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Discovery of Novel *p*-Hydroxybenzoate-*m*-hydroxylase, Protocatechuate 3,4 Ring-Cleavage Dioxygenase, and Hydroxyquinol 1,2 Ring-Cleavage Dioxygenase from the Filamentous Fungus *Aspergillus niger*

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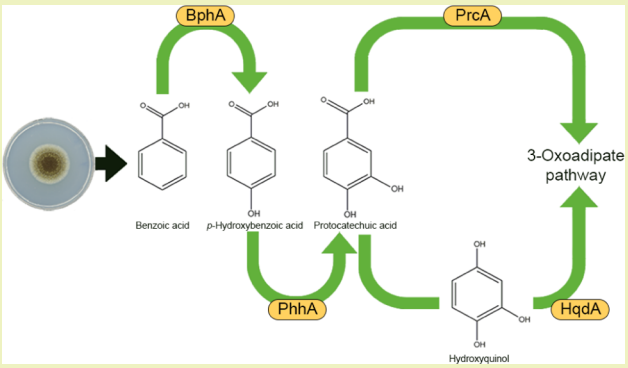
Supporting Information

ABSTRACT: The aromatic compound benzoic acid and its derivatives are frequently used preservatives in food and beverages due to their strong antimicrobial properties. Benzoic acid naturally occurs in plants and serves as a building block for primary and secondary metabolites. Several Aspergilli are able to degrade benzoic acid to *p*-hydroxybenzoic acid by *p*-hydroxylation followed by *m*-hydroxylation to protocatechuic acid. The aromatic ring of protocatechuic acid is then cleaved and further converted through the oxoadipate pathway where it is used as a carbon source. The benzoic acid pathway is well studied in the filamentous fungus *Aspergillus niger*; however, only one enzyme, benzoate-4-monooxygenase, has been characterized. In this study, three novel enzymes of the benzoic acid pathway were identified and characterized. Transcriptome analysis of *A. niger* on *p*-hydroxybenzoic acid, protocatechuic acid, and the related compounds *p*-coumaric acid and caffeic acid revealed two genes of unknown function, which were highly expressed on all four aromatic compounds. Analysis of *A. niger* deletion mutants of these genes revealed their essential role in the utilization of benzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid. Biochemical analysis confirmed that the corresponding enzymes function as the *p*-hydroxybenzoate-*m*-hydroxylase and protocatechuate 3,4 ring-cleavage dioxygenase in the benzoic acid pathway. In addition, a hydroxyquinol 1,2 ring-cleavage dioxygenase, involved in an alternative protocatechuic acid metabolic pathway, was identified.



KEYWORDS: *benzoic acid pathway, flavoprotein, intradiol ring fission*

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INTRODUCTION

The production of aromatic compounds from renewable sources such as lignin through metabolic engineering has great potential in many industries. Aromatic compounds like benzoic acid and vanillin can be used directly or can be further processed to create value-added building blocks such as *cis,cis*-muconic acid. Benzoic acid and its derivatives are widely used as preservatives in juices, soft drinks, acidic foods, and dairy and cosmetic products because of their antimicrobial properties.[1] Moreover, they are also used, e.g., as pH adjusters in cosmetic products, as precursors for plasticizers in plastic production, and as a building block for Vectran fibers.[2,3] Benzoic acid is a basic aromatic carboxylic acid, which naturally occurs in fruits, vegetables, and other plants where its role has been reported to be a defense mechanism against fungal infections.[4] However, many filamentous fungi produce a set of enzymes to counteract its toxicity by converting benzoic acid to less or nontoxic compounds, which they can eventually use as a carbon source.

Several benzoic acid pathways have been described in bacterial species,[5] but, currently, only one benzoic acid pathway has been described in filamentous fungi. In *Aspergillus niger*, benzoic acid is converted in three steps. (1) Benzoic acid is converted into *p*-hydroxybenzoic acid through hydroxylation by the cytochrome P450 enzyme, benzoate-4-monooxygenase (BphA), and its partner NADPH-cytochrome P450 reductase (CprA).[6,7] (2) *p*-Hydroxybenzoic acid is converted into 3,4-dihydroxybenzoic acid (protocatechuic acid) by a *p*-hydroxybenzoate-*m*-hydroxylase (Phh).[8,9] (3) Protocatechuic acid is converted into 3-carboxy-*cis,cis*-muconic acid by an unidentified

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intradiol ring-cleavage dioxygenase (Prc) and is further metabolized through the 3-oxoadipate pathway to finally enter the tricarboxylic acid cycle (TCA).[10,11]

In bacteria, both enzymes involved in the *m*-hydroxylation of *p*-hydroxybenzoic acid to protocatechuic acid and the intradiol cleavage of protocatechuic acid have been characterized. In *Pseudomonas aeruginosa*, this conversion is catalyzed by *p*-hydroxybenzoate-*m*-hydroxylase (PobA),[12] and the ring cleavage of protocatechuic acid is catalyzed by protocatechuate 3,4-dioxygenase α chain (PcaH) and β chain (PcaG).[13] A recent study of benzoic acid metabolism in *Aspergillus nidulans* proposed that *p*-hydroxybenzoic acid is converted into protocatechuic acid by the enzymes encoded by the genes AN10952 and AN0876, and protocatechuic acid is cleaved by AN8566.[11] However, the function of these putative enzymes has not been verified. Despite the knowledge of the benzoic acid pathway in fungi and the characterized bacterial enzymes, the fungal enzymes involved in the *m*-hydroxylation of *p*-hydroxybenzoic acid and the ring cleavage of protocatechuic acid remained to be characterized.

In this study, we used transcriptome analysis of *A. niger* N402 to identify candidate *phh* and *prc* genes, which were then verified by gene deletion and by characterization of the corresponding enzymes. In addition, double gene deletion of *prc* and a putative hydroxyquinol 1,2-dioxygenase gene (*hqd*) leads to the accumulation of hydroxyquinol and revealed an alternative protocatechuic acid metabolic pathway.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions. *A. niger* strains used in this study are shown in Table 1. The fungi were grown on complete medium (CM)[14] agar (1.5 % w/v) plates at 30 °C for 4 days. Spores were harvested with 10 mL of *N*-(2-acetamido)-2-aminoethanesulfonic acid buffer, and the minimal medium (MM)[13] agar (1.5% w/v) plates were inoculated with 10^3 freshly isolated spores. MM plates for growth profile experiments were supplemented with aromatic compounds as the sole carbon source. Due to the toxicity of the aromatic compounds, different concentrations were used for the growth profile, i.e., 2 mM for ferulic acid; 3 mM for benzoic acid and benzaldehyde; and 5 mM for 2,3-dihydroxybenzoic acid, benzyl alcohol, caffeic acid, catechol, cinnamic acid, *m*-hydroxybenzoic acid, *p*-anisic acid, *p*-anisyl alcohol, *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, protocatechuic aldehyde, salicylic acid, vanillic acid, and veratric acid. All aromatic compounds and chemicals were purchased from Sigma-Aldrich.

