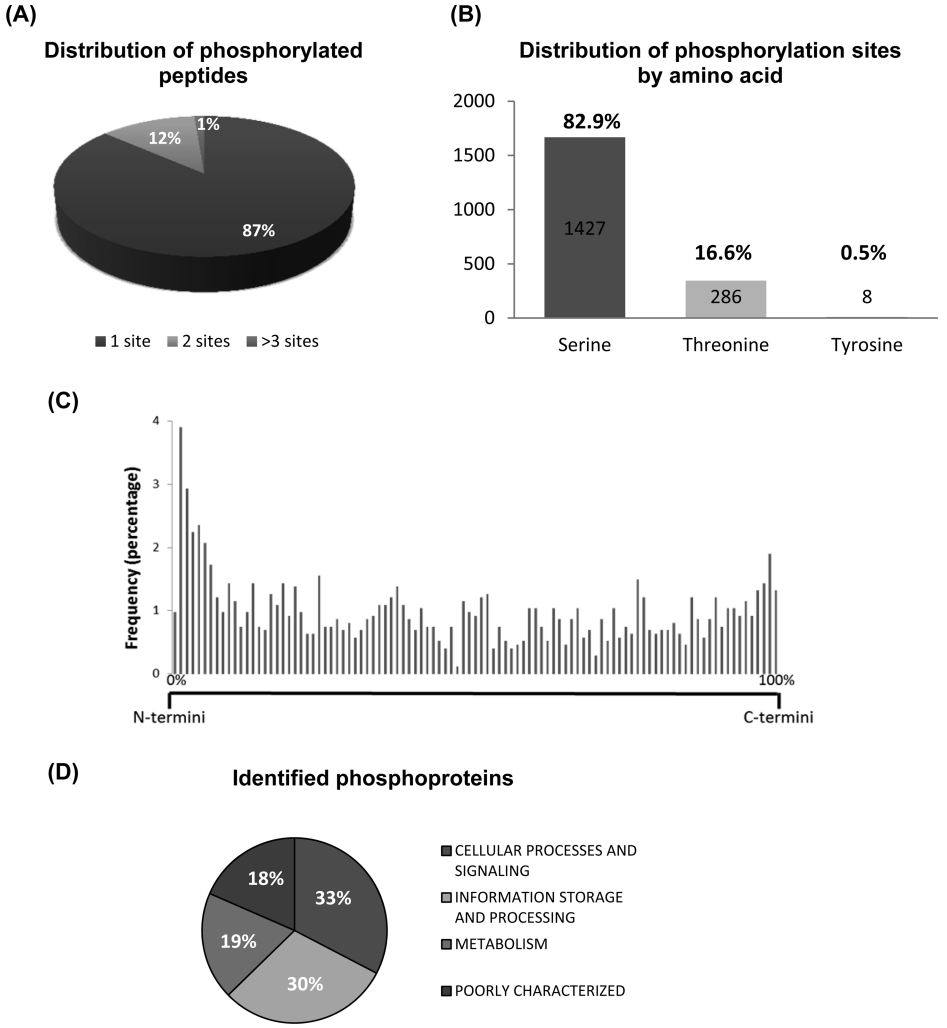

Figure 1. Results of large-scale phosphopeptide analysis. (A) Distribution of peptides with single and multiple phosphorylation. (B) Distribution of phosphorylation at S, T, and Y residues in the phosphoproteome of *T. reesei*. (C) Distribution of phosphorylated sites along the protein length in the phosphoproteome of *T. reesei*. (D) Distribution of the identified phosphoproteins in four functional classes according to KOG classification (http://genome.jgi-psf.org/).

cultures was detected at 2 and 5 min of exposure time (246 and 263 phosphopeptides, respectively). In the induction experiment with sophorose, only 4% of the differentially phosphorylated peptides were present at a lower level in the sophorose treated cultures as compared with the control cultures with none of the same phosphopeptides found in other time points. In the comparison of the spent-grain and control cultures, 18% represented significant downregulated phosphopeptides in distiller's spent-grain samples with 13 phosphopeptides represented in samples from 2 to 10 min.

All identified phosphopeptides with at least one significant difference in comparison with the control at any time point were analyzed by fuzzy c-means clustering to show trends in regulation of phosphorylation (Figure 3). At the first time point prior to introduction of any inducer there should be no expression difference. Four general trends were considered from the following clusters: 1, 2, 3, 6, and 8. We grouped our phosphorylation profiles with reference to fold-change difference in comparison with control: gradual increase in phosphorylation after 2 min (Figure 3A), gradual decrease in phosphorylation after 2 min (Figure 3B), a drop in phosphorylation at 5 min (Figure 3C), and peak in phosphorylation at 5 min (Figure 3D).

Cellulase and hemicellulase induction with sophorose designated 35 upregulated phosphopeptides that gradually increased with time, 21 decreased with time, 35 spiked at 5 min, and 97 phosphopeptides dropped at 5 min (see Supporting Information Figure S1 for protein names and phosphorylation sites). Furthermore, there were 16 proteins that displayed phosphorylations regulated in a contrasting manner (Supporting Information Table S-5); however, none of the phosphopeptides had a significant change throughout the time course of the sophorose experiment as compared with the control (from 2 to 10 min). The majority of the 366 phosphopeptides were significantly upregulated only after 10 min of induction (Figure 2).

Treatment with distiller's spent grain designated 37 upregulated phosphopeptides that increased with time, 106 decreased with time, 134 spiked at 5 min, and 54 phosphopeptides dropped at 5 min (see Supporting Information Figure S2 for protein names and phosphorylation sites). There were 34 proteins that displayed phosphorylation sites regulated in contrasting behavior (Supporting Information Table S-6). Treatment with distiller's spent grain indicated that 106 phosphopeptides were significantly regulated to control from 2 to 10 min (Supporting Information Table S-7).

