Structure–Activity Relationships Delineate How the Maize Pathogen *Cochliobolus heterostrophus* Uses Aromatic Compounds as Signals and Metabolites

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The necrotrophic maize pathogen *Cochliobolus heterostrophus* senses plant-derived phenolic compounds, which promote nuclear retention of the redox-sensitive transcription factor ChAP1 and alter gene expression. The intradiol dioxygenase gene *CCHD1* is strongly upregulated by coumaric and caffeic acids. Plant phenolics are potential nutrients but some of them are damaging compounds that need to be detoxified. Using coumaric acid as an inducer (16 to 160 μ M), we demonstrated the rapid and simultaneous up-regulation of most of the β -ketoadipate pathway genes in *C. heterostrophus*. A *cchd1* deletion mutant provided genetic evidence that protocatechuic acid is an intermediate in catabolism of a wide range of aromatic acids. Aromatics catabolism was slowed for compounds showing toxicity, and this was strongly correlated with nuclear retention of GFP-ChAP1. The activity of a structure series of compounds showed complementary requirements for upregulation of *CCHD1* and for ChAP1 nuclear retention. Thus, there is an inverse correlation between the ability to metabolize a compound and the stress response (ChAP1 nuclear retention) that it causes. The ability to metabolize phenolics and to respond to them as signals should be an advantage to plant pathogens and may explain the presence of at least two response pathways detecting these compounds.

Low molecular weight phenolic compounds play important roles in the molecular dialog between plants and their symbionts and pathogens. Phenolics are widely distributed in the cuticles and walls of aerial plant organs and accumulate in response to pathogen attack (Bennett and Wallsgrove 1994; Mandal et al. 2010; Nicholson and Hammerschmidt 1992). Plant phenolic and related compounds provide signals for communication between plant roots and the rhizosphere bacteria *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) and *Agrobacterium tumefaciens* (Bhattacharya et al. 2010; Ehrhardt et al. 1996; Fisher and Long 1992). *Agrobacterium* spp. detect phenolic compounds released from wounded plant tissues with a two-component phosphorelay (Brencic and Winans 2005; Winans 1991). Demethylation of phenolic inducers by virH2 converts them to less active forms and provides a gateway to their metabolism through the β -keto-

adipate pathway (Brencic et al. 2004). Studies on *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) showed that, in this soft-rot pathogen, expression of genes encoding the type III secretion system is induced by *ortho*-coumaric acid and *trans*-cinnamic acid (Yang et al. 2008). The same genes are suppressed by *para*-coumaric acid (Li et al. 2009), which implies that phenolics provide both positive and negative signals in bacteria–plant interactions.

Among the eukaryotic pathogens, parasitic plants respond to host-derived aromatics and quinones (Bandaranayake et al. 2010; Palmer et al. 2004). Less is known about phenolics as signals for development and gene expression in fungal pathogens of plants. Phenolics and related compounds can act as signals and initiate at least two pathways in *Cochliobolus heterostrophus*, a necrotrophic foliar pathogen of maize belonging to the family Dothideomycetes. The redox-sensitive transcription factor ChAP1 (Lev et al. 2005) is retained in the fungal nucleus in response to 2.5 mM cinnamic acid and related compounds. Despite nuclear retention, antioxidant response genes are not induced, suggesting that the response to phenolics does not occur by a direct redox mechanism. In addition, coumaric and caffeic acids in the micromolar range (16 to 160 μ M) induce expression of the intradiol dioxygenase gene *CCHD1*. The two response pathways are triggered by different concentration ranges of the inducers. Furthermore, the most effective compound tested for induction of ChAP1 nuclear retention, cinnamic acid, was relatively ineffective for expression of *CCHD1*, suggesting different sensory mechanisms (Shanmugam et al. 2010).

A substrate or related compound often induces expression of the enzymes for its catabolism. A classic example is the quinate gene cluster, studied extensively in *Neurospora crassa* and *Aspergillus nidulans*. In *Neurospora* spp., the use of quinic acid (QA) as a carbon source depends on five structural genes and two regulatory genes, linked to form the *qa* gene cluster (Giles et al. 1991; Patel et al. 1981; Tang et al. 2011). Although the order and orientation of the genes varies, proximity clustering is highly conserved (Hane et al. 2007). Dehydroshikimic acid (DHS) dehydratase converts DHS to protocatechuic acid (PCA). Based on homology, we predicted that the dioxygenase encoded by *CCHD1* could open the aromatic ring of PCA, forming β -carboxymuconate, which is catabolized via the β -ketoadipate pathway. DHS, alternatively, can enter the shikimic acid pathway, which preserves the aromatic ring, “recycling” it for biosynthesis of aromatic amino acids. Thus, plant phenolics could induce synthesis of the enzymes for either or both these branches, allowing the fungus to use phenolics as a carbon source and as a starting point for aromatic amino acid biosynthesis. Therefore, we identified the quinate cluster in

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C. heterostrophus and tested whether the *qa* genes and *CCHD1* are coregulated by phenolic compounds. We also identified the structural requirements of the molecules required for these two fungal responses.

RESULTS

Regulation of genes encoding enzymes of the β -ketoadipate and associated pathways.