Table 1. *A. niger* Strains Used in This Study

strain	CBS number	genotype	reference
N402	CBS 141247	*cspA1*	31
N593	CBS 144057	*cspA1*, *pyrG*	32
N593 Δ*ku70*	CBS 138852	*cspA1*, *pyrG*, Δ*kusA::amdS*	33
Δ*bphA*	CBS 145163	*cspA1*, *pyrG*, Δ*kusA::amdS*, Δ*bphA::hph*	this study
Δ*phhA*	CBS 145164	*cspA1*, *pyrG*, Δ*kusA::amdS*, Δ*phhA::hph*	this study
Δ*prcA*	CBS 145165	*cspA1*, *pyrG*, Δ*kusA::amdS*, Δ*prcA::hph*	this study
Δ*hqdA*	CBS 145839	*cspA1*, *pyrG*, Δ*kusA::amdS*, Δ*hqdA::pyrG*	this study
Δ*prcA* Δ*hqdA*	CBS 145840	*cspA1*, *pyrG*, Δ*kusA::amdS*, Δ*prcA::hph*, Δ*hqdA::pyrG*	this study
phhA	CBS 145161	*cspA1*, *pyrG*, *gpdA::phhA-his::pyrG*	this study
prcA	CBS 145162	*cspA1*, *pyrG*, *gpdA::prcA-his::pyrG*	this study

For transfer experiments, *A. niger* spores (1 x 10^6 spores/mL) were inoculated to 200 mL of CM with 2% fructose and incubated overnight on a rotary shaker at 30 °C, 250 rpm. Freshly germinated mycelia were harvested on Miracloth (Merck KGaA, Darmstadt, Germany) and washed with MM. Equal portions of mycelia were transferred to flasks containing 50 mL of MM and 0.02% (w/v) aromatic compound. The cultures were incubated on a rotary shaker for 2 h at 30 °C, 250 rpm. Mycelia were harvested, dried between a tissue paper to remove excess liquid, and frozen in liquid nitrogen.

$FeCl_3$ Assay. MM agar plates (1.5 % w/v) supplemented with 5 mM protocatechuic acid and 1.22 mg/mL uridine were used for this assay. Sterilized polycarbonate (PC) membranes (Maine Manufacturing LLC, Sanford, ME) were placed on the MM plates after which they were inoculated with 10^3 freshly isolated spores and incubated at 30 °C for 5 days. The PC-membrane containing the mycelia was removed from agar plates and stained with 5 mL of 20% $FeCl_3$ solution for 5 min. Thereafter, the $FeCl_3$ solution was removed and pictures were taken.

RNA Extraction and RNA Sequencing. The frozen mycelia were ground using the tissue lyser (Qiagen, Hilden, Germany), and total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and RNA isolation kit (NucleoSpin RNA, Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendation. The quality and quantity of RNA were determined by gel electrophoresis and RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA sequencing was conducted by BGI Tech Solutions (Tai Po, Hong Kong) using Illumina Hiseq 2000 platform (Illumina Inc., San Diego, CA). Transcriptome analysis was performed as described in Kowalczyk et al.[15] The transcriptome data was stored at the NCBI Gene expression omnibus, under the GEO accession number GSE134999.

Construction of Gene Deletion Cassettes and Transformation of *A. niger*. The gene deletion cassettes were constructed using fusion PCR.[15] The flanking regions contained 900−1000 bp upstream and downstream of the gene fragment to be deleted including an overlap of the selection marker. The selection markers orotidine 5′-phosphate decarboxylase (*pyrG*) from *Aspergillus oryzae* RIB40 and hygromycin B (*hph*) from *Escherichia coli* were amplified from the plasmid pAN7.1.[16] These three fragments were combined in a PCR fusion reaction using the GoTaq Long PCR Master Mix (Promega, Madison, WI). The fusion PCR mixture contained 0.4 μL of each amplified product, 0.6 μL of 10 mM upstream and downstream primers, and 12.5 μL of GoTaq Long PCR Master Mix in a total volume of 25 μL. The following PCR conditions were used: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 5 min, and a final extension at 72 °C for 10 min. *A. niger* N593 Δ*ku*70 was transformed through protoplast-mediated transformation, and purification of the different transformants was performed as described in Kowalczyk et al.[15]

Construction of *phhA* and *prcA* Overexpression Plasmids. Plasmids for overexpression of *phhA* and *prcA* genes were created by replacing the *hph* gene in pAN7.1 with the gene of interest including a C-terminal His-tag through homologous recombination based on Jacobus and Gross.[17] The *phhA* and *prcA* genes were amplified from the genomic DNA of *A. niger* N593 using the gene-specific primers containing a 20−30 bp overlap with pAN7.1 *gpdA* promoter and primers containing a hexa His-tag and 20−30 bp overlap with *trpC* terminator (Table S1). The backbone of the plasmid was amplified from pAN7.1 flanking *hph*, resulting in a linear amplification product without *hph* (Table S1). The reactions were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and co-transformed into *E. coli* strain TOP10 (Thermo Fisher Scientific, Waltham, MA). The transformants were selected on Luria Bertani (LB) medium supplemented with 50 μg/mL ampicillin. Positive colonies were verified by colony PCR using gene-specific primers, after which the selected ones were fully sequenced (Macrogen, Amsterdam, The Netherlands). The pAN7.1-*phhA* or pAN7.1-*prcA* plasmids were co-transformed with pRV1005 plasmid,[18] containing *pyrG* of *A. oryzae*, into *A. niger* N593. Protoplast-mediated transformation and purifica-