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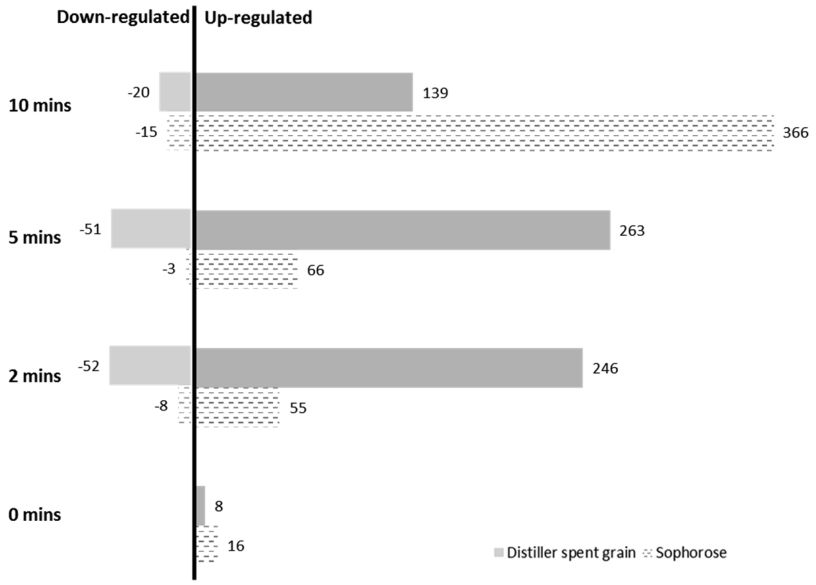

Figure 2. Significantly regulated phosphopeptides for each time point in comparison with control upon treatment with sophorose or distiller's spent grain. Statistical analysis calculated using ANOVA with multiple comparisons. $P \leq 0.05$ with a power ≥ 0.8.

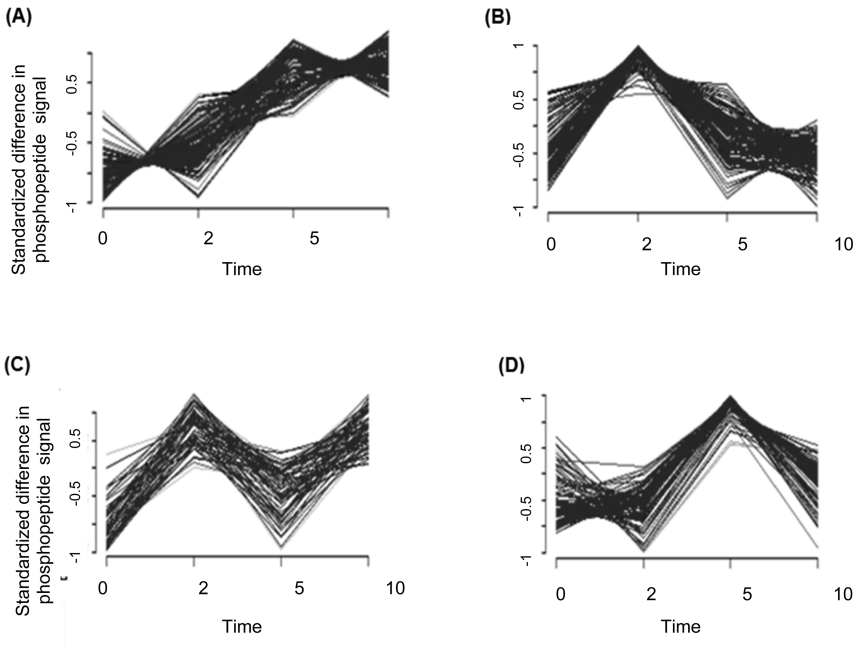

Figure 3. Four time-course clusters found using Mfuzz *c*-means clustering of the time-series data in all phosphopeptides. (A) Gradual increase in phosphorylation. (B) Gradual decrease in phosphorylation. (C) 5 min decrease in phosphorylation. (D) 5 min in spike phosphorylation.

Phosphoproteome Functional Pathway Analysis

Functional classification (eukaryotic orthologous groups) of the proteins with significant differential phosphorylation upon treatment with sophorose or distiller's spent grain is represented in Figure 4. The significantly phosphorylated proteins in the induction experiments were mostly related to signaling and transcription mechanisms. In addition, proteins related to cellular structure, organization, and trafficking as well as proteins related to carbon, lipid, and amino acid metabolism were abundant among the ones with altered phosphorylation pattern in both the sophorose- and spent-grain-induced cultures. The sophorose-induced cultures showed a larger number (2−5 fold higher number of proteins in the class) of significantly phosphorylated proteins with functions related to protein translation machinery, cell cycle, nucleotide metabolism, and DNA replication, recombination, and repair, as compared with the cultures induced with distiller's spent grain, which may reflect a more pronounced effect of sophorose addition on the growth of the fungus. Manually curated annotations could be retrieved from JGI (http://genome.jgi-psf.org/Trire2/Trire2.home.html) for 33% of the significantly phosphorylated peptides. The signal transduction pathway components that were significantly phosphorylated in both the sophorose and distiller's spent-grain cultures included the MAP kinase TMK3 (JGI ID 45018), MAP kinase kinase 1 (JGI ID 21168), a hypothetical cAMP-dependent protein kinase (JGI ID 119614), a candidate conidiation-related kinase (JGI ID 80758), and a hypothetical

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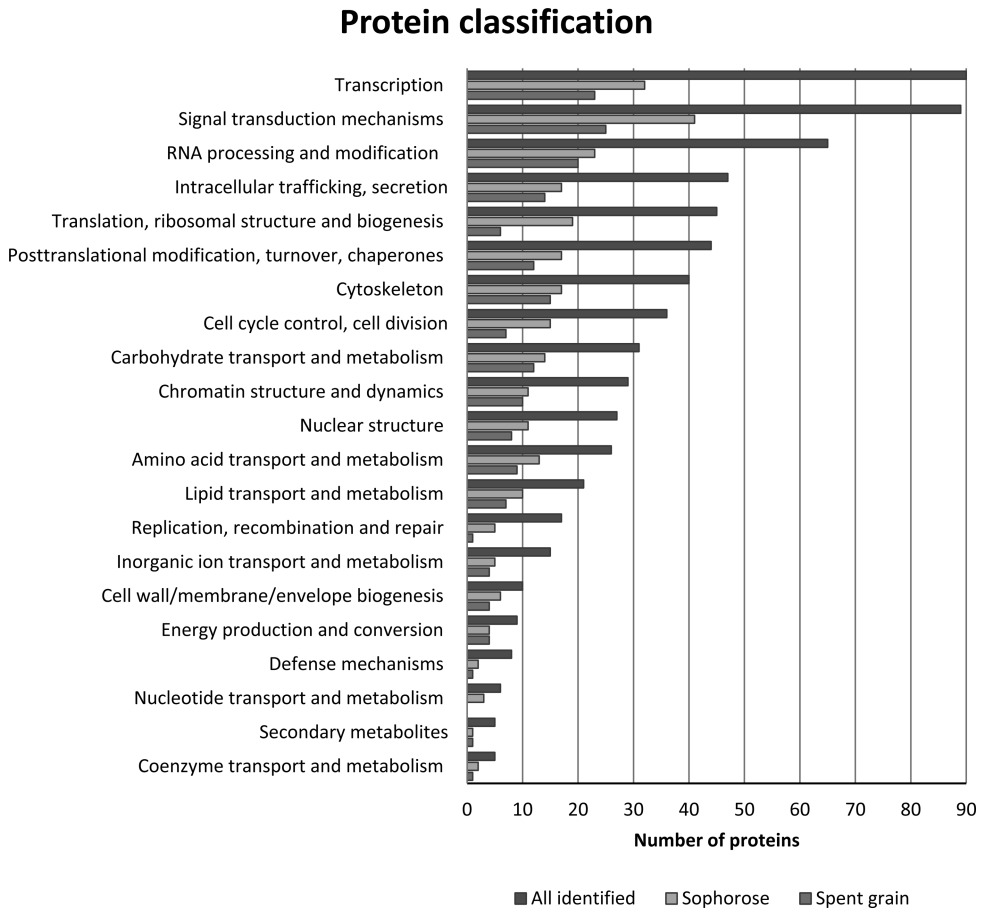

Figure 4. KOG-based classification of the differential phosphorylated proteins in cultures treated with sophorose or distiller's spent grain compared with the control cultures.

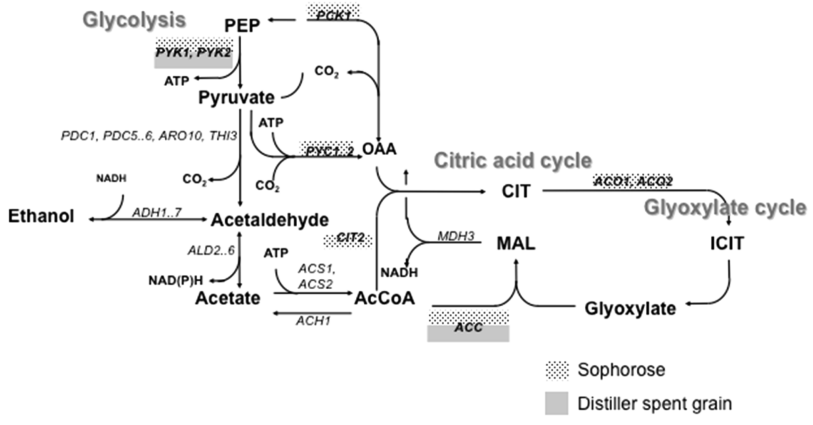

Figure 5. Pathway for glucose catabolism. The differentially phosphorylated proteins in cultures treated with sophorose or distiller's spent grain are indicated by shaded boxes: enzymes with increased phosphorylation at 10 min after sophorose addition and 2 to 10 min after the addition of distiller's spent grain.

RHO-family GTPase (JGI ID 4751). Multiple components of signaling cascades were significantly phosphorylated only in the cultures induced with sophorose. The adenylate cyclase (ACY1), a regulatory subunit of protein kinase A (PKAR1), and the light-sensing protein VeA were identified. ACYI and protein kinase A have previously been implicated to have a role in light-modulated regulation of cellulase production.[12] In addition two homologues of casein kinases (HHP1 ID 109876, CBK1 ID 124117), which in other organisms have been suggested to be involved in circadian clock regulation, were also significantly phosphorylated. In the presence of spent-grain extract, a histidine kinase (ID 78012), two guanine nucleotide exchange factors for Ras-like GTPases (IDs 34726 and 70548), and a hypothetical GTPase activator protein for Ras-like GTPase (ID 62426) were significantly phosphorylated. Details of the proteins are presented in Supporting Information Tables S-2, S-3, and S-4.

Carbon source sensing, uptake, and metabolism, together with the subsequent signaling events, is expected to have an impact