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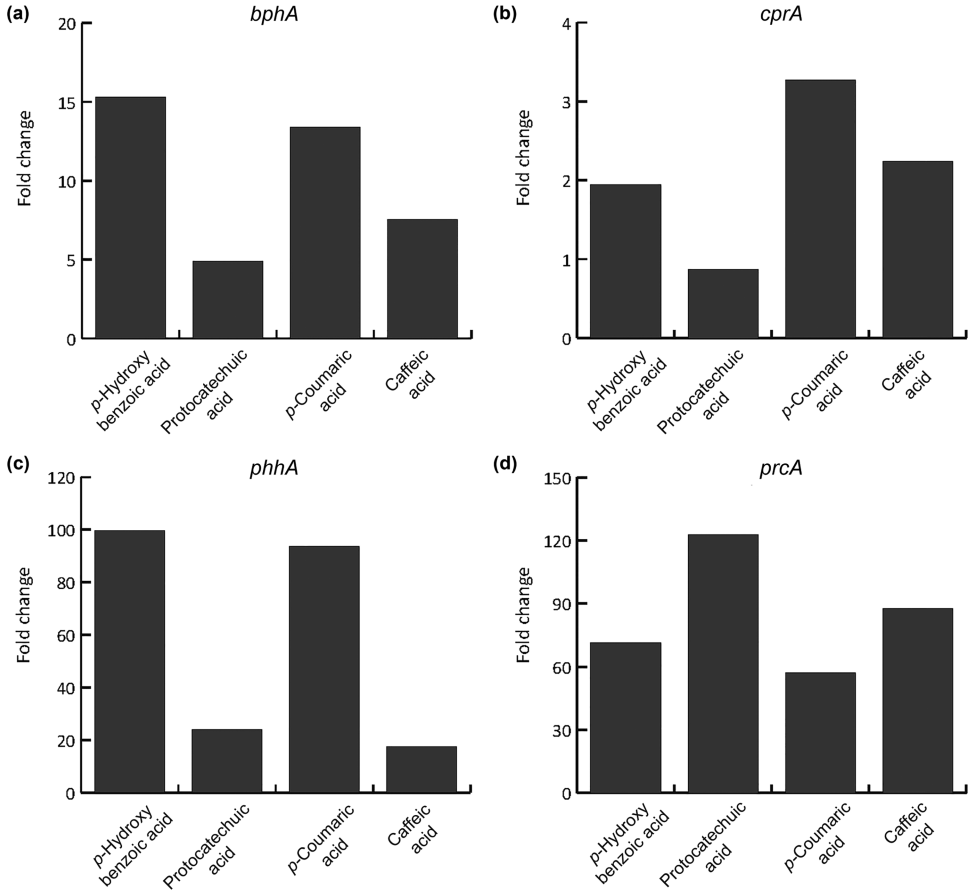

Figure 1. Fold change compared to a no carbon source control of the DESeq2 value of the expression of (a) bphA, (b) cprA, (c) putative phhA (NRRL3_8551), and (d) putative prcA (NRRL3_1405) in *A. niger* grown on p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, and caffeic acid.

tion of the selected transformants were performed as described in Kowalczyk et al.[15]

Construction of *hqdA* Overexpression Plasmid. The overexpression plasmid for *hqdA* containing an N-terminal hexa His-tag was created by inserting the gene in pET28a through homologous recombination based on Jacobus and Gross.[17] The plasmid was amplified from pET28a flanking the multiple cloning site (Table S1). The reactions were purified with Wizard SV Gel and PCR Clean-Up System and co-transformed into *E. coli* strain TOP10 and selected on LB medium supplemented with 50 μg/mL kanamycin. The plasmid was isolated and verified for the correctness by sequencing. The pET28a-*hqdA* plasmid was transformed into the *E. coli* protein production strain BL-21 DE3 (New England Biolabs, Ipswich, MA).

Production of Recombinant *p*-Hydroxybenzoate-*m*-hydroxylase (PhhA) and Protocatechuate 3,4 Ring-Cleavage Dioxygenase (PrcA). *E. coli* BL-21 DE3 was the preferred production host for this study and used for hydroxyquinol 1,2 ring-cleavage dioxygenase (HqdA). As we were not able to produce PhhA or PrcA in this host, these proteins were produced using *A. niger* N593. For protein production, CM was inoculated with 1×10^6 spores/mL of the selected *A. niger* N593 containing overexpressing *phhA* or *prcA* plasmid and incubated at 30 °C for 24 h, 250 rpm. The mycelia were harvested as described above. The frozen mycelium was ground using the tissue lyser. The proteins were extracted using 10 mL per gram mycelium YeastBuster Protein Extraction Reagent (Novagen) containing 1.5 μM tris(hydroxypropyl) phosphine, 25 U Benzonase Nuclease, and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). The mixtures were incubated at 4 °C for 20 min with rotation and centrifuged at 18 000*g*, 4 °C for 10 min. The supernatant was collected, filtered, and proceeded to further analysis or stored at −20 °C.

Production of Recombinant HqdA. *E. coli* BL-21 DE3 pET28a-*hqdA* was grown in LB medium with 50 μg/mL kanamycin at 37 °C, 160 rpm, until the OD_{600} of 0.4−0.8 was reached. Thereafter, IPTG was added, with a final concentration of 100 μM, to the culture and further incubated for 24 h at 12 °C, 160 rpm. The culture was centrifuged at 3.2*g*, 4 °C for 10 min. The pellet was dissolved in 5 mL (per 100 mL culture) of BugBuster Protein Extraction Reagent (Novagen), containing 1 KU lysozyme/mL (Sigma-Aldrich), 25 U Benzonase Nuclease, and cOmplete, EDTA-free Protease Inhibitor Cocktail, and was incubated for 20 min at 4 °C with shaking. The cell debris was centrifuged at 4 °C, and the supernatant containing the soluble fraction of proteins was frozen for further use.

Protein Purification. The supernatant containing proteins was applied to an ÄKTA start system (GE Healthcare Life Sciences, Uppsala, Sweden) equipped with a 1 mL HisTrap FF column (GE Healthcare Life Sciences), equilibrated with 20 mM HEPES, 0.4 M NaCl, and 20 mM imidazole, pH 7.5, with a flow rate of 1 mL/min. After washing with the equilibrating buffer, the protein was eluted with 5 mL of 20 mM HEPES, 0.4 M NaCl, and 400 mM imidazole, pH 7.5. Fractions containing the enzyme, confirmed by SDS-PAGE, were pooled, concentrated, and buffer-exchanged to 20 mM HEPES, pH 7.0. All purification steps were performed at 4 °C.

Protein Analysis. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Theoretical molecular masses were calculated by ExPASy−ProtParam tool (http://www.expasy.ch/tools/protparam.html). Molecular mass was estimated by SDS-PAGE (12% w/v, polyacrylamide gel) using Mini-PROTEAN1 Tetra Cell (Bio-Rad, Hercules, CA) and molecular mass standard (Precision Plus Protein Prestained Standards, Bio-Rad). The gel was stained by Coomassie Brilliant Blue G-250 (Thermo Scientific) or used for Western blotting. For Western blotting, the

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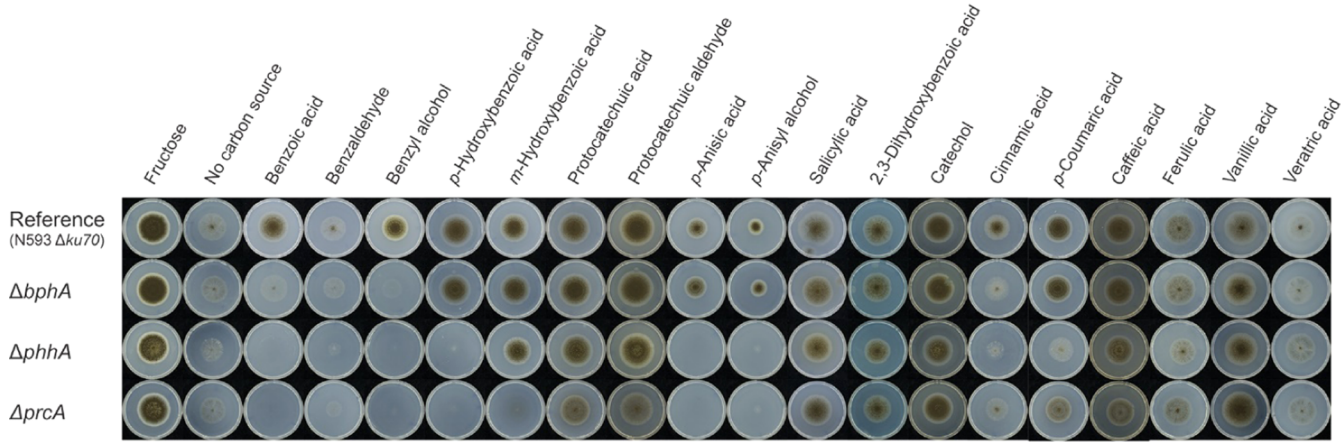

Figure 2. Growth profile of the *A. niger* deletion mutants Δ*bphA*, Δ*phhA*, and Δ*prcA*, and the reference strain N593 Δ*ku*70. Growth was examined on selected aromatic compounds related to the benzoic acid pathway. Agar plates were incubated at 30 °C for 10 days and supplemented with uridine.

proteins were transferred to a nitrocellulose blotting membrane (GE Healthcare, Chicago, IL) and subsequently blocked with 5% (w/v) skimmed milk in phosphate-buffered saline (PBS) for 1 h. His-tagged proteins were detected using Mouse monoclonal anti-Histidine Tag antibody (Bio-Rad) conjugated with alkaline phosphatase (Bio-Rad). Proteins were visualized with the BCIP/NBT colorimetric assay.[19]

Enzyme Activity Assays. The *p*-hydroxybenzoic acid *m*-hydroxylase activity assay was performed in a total volume of 500 μL containing 50 μM *p*-hydroxybenzoic acid, 10 μM FAD, 200 μM NADH, and 50 μL of cell-free extract or purified protein in 50 mM HEPES, pH 7.0 or pH 8.0. The protocatechuate ring cleaving assay was performed in a total volume of 500 μL containing 50 μM protocatechuic acid, 2.5 μM $Fe_2(SO_4)_3$, and 50 μL of cell-free extract or purified protein in 50 mM HEPES, pH 7.0 or pH 8.0. Reaction mixtures were incubated at 30 °C for 16 h and mixed with 100% acetonitrile (1:5, v/v). The hydroxyquinol ring cleaving assay was performed in a total volume of 500 μL containing 50 μM hydroxyquinol, 2.5 μM $Fe_2(SO_4)_3$, and 50 μL of cell-free extract or purified protein in 50 mM phosphate buffer, pH 6.0. The reduction of hydroxyquinol, *p*-hydroxybenzoic acid, and protocatechuic acid, or formation of maleylacetate, protocatechuic acid, and 3-carboxy-*cis,cis*-muconic acid, was monitored by HPLC (Dionex ICS-5000+ chromatography system; Thermo Scientific, Sunnyvale, CA) equipped with an Acclaim Mixed-Mode WAX-1 LC Column (3 × 150 mm; Thermo Scientific) and a UV detector (225, 250, or 280 nm, Thermo Scientific).[20]

RESULTS

Identification of Candidate Genes Encoding Putative *p*-Hydroxybenzoic Acid *m*-Hydroxylase and Protocatechuic Acid Ring-Cleavage Dioxygenase in *A. niger*. To identify candidate genes involved in the metabolism of *p*-hydroxybenzoic acid and protocatechuic acid, transcriptomes of *A. niger* N402 grown on *p*-hydroxybenzoic acid and protocatechuic acid were generated. In addition, transcriptomes of *A. niger* N402 grown on *p*-coumaric acid and caffeic acid were included in this study because of the structural resemblance to *p*-hydroxybenzoic acid and protocatechuic acid. The data set from *A. niger* N402 grown on MM without a carbon source was used as a control. The transcriptome data revealed 309 genes that were induced (fold change ≥2, *p*-value ≤ 0.01) on all four aromatic compounds tested (Figure S1). As part of these 309 induced genes, *bphA* was induced on all tested compounds, while its partner *cprA* was induced only by *p*-coumaric acid and to a lesser extent caffeic acid (Figure 1). In addition, NRRL3_2586, NRRL3_837, and NRRL3_1886 (https://mycocosm.jgi.doe.gov/Aspni_NRRL3_1), which are the homologues of the *A. nidulans* genes (http://www.aspgd.org/) AN1151 (carboxy-*cis,cis*-muconate cyclase), AN5232 (3-carboxymuconolactone hydrolase), and AN10495 (succinyl-CoA:3-oxoadipate CoA-transferase), respectively, involved in the 3-oxoadipate pathway,[11] were induced by *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, and caffeic acid (data not shown). Five other genes were highly induced (fold change ≥ 50, *p*-value ≤ 0.01) on *p*-hydroxybenzoic acid, including a FAD-binding domain-containing protein (NRRL3_8551) and an intradiol ring-cleavage dioxygenase (NRRL3_1405). NRRL3_8551 was selected as a putative *phh* because it is the only gene in the data set that encodes an enzyme containing a FAD-binding site, which is essential for NADPH binding, needed for the hydroxylation of aromatic compounds. NRRL3_1405 was selected because it was also highly induced by protocatechuic acid and caffeic acid and encodes a putative intradiol ring-cleavage dioxygenase. NRRL3_8551 and NRRL3_1405 showed high amino acid sequence similarity to AN10952 (83.5%) and AN8566 (94.3%), respectively, from *A. nidulans*.[11] These were suggested to encode putative *p*-hydroxybenzoic acid *m*-hydroxylase (PhhA) and protocatechuic acid ring-cleavage dioxygenase (PrcA).