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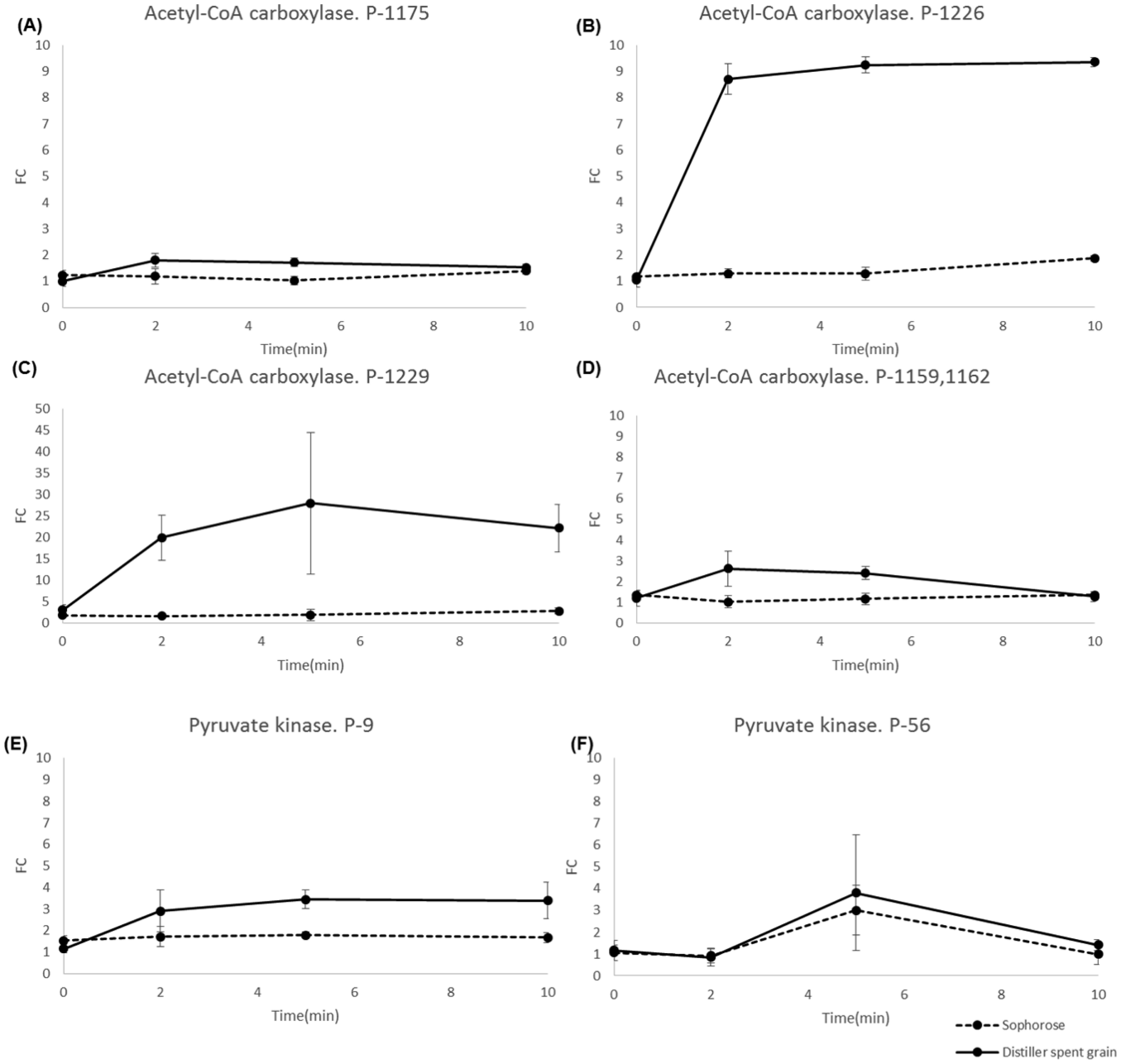

Figure 6. Phosphorylation of Acetyl-CoA carboxylase and pyruvate kinase. Fold change is shown as the average of three biological replicates for each time point of the phosphopeptide signal upon treatment with sophorose or distiller's spent-grain cultures to the signal in the control cultures. (A) Phosphorylation at amino acid 1175. (B) Phosphorylation at amino acid 1226. (C) Phosphorylation at amino acid 1229. (D) Phosphorylation at amino acid 1159 and 1162. (E) Phosphorylation at amino acid 9. (F) Phosphorylation at amino acid 56.

on the regulation of the genes encoding the carbohydrate active enzymes. Thus, it is of interest that the significantly phosphorylated proteins in the induced cultures included candidate sugar transporters (ID 50618 in both of the culture types, 47710 in sophorose cultures, and 58712 in spent-grain cultures). Furthermore, a homologue of the *S. cerevisiae* dual-specificity protein kinase YAK1 (ID 44330), most likely part of a glucose-sensing system involved in growth control in response to glucose availability, was significantly phosphorylated under both the inducing conditions. The significantly phosphorylated proteins included proteins of central carbon metabolism: ATP citrate lyase (ACL1, ID 121824), acetyl-CoA carboxylase (ID 81110), pyruvate kinase (ID 78439), pyruvate dehydrogenase subunit (ID 122987), and pyruvate carboxylase (PYC1, ID 21957) (Figure 5). Proteins related to carbon storage, transport, and signaling were also identified: glycogen synthase (gys1, ID 44529), glycogen synthase kinase 3 (gsk3, ID 74400), protein containing a starch/glycogen-binding module (ID 123502), glycogen phosphorylase 1 (gph1), candidate trehalase (ID 120676), and candidate bifunctional trehalose-6-phosphate synthases (ID 75295 and ID 48707).

Phosphopeptides associated with carbon metabolism were further investigated. Acetyl-CoA carboxylase revealed a significant increase in phosphopeptides at five different sites (1175, 1226, 1229, 1159, and 1162) upon treatment with distiller's spent grain after 2 min (Figure 6). Serine residues 1226 and 1229 of this enzyme were extensively phosphorylated with an increase of 9- and 25-fold, respectively. Phosphorylation levels upon