Deletion of *bphA* and the Putative *phhA* and *prcA* Genes Results in Reduced Growth on Benzoic Acid. To verify that the candidate genes encode the enzymes involved in the benzoic acid pathway, three *A. niger* deletion mutants, Δ*bphA*, Δ*phhA*, and Δ*prcA*, were created and cultivated for 10 days on benzoic acid and related metabolites of the benzoic acid pathway (Figure 2). As expected, Δ*bphA* resulted in reduced growth on benzoic acid. Growth was also reduced on benzaldehyde, benzyl alcohol, and cinnamic acid (Figure 2). Growth of Δ*phhA* was abolished on benzoic acid, benzaldehyde, benzyl alcohol, *p*-anisic acid, *p*-anisyl alcohol, and *p*-hydroxybenzoic acid and reduced on p-coumaric acid and cinnamic acid. Δ*prcA* resulted in a similar phenotype as Δ*phhA*, but, in addition, growth on *m*-hydroxybenzoic acid was abolished and reduced on protocatechuic acid, *p*-coumaric acid, and caffeic acid. After 5 days of growth, the phenotype of Δ*prcA* on protocatechuic acid was more different than that of the reference (Figure 3). Surprisingly, after 10 days, Δ*prcA* recovered on protocatechuic acid and showed almost the same radial size as the reference and the other mutants (Figure 2). However, the mycelium of Δ*prcA* on protocatechuic acid was thinner, sporulated less, and was lighter in color than the reference.

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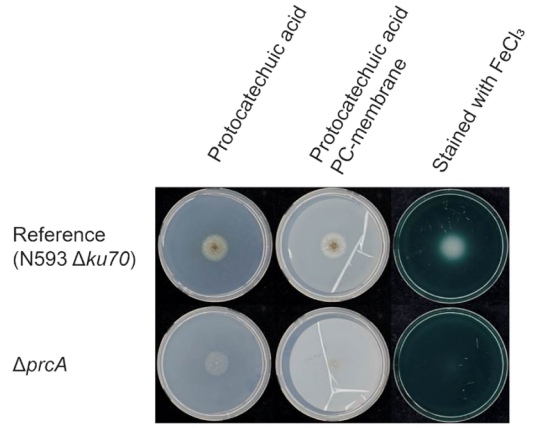

Figure 3. Growth profile of Δ*prcA* and N593 Δ*ku*70 on minimal medium agar plates containing protocatechuic acid after 5 days of growth with and without polycarbonate (PC) membrane and after staining with $FeCl_3$. All plates were supplemented with uridine.

Degradation of protocatechuic acid was determined by a $FeCl_3$ assay (Figure 3). Protocatechuic acid can reduce Fe^{3+} to Fe^{2+}; hence, addition of $FeCl_3$ to the medium containing protocatechuic acid turns the color of the medium green. The reference strain revealed a clear white halo, indicating that protocatechuic acid was degraded, while Δ*prcA* had a weaker halo. The recovered growth after 10 days suggests that an alternative ring-cleavage dioxygenase is present in *A. niger*. These results strongly support that the candidate genes, *phhA* and *prcA*, encode *p*-hydroxybenzoate-*m*-hydroxylase and protocatechuate 3,4 ring-cleavage dioxygenase, respectively.

Identification of an Additional Intradiol Ring Dioxygenase. The RNAseq data was mined to identify alternative ring-cleavage dioxygenases that could be involved in the cleavage of protocatechuic acid. Only one candidate intradiol ring-cleavage dioxygenase (NRRL3_2644), annotated as a hydroxyquinol 1,2 ring-cleavage dioxygenase (HqdA), was induced by protocatechuic acid, *p*-hydroxybenzoic acid, and caffeic acid (fold changes of 4.5, 9.7, and 4.8, respectively, compared to no carbon source control). To study the involvement of *hqdA* in the benzoic acid pathway, a single deletion mutant (Δ*hqdA*) and a double knockout (Δ*prcA*Δ*hqdA*) were created and grown on several aromatic compounds (Figure 4). Deletion of Δ*hqdA* did not result in a phenotype on any of the tested aromatic compounds. However, growth of the double deletion mutant Δ*prcA*Δ*hqdA* on protocatechuic acid was reduced compared to Δ*prcA*, indicating that *hqdA* is part of an alternative protocatechuic acid pathway. In addition, the formation of a brown product was observed on protocatechuic acid, *p*-coumaric acid, caffeic acid, and cinnamic acid, suggesting that these compounds are connected to the benzoic acid pathway (Figure 4).

Extraction and Identification of the Accumulated Compound Produced by Δ*prcA*Δ*hqdA*. To identify the accumulated product produced by the double deletion mutant Δ*prcA*Δ*hqdA*, a transfer experiment was performed. Spores of *A. niger* N593 Δ*ku*70, Δ*prcA*, Δ*hqdA*, and Δ*prcA*Δ*hqdA* were germinated overnight in CM with fructose and transferred to flasks containing MM with 5 mM protocatechuic acid. After 6 h of incubation, the formation of a red product was observed in Δ*prcA*Δ*hqdA* cultures, which turned brown within 24 h (Figure S2). The culture supernatant was extracted with ethyl acetate and methanol. HPLC analysis of the ethyl acetate fraction resulted in the identification of hydroxyquinol, while the methanol fraction contained an unknown compound.

Characterization of the Enzyme Activity of HqdA, PhhA, and PrcA Confirmed Their Predicted Function. To confirm their enzymatic function, PhhA and PrcA were overproduced in *A. niger* N593. The cell-free extracts containing PhhA showed statistically significant removal of *p*-hydroxybenzoic acid from the assay mixture (Figure 5), whereas those containing PrcA, supplemented with $Fe_2(SO_4)_3$, showed

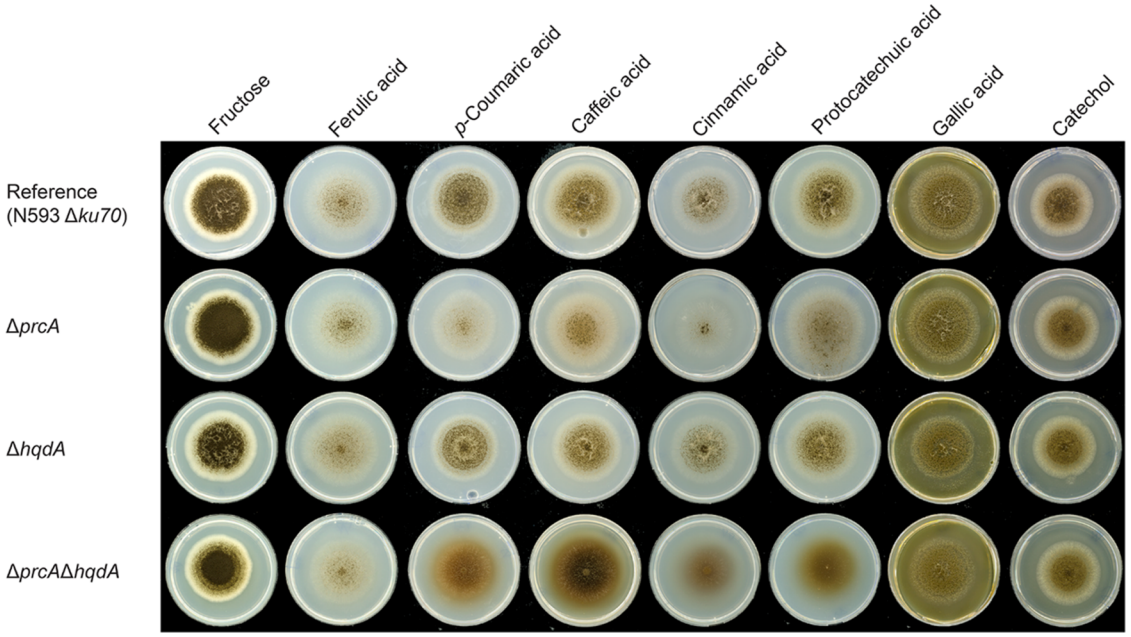

Figure 4. Growth profile of the *A. niger* deletion mutants Δ*prcA*, Δ*hqdA*, and Δ*prcA*Δ*hqdA* and the reference strain N593 Δ*ku*70 on selected aromatic compounds related to the benzoic acid pathway. Agar plates were incubated at 30 °C for 8 days. Agar plates of the reference strain N593 Δ*ku*70 and Δ*prcA* were supplemented with uridine.

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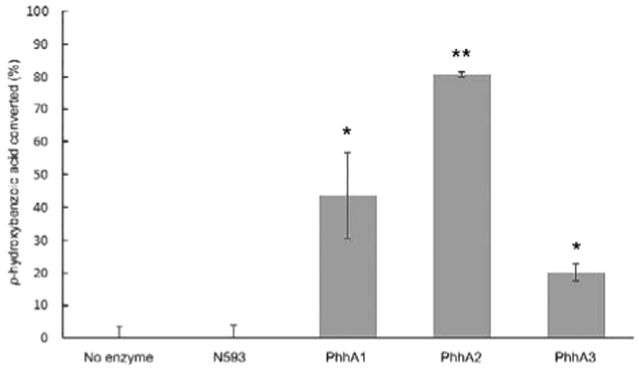

Figure 5. PhhA activity toward *p*-hydroxybenzoic acid of *A. niger* mutants overexpressing phhA. The assay was performed with three independent phhA overexpression mutants, PhhA1, PhhA2, and PhhA3. Error bars represent the standard deviation of three technical replicates. Asterisks indicate significant differences compared with *A. niger* N593 (Student's *t* test, *p-value ≤ 0.05, **p-value ≤ 0.01).

statistically significant removal of protocatechuic acid (Figure 6). The conversion products of PhhA and PrcA, protocatechuic

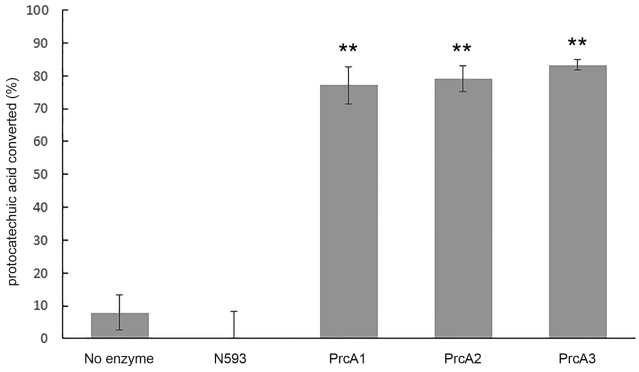

Figure 6. PrcA activity toward protocatechuic acid of *A. niger* mutants overexpressing prcA. The assay was performed with three independent prcA overexpression mutants, PrcA1, PrcA2, and PrcA3. Error bars represent the standard deviation of three technical replicates. Asterisks indicate significant differences compared with *A. niger* N593 (Student's *t* test, ** = p-value ≤ 0.01).

acid and 3-carboxy-*cis,cis*-muconic acid, respectively, were detected by HPLC (data not shown). The extract from the parental strain *A. niger* N593 grown under the same conditions was used as a negative control, which showed no removal of *p*-hydroxybenzoic acid or protocatechuic acid. We confirmed the production of recombinant PhhA and PrcA in the cell-free extracts by Western blotting using a monoclonal antibody raised against the Histidine tag (Figure S3). The detected size of PhhA and PrcA corresponded well with their expected mass (71.8 and 36 kDa, respectively). In addition, a protein of 72 kDa was observed, indicating possible dimerization of PrcA. PhhA and PrcA were partially purified by Histidine tag purification from the most active overexpressing mutants (Figure S3). The purified PrcA showed activity toward protocatechuic acid (Table 2), whereas the purified PhhA showed no activity after the purification. This could be because the protein was unstable under the purification conditions or a required co-enzyme is present only in the cell-free extract.

The putative HqdA was produced with an N-terminal Histidine tag in *E. coli* and isolated and purified. The presence

Table 2. Ring Cleavage of Aromatic Compounds by *A. niger* PrcA and HqdA[a,c]

substrate	PrcA	HqdA
protocatechuic acid	≥99%	0
hydroxyquinol[b]	0	≥99%
catechol	0	≥99%
protocatechuic aldehyde	0	n.d.
methoxyhydroquinone	0	0

[a]Reaction condition using purified enzymes: 250 μM substrate, 100 mM HEPES, pH 7.0, 30 °C, 180 rpm, 18 h. Conversion values are determined by HPLC. [b]Reaction in 100 mM phosphate buffer, pH 6.0. [c]n.d. = not determined.

of recombinant HqdA in the purified fractions was confirmed by SDS-PAGE and Western blotting using the monoclonal Histidine tag antibody (Figure S4). The detected size of HqdA-His corresponded with the expected mass of 37.5 kDa. In addition, a protein of 75 kDa was observed, indicating possible dimerization of HqdA. Enzyme assays of HqdA revealed that it was active on hydroxyquinol and catechol but not on protocatechuic acid (Table 2).