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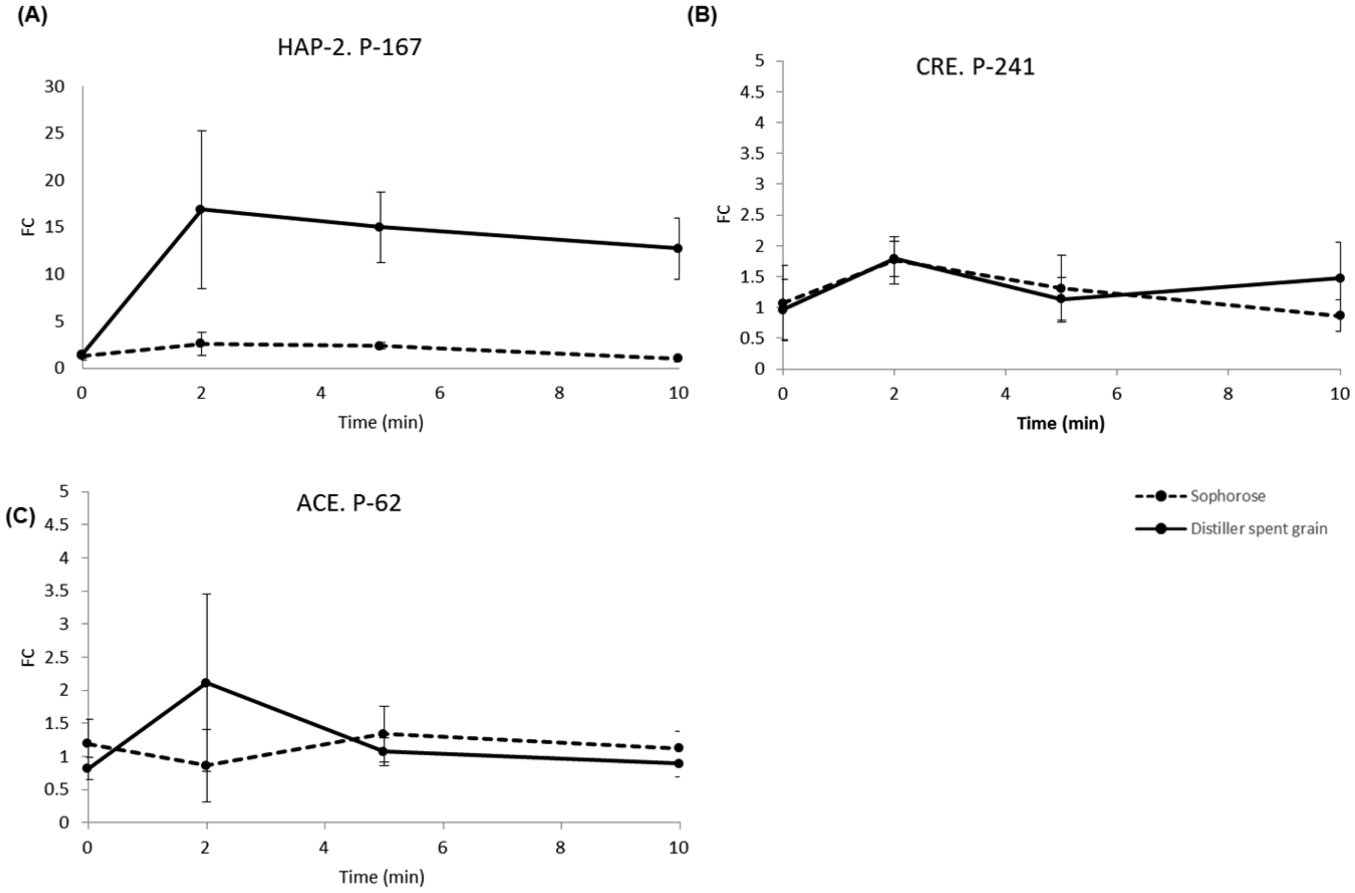

Figure 7. Phosphorylation of HAP2, CREI, and ACEI. Fold change is shown as the average of three biological replicates for each time point of the phosphopeptide signal upon treatment with sophorose or distiller's spent-grain cultures to the signal in the control cultures. (A) HAP2. Phosphorylation at amino acid 167. (B) CRE1. Phosphorylation at amino acid 241. (C) ACE1. Phosphorylation at amino acid 62.

induction with sophorose were close to the levels detected in the control cultures (Figure 6A–D). Pyruvate kinase was phosphorylated at an increasing level at serine residue 9 after 2 min and onward in spent-grain cultures. Phosphorylation of the site in the sophorose cultures was at a more constant level throughout the induction period (Figure 6E). In both culture types, the phosphorylation of serine residue 56 is transiently increased at 5 min after the addition of sophorose or spent grain (Figure 6F).

Phosphopeptides of known transcriptional regulators of cellulose gene expression[7] were profiled. Phosphorylation of the HAP2 subunit of the CAAT-binding complex, CREI, and ACEI was detected. Upon treatment with distiller's spent grain, the phosphorylation of the serine residue at position 167 of HAP2 increases 10-fold in comparison with control after 2 min (Figure 7A). Phosphorylation of CREI at position 241 fluctuates over time in both cultures (Figure 7B). The serine residue at position 62 in ACEI was phosphorylated, but there was no significant difference upon induction with sophorose or distiller spent grain (Figure 7C).

DISCUSSION

This is the first report of extensive phosphoproteomic profiling performed in *T. reesei* to study the regulation of phosphorylation in the presence of substrates inducing cellulase and hemicellulase production. The methods used enable direct profiling of phosphorylation changes in a time-dependent manner. In addition to phosphorylation by kinases, the overall level of phosphorylated proteins is affected by phosphatases, protein degradation, and translocation of protein. These events together contribute to the dynamic fluctuation of phosphorylation on proteins.

In the sophorose cultures, the phosphorylation signaling was most induced only after 10 min of addition of sophorose in the cultures. The addition of distiller's spent grain induced phosphorylation as early as 2 min after addition. Our data indicate a complex regulation pathway that continues from 2 to 10 min upon induction with distiller's spent grain. Sophorose induction is affected by various parameters, such as its concentration and rate of uptake.[29,30] Sophorose import via a permease with high K_m and low V_{max}[31,32] could explain the delayed signaling by phosphorylation seen in our sophorose-induced samples. While induction of cellulase and hemicellulase genes by sophorose has been extensively studied, the inducing components in distiller's spent grain are poorly characterized. In accordance with the complexity of the spent-grain material, our study demonstrates a complex mechanism of phosphorylation with significant differences acting in opposing trends. These results suggest a series of off and on triggers by phosphorylation of different biological mechanisms.

The large-scale phosphopeptide analysis in our study demonstrates that signaling and regulation by phosphorylation signaling in fungi can be characterized in a time-dependent manner. The major constraint to the study is the limited amount of manually curated annotations available as well as relatively scarce experimental data to support the functional predictions. From the functional predictions available, it can be concluded that the major group of peptides showing differential

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phosphorylation in the cultures in the presence of sophorose or spent grain were involved in signal transduction or other regulatory functions, such as transcription. A multitude of signaling cascades were affected by the addition of both the sophorose and spent-grain substrates.