Presence of HqdA, PhhA, and PrcA Encoding Genes Is Conserved in Ascomycete Genomes. In the *A. niger* NRRL3 genome database (https://mycocosm.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html), HqdA is annotated as hydroxyquinol 1,2-dioxygenase, PhhA is annotated as a FAD-binding monooxygenase, and PrcA is annotated as a catechol 1,2-dioxygenase. *A. niger* PhhA has low amino acid sequence similarity with the bacterial *p*-hydroxybenzoate-*m*-hydroxylase enzyme PobA, (https://www.uniprot.org/uniprot/P00438) (18.0%), but shares a higher amino acid sequence similarity with the *m*-hydroxybenzoic acid *p*-hydroxylase (MobA, https://www.uniprot.org/uniprot/Q6SSJ6) of *Pseudomonas testosteroni* (32.7%). A comparison of the amino acid sequence of *A. niger* PrcA and the bacterial protocatechuate 3,4-dioxygenase β and α chains, PcaG (https://www.uniprot.org/uniprot/P00436) and PcaH (https://www.uniprot.org/uniprot/P00437), revealed that these enzymes share low similarities of 17.2 and 23.5%, respectively. *A. niger* HqdA compared to the characterized hydroxyquinol 1,2-dioxygenase ChqA (https://www.uniprot.org/uniprot/Q3KZ33) of *Nocardioides simplex* and NpcC (https://www.uniprot.org/uniprot/Q6F4M7) of *Rhodococcus oparus* revealed similarities of 40.0 and 40.2%, respectively. Phylogenetic analysis revealed that HqdA, PhhA, and PrcA are conserved in *Aspergillus* species (Figures S5 and S6). In addition, high homology of PhhA was also observed in industrially important fungi such as *Penicillium*, *Myceliophthora thermophila*, *Neurospora crassa*, *Podospora anserina*, and *Trichoderma reesei* (Figure S5). Similar results were observed with PrcA (Figure S6). It appears that catechol 1,2-dioxygenase and HqdA cluster separately from PrcA. The high amino acid sequence similarities of PhhA and PrcA to AN10952 and AN8566 encoding genes,[11] respectively, indicate that these encode the PhhA and PrcA enzymes of *A. nidulans*.

DISCUSSION

The benzoic acid pathway has been studied for decades in bacteria and fungi,[5,9,10,21] but only BphA, which catalyzed the first step of the benzoic acid pathway, was characterized in fungi.[6,7] In this study, we identified and characterized three novel enzymes of the benzoic acid pathway in *A. niger*, HqdA, PhhA, and PrcA. Phylogenetic analysis of HqdA, PhhA, and

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PrcA revealed that these enzymes are conserved in many ascomycetes, indicating that the benzoic acid pathway is present in many ascomycetes including the industrially relevant species *N. crassa*, *Potentilla anserina,* and *T. reesei*.

Transcriptomes of *A. niger* grown on caffeic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and protocatechuic acid revealed that *bphA, phhA*, and *prcA* were induced by these compounds. The expression of *bphA* showed a similar trend as in a previous transcription analysis of *A. niger*.[14] In addition, our results showed that *bphA*, *phhA*, and *prcA* were also induced on benzoic acid (Lubbers et al., unpublished data). The *hqdA* gene was slightly induced on *p*-hydroxybenzoic acid, protocatechuic acid, and caffeic acid. The transcription factor that regulates the benzoic acid pathway has not yet been identified, but two suggestions for the benzoic acid transcription factor binding site of *bphA* have been made previously.[22,23] One of the suggested binding sites, TGACTA,[22] is present at the −950 position of *phhA* and the −1916 position of *prcA* but not in the promotor region of *hqdA*. The promoter of *cprA* contains a TGACTA binding site at the −2664 position and is induced by benzoic acid but not by *p*-hydroxybenzoic acid and protocatechuic acid.[22,24] The distant location of the binding site in the promotor of *prcA* and *cprA* makes it unlikely that they are regulated by the same transcription factor as *bphA*. In addition, the differences of gene expression indicate that there are multiple inducers of the benzoic acid pathway and that there is a second transcription factor regulating this pathway. This confirmed a previous study that showed the induction of *bphA* by *p*-coumaric acid and *p*-hydroxybenzoic acid in *A. niger*.[14] The same study also showed that *bphA* was highly induced by *p*-anisyl alcohol. In our study, deletion of *bphA* did not result in a growth reduction on *p*-anisyl alcohol or *p*-anisic acid (Figure 3). However, Δ*phhA* deletion strain revealed that these compounds are converted into *p*-hydroxybenzoic acid and further into protocatechuic acid.

Deletion of *hqdA* did not have an effect on *A. niger* growth on protocatechuic acid, while the growth of Δ*prcA* was delayed but recovered over time. The double deletion mutant Δ*prcA*Δ*hqdA* resulted in further reduced growth on protocatechuic acid compared to Δ*prcA* as well as the formation of hydroxyquinol and an unidentified brown compound. It has been reported that hydroxyquinol can be autoxidized to hydroxy-1,4-benzoquinone,[25] which corresponds to the brown compound detected in our experiments. Purified *A. niger* HqdA revealed activity on hydroxyquinol and catechol, which corresponds with the activity of the hydroxyquinol 1,2-dioxygenase from the basidiomycete fungi *Trichosporon cutaneum* and *Phanerochaete chrysosporium*.[25,26] Deletion of *hqdA* did not result in reduced growth on catechol, suggesting that HqdA is not involved in catechol conversion in vivo, despite its ability to convert this compound in vitro. Phylogenetic analysis of HqdA and PrcA revealed a gene (NRRL3_4787), which separated from the clades of HqdA and PrcA (Figure S6) and is likely to encode the catechol 1,2-dioxygenase of *A. niger*. HqdA was not active on protocatechuic acid, revealing that protocatechuic acid is decarboxylated to hydroxyquinol by an as yet unidentified enzyme. Based on the HqdA substrate specificity, delayed growth of Δ*prcA* on protocatechuic acid, and low induction levels of *hqdA*, we suggest that this metabolic pathway is an alternative pathway regulated by another transcription factor.

Phenotypic analysis of Δ*bphA*, Δ*phhA*, Δ*prcA*, and Δ*prcA*Δ*hqdA* revealed that *p*-coumaric acid and caffeic acid are degraded through the benzoic acid pathway. The reduced growth of deletion mutants Δ*phhA*, Δ*prcA*, and Δ*prcA*Δ*hqdA* on *p*-coumaric acid indicates that *p*-coumaric acid is converted into *p*-hydroxybenzoic acid as was suggested in *Aspergillus flavus* and *Aspergillus japonicus*.[27,28] Growth reduction was observed on caffeic acid by Δ*prcA* and Δ*prcA*Δ*hqdA*. The conversion of caffeic acid into protocatechuic acid in multiple steps was suggested in *A. japonicus*.[27] In addition, Δ*prcA*Δ*hqdA* grown on caffeic acid and *p*-coumaric acid resulted in the accumulation of hydroxy-1,4-benzoquinone, confirming that both compounds are converted into protocatechuic acid and hydroxyquinol. Furthermore, the *A. niger* homologues of the *A. nidulans* genes AN3895 (NRRL3_2586), AN3895 (NRRL3_837), and AN4061 (NRRL3_1886), involved in the 3-oxoadipate pathway,[11] were induced by *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, and caffeic acid. This supports that *p*-coumaric acid and caffeic acid are utilized through the 3-oxoadipate pathway, which is the final step in the benzoic acid pathway before entering the TCA cycle. Based on these phenotypes and transcriptome data, we conclude that *A. niger* converts *p*-coumaric acid and caffeic acid into *p*-hydroxybenzoic acid and protocatechuic acid, respectively.