MAP kinase pathways represent one of the most prominent signal transduction systems encompassing serine/threonine protein kinases acting in a series.[33,34] A model for MAP kinase cascades in *H. jecorina* was proposed[35] identifying nine members of MAP-kinase cascades and additional upstream components. On the basis of homologues of these members, the MAP kinases are predicted to function in (i) pheromone response and biocontrol, (ii) stress response and protein degradation, and (iii) osmosensing. MAP kinases have also been suggested to have a role in carbon source signaling. Specifically, the TMK2 and TMK3 have been shown to have an effect on cellulase production.[14,15] In our study, increased phosphorylation of members of all three proposed MAP kinase cascades[26] were detected. In the cascade indicated as a pheromone response and biocontrol, the MAPKK (ID 75872) was significantly phosphorylated in spent-grain-induced cultures and the upstream component, ID 104364, in the sophorose induced cultures. The latter is annotated as a homologue of STE20 of *Neurospora crassa*, which is also involved in regulation of cell polarity and cell cycle. In the cascade related to stress response, the MAP kinase kinase MKK1 was significantly phosphorylated in both the sophorose and spent-grain induced cultures and the upstream factor PKC1 (protein kinase C) was significantly phosphorylated in the sophorose-induced cultures. TMK3, involved in the osmosensing cascade and indicated to have a positive role in cellulase production,[14] was significantly phosphorylated in both the sophorose and spent-grain cultures.

cAMP signaling pathway is involved in light-mediated cellulase regulation.[35] Our finding of the increased phosphorylation of cAMP signaling pathway components (adenylate cyclase 1, regulatory subunit of protein kinase A) under the cellulase-inducing condition on sophorose further supports the idea that the mechanisms related to cellulase regulation in response to different stimuli share common components. Ras small GTPases are known to regulate various cellular signaling pathways: cell growth, differentiation, proliferation, and apoptosis.[36] In addition, Ras signaling of glucose availability has been shown in other organisms (e.g., *S. cerevisiae*[37]) and suggested to target MAP kinases.[33,38] In *T. reesei*, Ras signaling components, especially TrRas2, have an effect on cellulase production.[13] Our data showed increased phosphorylation of Ras-like GTPases, GTPase activators, and guanine nucleotide exchange factors for Ras-like GTPases; however, TrRas2 was not among the ones detected. Histidine kinases, also identified in the spent-grain cultures, are known to function in a two-component phosphorelay system that provides the fungus with a sophisticated means to integrate numerous environmental signals.[39]

Phosphorylation of CREI affects its repressing activity on the cellulase and hemicellulase genes under carbon catabolite repression.[40] Our data show fluctuation in phosphorylation of CREI after the addition of sophorose or spent-grain extract to the cultures of *T. reesei*, suggesting that an early response by phosphorylation of CREI may play a role in sensing the carbon source and the onset of the induction. The CAAT binding complex HAP2/3/5 interacting with the promoter of the cellulase gene *cel6a* is required for full induction of the gene.[41,42] Phosphorylation of amino acid position 167 of HAP2 was identified, which may form a novel component of the regulatory system. The CAAT binding complex HAP2/3/5 has been shown to play a role in the induction of cellulase gene expression and the binding of the complex to the promoter of the cellulase gene *cel6a* to be required for full induction of the gene.[41,42] Our data showed phosphorylation of amino acid position 167 of HAP2, which may form a novel component of the regulatory system.

Phosphorylation of pyruvate kinase and Acetyl-CoA carboxylase, two enzymes involved in glucose catabolism, upon treatment with sophorose and distiller spent grain were further investigated in comparison with control. Transcriptomic and proteomic studies have proposed that flux through early glycolysis or the TCA cycle is among the fundamental determining factors in cellulase production.[6] Phosphorylation of both of these enzymes is known to render the enzymes inactive in *H. sapiens*. In our study, Acetyl-CoA carboxylase was differentially phosphorylated at multiple sites upon treatment by distiller's spent grain. Increased phosphorylation was detected in three singly phosphorylated peptides and in one doubly phosphorylated peptide. Phosphorylation at positions 1226 and 1229 was increased over 5-fold upon the addition of spent grain, whereas phosphorylation at the other sites was approximately at the same level as in sophorose induced cultures. Phosphorylation of pyruvate kinase at serine residue at position 9 was three times more abundant in cultures with spent grain added as compared with sophorose cultures (Figure 6E). These results suggest a possible repression of glucose catabolism signaled by increased phosphorylation upon treatment of distiller's spent grain at 2 min that is not seen with sophorose. Differential phosphorylation of proteins related to carbon storage, which may be connected to the changes in glucose metabolism, was also indicated.

The multiple signaling cascades affected by the addition of sophorose or spent grain included proteins predicted to affect cell polarity, cell cycle, and growth. In accordance with this, proteins with predicted roles in actin skeleton organization and intracellular trafficking were found among the targets for differential phosphorylation. The responses in growth, cellular polarity, and trafficking could either reflect the effect of the added carbon source on growth or it may have an impact on transport of the secreted gene products under the inducing conditions.

■ CONCLUSIONS

Our study describes extensive phosphorylation activity and signaling in *Trichoderma reesei* upon treatment of sophorose and distiller spent grain in a time-dependent manner. It is also the first study that compares the induction behavior of sophorose and distiller spent grain. Observation of the data reveals a complex signaling network that is activated upon induction and involves components related to light-mediated cellulase induction, circadian clock, osmoregulation, carbon sensing, cell polarity, and cell cycle. The results indicate an intertwined signaling response by light and carbon sources that modulate the growth in *T. reesei*. Altered phosphorylation of HAP2 and the carbon catabolite repressor CREI proteins, regulators of cellulase and hemicellulase gene expression, were also observed. Furthermore, changes in the phosphorylation pattern of acetyl-CoA and pyruvate kinase suggest a possible mechanism for inhibition of glucose catabolism with effects on carbon storage. The changes of phosphorylation of predicted and putative proteins are provided in the Supporting Information tables as a reference for further examination.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00796. The mass spectrometry raw data and Proteome Discoverer.msf files have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD003219. For viewing the msf files, which contain identification results and annotated spectra, a free Proteome Discoverer viewer is available from the Thermo Omics Software Portal (http://portal.thermo-brims.com).