Phenotypic analysis also revealed that other aromatic compounds are funneled to the benzoic acid pathway by *A. niger*. Deletion of *prcA* resulted in abolished growth on benzaldehyde, benzyl alcohol, *m*-hydroxybenzoic acid, *p*-anisyl alcohol, and *p*-anisic acid, indicating that these compounds are degraded via protocatechuic acid. As the deletion of *phhA* did not result in growth reduction on *m*-hydroxybenzoic acid, PhhA is not responsible for the *p*-hydroxylation of *m*-hydroxybenzoic acid. In addition, abolished growth of Δ*prcA* on *m*-hydroxybenzoic acid supports a previous study showing that *m*-hydroxybenzoic acid is converted into protocatechuic acid in *A. niger*.[29] Therefore, *A. niger* contains an *m*-hydroxybenzoate-*p*-hydroxylase that remains to be identified. Reduced growth was observed on cinnamic acid by Δ*bphA*, Δ*phhA*, Δ*prcA*, and Δ*prcA*Δ*hqdA*, indicating that cinnamic acid can be converted in multiple steps into benzoic acid and further degraded through the benzoic acid pathway. Also, the double deletion mutant Δ*prcA*Δ*hqdA* grown on cinnamic acid resulted in the accumulation of hydroxy-1,4-benzoquinone, supporting that cinnamic acid is degraded through the benzoic acid pathway. This is in agreement with a previous study, which revealed that *bphA*, *phhA*, and *prcA* are induced by cinnamic acid and cinnamyl alcohol (Lubbers et al., unpublished data).

Phenotypic analysis of Δ*bphA*, Δ*phhA*, and Δ*prcA* also revealed that in *A. niger* various aromatic compounds are not converted through the benzoic acid pathway. No phenotypes were observed on salicylic acid, 2,3-dihydroxybenzoic acid, and catechol. This agrees with a previous study where *A. japonicus* did not show any protocatechuic acid 3,4-dioxygenase activity toward salicylic acid or catechol[27] and that these genes were not induced by salicylic acid (Lubbers et al., unpublished data). Furthermore, we observed no growth reduction for Δ*prcA* on ferulic acid, vanillic acid, and veratric acid, indicating that they are not converted into protocatechuic acid, as was suggested previously in *A. japonicus*.[27] It is therefore more likely that these metabolites are converted into 2-methoxyhydroquinone as observed in *A. niger* C28B25.[30] Based on the phenotypic profile, enzyme assays, and transcriptome data, we suggested the updated version of the benzoic acid pathway in *A. niger* (Figure 7).

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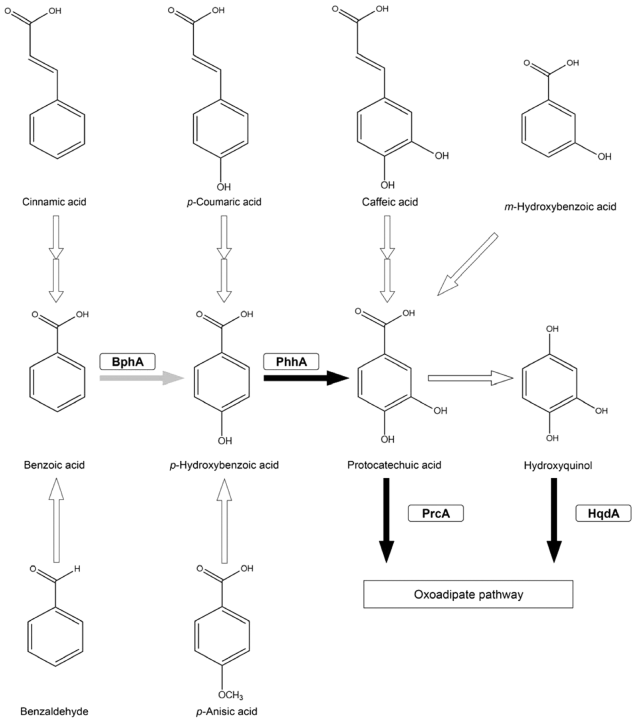

Figure 7. Proposed benzoic acid pathway in *A. niger* based on the results of this study. The characterized pathways are shown with gray arrows, the pathways proven biochemically in this study with black arrows, and the suggested pathways with white arrows.

CONCLUSIONS

In fungi, many aromatic compounds are degraded through the benzoic acid pathway. The identification and characterization of the novel *A. niger* enzymes PhhA, PrcA, and HqdA contribute greatly to a better understanding of the fungal aromatic metabolic pathways and the detoxification of toxic aromatic compounds by fungi. In addition, this study improves the understanding of the conversion of lignin-derived and non-lignin-derived aromatic compounds by fungi and can be applied to create new strategies to produce aromatic compounds or chemical building blocks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b04918.

Primers used in this study (Table S1); Venn diagram of the induced genes of *A. niger* N402 grown on *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, and protocatechuic acid for 2 h (Figure S1); accumulation of hydroxyquinol in *A. niger* deletion mutants (Figure S2); SDS-PAGE gels and Western blots of purified fractions of *A. niger* PhhA and PrcA (Figure S3); SDS-PAGE gel of purified fractions of *A. niger* HqdA (Figure S4); maximum likelihood phylogenetic tree of *A. niger* PhhA compared to selected fungal genomes (Figure S5); maximum likelihood phylogenetic tree of *A. niger* HqdA and PrcA compared to selected fungal genomes (Figure S6) (PDF)

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Author Contributions

R.J.M.L. conducted the experiments, analyzed the data, and wrote the manuscript. M.P. processed and analyzed RNA sequencing data. R.P.d.V. conceived and supervised the overall project. All authors commented on the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

BphA,benzoate-4-monooxygenase A; HqdA,hydroxyquinol 1,2 ring-cleavage dioxygenase A; MobA,*m*-hydroxybenzoate-*p*-hydroxylase; PhhA,*p*-hydroxybenzoate-*m*-hydroxylase; PobA,*p*-hydroxybenzoate-*m*-hydroxylase; PrcA,protocatechuic acid 3,4 ring-cleavage dioxygenase

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