Table S2: Pannzer, JGI manual annotation, Interpro, and KOG definition of all identified proteins. Table S3: List of significant differential phosphorylations for each time point in comparison to control upon treatment with sophorose. Table S4: List of significant differential phosphorylations for each time point in comparison to control upon treatment with distiller's spent grain. Figure S1: FC of phosphopeptides for each time point in comparison to control upon treatment with sophorose of designated regulation trend. Table S5: Protein showing differential regulation of phosphorylations upon treatment with sophorose. Figure S2: FC of phosphopeptides for each time point in comparison to control upon treatment with distiller's spent grain of designated regulation trend. Table S6: Protein showing differential regulation of phosphorylations upon treatment with distiller's spent grain. Table S7: List of 106 significant phosphorylations found in all time points in comparison to control upon treatment with distiller's spent grain. (PDF)

Table S1: Quantitative results of phosphopeptides. (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Schuster, A.; Schmoll, M. Biology and biotechnology of Trichoderma. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 787−99.
(2) Martinez, D.; Berka, R. M.; Henrissat, B.; Saloheimo, M.; Arvas, M.; Baker, S. E.; Chapman, J.; Chertkov, O.; Coutinho, P. M.; Cullen, D.; Danchin, E. G.; Grigoriev, I. V.; Harris, P.; Jackson, M.; Kubicek, C. P.; Han, C. S.; Ho, I.; Larrondo, L. F.; de Leon, A. L.; Magnuson, J. K.; Merino, S.; Misra, M.; Nelson, B.; Putnam, N.; Robbertse, B.; Salamov, A. A.; Schmoll, M.; Terry, A.; Thayer, N.; Westerholm-Parvinen, A.; Schoch, C. L.; Yao, J.; Barabote, R.; Barbote, R.; Nelson, M. A.; Detter, C.; Bruce, D.; Kuske, C. R.; Xie, G.; Richardson, P.; Rokhsar, D. S.; Lucas, S. M.; Rubin, E. M.; Dunn-Coleman, N.; Ward, M.; Brettin, T. S. Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nat. Biotechnol.* **2008**, *26*, 553−60.
(3) Häkkinen, M.; Arvas, M.; Oja, M.; Aro, N.; Penttilä, M.; Saloheimo, M.; Pakula, T. M. Re-annotation of the CAZy genes of Trichoderma reesei and transcription in the presence of lignocellulosic substrates. *Microb. Cell Fact.* **2012**, *11*, 134.
(4) Aro, N.; Pakula, T.; Penttilä, M. Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev.* **2005**, *29*, 719−39.
(5) Pakula, T. M.; Salonen, K.; Uusitalo, J.; Penttilä, M. The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus Trichoderma reesei. *Microbiology* **2005**, *151* (Pt 1), 135−43.
(6) Arvas, M.; Pakula, T.; Smit, B.; Rautio, J.; Koivistoinen, H.; Jouhten, P.; Lindfors, E.; Wiebe, M.; Penttilä, M.; Saloheimo, M. Correlation of gene expression and protein production rate - a system wide study. *BMC Genomics* **2011**, *12*, 616.
(7) Kubicek, C. P.; Mikus, M.; Schuster, A.; Schmoll, M.; Seiboth, B. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. *Biotechnol. Biofuels* **2009**, *2*, 19.
(8) Kubicek, C. P. Systems biological approaches towards understanding cellulase production by Trichoderma reesei. *J. Biotechnol.* **2013**, *163*, 133−42.
(9) Tisch, D.; Schmoll, M. Targets of light signalling in Trichoderma reesei. *BMC Genomics* **2013**, *14*, 657.
(10) Häkkinen, M.; Valkonen, M. J.; Westerholm-Parvinen, A.; Aro, N.; Arvas, M.; Vitikainen, M.; Penttilä, M.; Saloheimo, M.; Pakula, T. M. Screening of candidate regulators for cellulase and hemicellulase production in Trichoderma reesei and identification of a factor essential for cellulase production. *Biotechnol. Biofuels* **2014**, *7*, 14.
(11) Tisch, D.; Schuster, A.; Schmoll, M. Crossroads between light response and nutrient signalling: ENV1 and PhLP1 act as mutual regulatory pair in Trichoderma reesei. *BMC Genomics* **2014**, *15*, 425.
(12) Schuster, A.; Tisch, D.; Seidl-Seiboth, V.; Kubicek, C. P.; Schmoll, M. Roles of protein kinase A and adenylate cyclase in light-modulated cellulase regulation in Trichoderma reesei. *Appl. Environ. Microbiol.* **2012**, *78*, 2168−78.
(13) Zhang, J.; Zhang, Y.; Zhong, Y.; Qu, Y.; Wang, T. Ras GTPases modulate morphogenesis, sporulation and cellulase gene expression in the cellulolytic fungus Trichoderma reesei. *PLoS One* **2012**, *7*, e48786.
(14) Wang, M.; Zhao, Q.; Yang, J.; Jiang, B.; Wang, F.; Liu, K.; Fang, X. A mitogen-activated protein kinase Tmk3 participates in high osmolarity resistance, cell wall integrity maintenance and cellulase production regulation in Trichoderma reesei. *PLoS One* **2013**, *8*, e72189.
(15) Wang, M.; Dong, Y.; Zhao, Q.; Wang, F.; Liu, K.; Jiang, B.; Fang, X. Identification of the role of a MAP kinase Tmk2 in Hypocrea jecorina (Trichoderma reesei). *Sci. Rep.* **2014**, *4*, 6732.
(16) Adav, S. S.; Ravindran, A.; Chao, L. T.; Tan, L.; Singh, S.; Sze, S. K. Proteomic analysis of pH and strains dependent protein secretion of Trichoderma reesei. *J. Proteome Res.* **2011**, *10*, 4579−96.
(17) Adav, S. S.; Chao, L. T.; Sze, S. K. Quantitative secretomic analysis of Trichoderma reesei strains reveals enzymatic composition for lignocellulosic biomass degradation. *Mol. Cell. Proteomics* **2012**, *11*, M111.012419.

DOI: 10.1021/acs.jproteome.5b00796
J. Proteome Res. 2016, 15, 457−467

(18) Adav, S. S.; Chao, L. T.; Sze, S. K. Protein abundance in multiplexed samples (PAMUS) for quantitation of Trichoderma reesei secretome. *J. Proteomics* **2013**, *83*, 180−96.
(19) Do Vale, L. H.; Gómez-Mendoza, D. P.; Kim, M. S.; Pandey, A.; Ricart, C. A.; Edivaldo, X. F. F.; Sousa, M. V. Secretome analysis of the fungus Trichoderma harzianum grown on cellulose. *Proteomics* **2012**, *12*, 2716−28.
(20) Gómez-Mendoza, D. P.; Junqueira, M.; do Vale, L. H.; Domont, G. B.; Ferreira Filho, E. X.; Sousa, M. V.; Ricart, C. A. Secretomic survey of Trichoderma harzianum grown on plant biomass substrates. *J. Proteome Res.* **2014**, *13*, 1810−22.
(21) Jun, H.; Guangye, H.; Daiwen, C. Insights into enzyme secretion by filamentous fungi: comparative proteome analysis of Trichoderma reesei grown on different carbon sources. *J. Proteomics* **2013**, *89*, 191−201.
(22) Rampitsch, C.; Tinker, N. A.; Subramaniam, R.; Barkow-Oesterreicher, S.; Laczko, E. Phosphoproteome profile of Fusarium graminearum grown in vitro under nonlimiting conditions. *Proteomics* **2012**, *12*, 1002−5.
(23) Mandels, M.; Parrish, F. W.; Reese, E. T. Sophorose as an inducer of cellulase in Trichoderma viride. *J. Bacteriol.* **1962**, *83*, 400−8.
(24) Imanishi, S. Y.; Kochin, V.; Ferraris, S. E.; de Thonel, A.; Pallari, H. M.; Corthals, G. L.; Eriksson, J. E. Reference-facilitated phosphoproteomics: fast and reliable phosphopeptide validation by microLC-ESI-Q-TOF MS/MS. *Mol. Cell. Proteomics* **2007**, *6*, 1380−91.
(25) Kumar, L.; Futschik, M. E. Mfuzz: a software package for soft clustering of microarray data. *Bioinformation* **2007**, *2*, 5−7.
(26) Saeed, A. I.; Sharov, V.; White, J.; Li, J.; Liang, W.; Bhagabati, N.; Braisted, J.; Klapa, M.; Currier, T.; Thiagarajan, M.; Sturn, A.; Snuffin, M.; Rezantsev, A.; Popov, D.; Ryltsov, A.; Kostukovich, E.; Borisovsky, I.; Liu, Z.; Vinsavich, A.; Trush, V.; Quackenbush, J. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **2003**, *34*, 374−8.
(27) Chi, A.; Huttenhower, C.; Geer, L. Y.; Coon, J. J.; Syka, J. E.; Bai, D. L.; Shabanowitz, J.; Burke, D. J.; Troyanskaya, O. G.; Hunt, D. F. Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 2193−8.
(28) Koskinen, P.; Toronen, P.; Nokso-Koivisto, J.; Holm, L. PANNZER: high-throughput functional annotation of uncharacterized proteins in an error-prone environment. *Bioinformatics* **2015**, *31*, 1544−52.
(29) Sternberg, D.; Mandels, G. R. Induction of cellulolytic enzymes in Trichoderma reesei by sophorose. *J. Bacteriol.* **1979**, *139*, 761−9.
(30) Sternberg, D.; Mandels, G. R. Regulation of the cellulolytic system in Trichoderma reesei by sophorose: induction of cellulase and repression of beta-glucosidase. *J. Bacteriol.* **1980**, *144*, 1197−9.
(31) Kubicek, C. P.; Messner, R.; Gruber, F.; Mach, R. L.; Kubicek-Pranz, E. M. The Trichoderma cellulase regulatory puzzle: from the interior life of a secretory fungus. *Enzyme Microb. Technol.* **1993**, *15*, 90−99.
(32) Loewenberg, J. R. Sophorose induction of an intracellular b-glucosidase in Trichoderma. *Arch. Microbiol.* **1984**, *137*, 53−7.
(33) Chen, R. E.; Thorner, J. Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. *Biochim. Biophys. Acta, Mol. Cell Res.* **2007**, *1773*, 1311−40.
(34) Gustin, M. C.; Albertyn, J.; Alexander, M.; Davenport, K. MAP kinase pathways in the yeast Saccharomyces cerevisiae. *Microbiol Mol. Biol. Rev.* **1998**, *62*, 1264−300.
(35) Schmoll, M. The information highways of a biotechnological workhorse−signal transduction in Hypocrea jecorina. *BMC Genomics* **2008**, *9*, 430.
(36) Zhang, B.; Zhang, Y.; Shacter, E.; Zheng, Y. Mechanism of the guanine nucleotide exchange reaction of Ras GTPase−evidence for a GTP/GDP displacement model. *Biochemistry* **2005**, *44*, 2566−76.
(37) Thevelein, J. M.; de Winde, J. H. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. *Mol. Microbiol.* **1999**, *33*, 904−18.
(38) Levin, D. E. Cell wall integrity signaling in Saccharomyces cerevisiae. *Microbiol Mol. Biol. Rev.* **2005**, *69*, 262−91.
(39) Catlett, N. L.; Yoder, O. C.; Turgeon, B. G. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. *Eukaryotic Cell* **2003**, *2*, 1151−61.
(40) Cziferszky, A.; Mach, R. L.; Kubicek, C. P. Phosphorylation positively regulates DNA binding of the carbon catabolite repressor Cre1 of Hypocrea jecorina (Trichoderma reesei). *J. Biol. Chem.* **2002**, *277*, 14688−94.
(41) Zeilinger, S.; Ebner, A.; Marosits, T.; Mach, R.; Kubicek, C. P. The Hypocrea jecorina HAP 2/3/5 protein complex binds to the inverted CCAAT-box (ATTGG) within the cbh2 (cellobiohydrolase II-gene) activating element. *Mol. Genet. Genomics* **2001**, *266*, 56−63.
(42) Zeilinger, S.; Schmoll, M.; Pail, M.; Mach, R. L.; Kubicek, C. P. Nucleosome transactions on the Hypocrea jecorina (Trichoderma reesei) cellulase promoter cbh2 associated with cellulase induction. *Mol. Genet. Genomics* **2003**, *270*, 46−55.

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J. Proteome Res. 2016, 15, 457−467