The pathway of aerobic catabolism of coumaric acid through the β -ketoadipate pathway in *Neurospora* spp. is shown in Figure 1. Preliminary results from a microarray study (*unpublished data*) identified candidate genes belonging to the upper β -ketoadipate pathway as upregulated by exposure to coumaric acid. The expression of these genes was studied by quantitative polymerase chain reaction (PCR) (Figs. 1 and 2). Joint Genome Institute (JGI) protein ID 31373 was the only aldehyde dehydrogenase significantly upregulated by coumaric acid, suggesting that it is 4-hydroxybenzaldehyde dehydrogenase. Monooxygenase (JGI protein ID 103177) was upregulated by coumaric acid and 4-hydroxybenzoic acid (4-HBA) (Fig. 2A).

The additional branch of the upper β -ketoadipate pathway converts (nonaromatic) QA to PCA (Fig. 1A). PCA is produced from QA by quinate 5-dehydrogenase (encoded by *qa-3*), 3-dehydroquininate dehydratase (encoded by *qa-2*), and dehydroshikimate dehydratase (encoded by *qa-4*), all members of the quinate cluster. QA permease is encoded by *qa-y*, the QA transcriptional regulator by *qa-F1*, and inositol phosphatase by *qa-x*. The *C. heterostrophus* genome has a quinate cluster located on Scaffold_15 of the JGI assembly (Fig. 1B; Table 1), containing seven genes that are collinear with the *Stagonospora nodorum* cluster (Hane et al. 2007). Fold induction of several of these genes by QA, coumaric acid, and PCA in the wild type (WT) and a *qa-F1* deletion mutant is shown in Figure 2B. As expected, these genes are upregulated by quinate in the WT and not in a *C. heterostrophus* Δqaf mutant, in which *qa-F1* was replaced by the hygromycin resistance cassette. Δqaf shows almost no growth on minimal medium, with 0.3% QA as sole carbon source (Supplementary Fig. S1A). Quinate cluster genes were also upregulated by PCA and coumaric acid, which are not directly related to quinate catabolism, suggesting co-regulation of the quinate and β -ketoadipate pathway genes.

Fig. 1. Pathway, regulation, and organization of genes encoding enzymes of the β -ketoadipate and associated pathways. **A**, Scheme of pathways initiating metabolism of phenolics, based on *Neurospora crassa* literature (Giles et al. 1991; Tang et al. 2011). The lower pathway includes intradiol dioxygenase CCHD1, β -carboxymuconate cyclase CMLE, 3-oxoadipate enol lactonase OEL, 4-carboxymuconalactone decarboxylase CMDE, and β -ketoadipyl CoA thiolase; the remainder of the pathway is referred as the “upper” part and includes the main catabolic pathway and an additional branch metabolizing quinic acid. Numbers and arrows indicate fold induction compared with control. **B**, Organization of the predicted quinate cluster. *qa-1F*, activator; *qa-1S*, repressor; *qa-y*, permease; *qa-x*, inositol phosphatase; *qa-2*, 3-dehydroquininate dehydratase; *qa-3*, quinate 5-dehydrogenase; *qa-4*, dehydroshikimic acid dehydratase.

Most of the genes of the lower β -ketoadipate pathway were upregulated by 0.16 mM coumaric acid as well as by caffeic acid (CA), 4-HBA, and ferulic acid (FA) (Fig. 2A). The strongest induction was observed for *CCHD1*, encoding the first enzyme of the lower β -ketoadipate pathway. Previously, we had (Shanmugam et al. 2010) failed to observe strong induction of *CMLE*, *OEL*, and *CMDE* by 3 mM CA, perhaps due to a different inducer concentration (0.16 mM in this study) and different levels of basal expression. Induction of *CCHD1* was very robust regardless of the conditions and was chosen here as a reporter. Following the hypothesis that the substrate or a related compound induces expression of genes encoding for enzymes for its catabolism, we explored the relationship between the structure of *CCHD1* inducers and their inducing activity. The induction by each compound at 0.16 mM is shown in Figure 3. Higher concentrations of 1.6, 3, and 5 mM did not cause a significantly higher induction level (for a concentration curve with increasing coumaric and CA concentrations, see Shanmugam and associates [2010]); other compounds were tested in preliminary experiments for this study [data not shown]. Inspection of the structures shows that the hydroxyl group in the position *para* to

the carboxyl side chain is absolutely required for induction of the *CCHD1* gene. Thus, PCA, CA, coumaric acid, and 4-HBA are the strongest inducers, whereas compounds lacking the *para*-hydroxy group or their *para*-methoxy substituted derivatives—benzoic acid (BA), cinnamic acid, 4-methoxycinnamic acid (4-MCA), and 3,4-dimethoxycinnamic acid (3,4-DMC)—have a near-basal level of *CCHD1* transcripts. The substituents at the *meta* position (-OH versus -H or -OCH₃) are not vital for inducing activity even though 3-methoxy compounds were much less active than their 3-hydroxy or unsubstituted analogues (PCA versus vanillic acid [VA], CA versus FA, or phloretic acid versus dihydroferulic [DHF]). All variations in the carboxylic side chain structure that we analyzed (COOH, CH₂-COOH, and CH=CH-COOH) were approximately equal in the extent to which they induced *CCHD1* expression, whereas catechol or chlorocatechol were ineffective.

Metabolism of phenolics.

PCA is a strong inducer; thus, induction of *CCHD1* may also result from production of PCA by upper pathway enzyme activity. To demonstrate the enzymatic conversion of *CCHD1*

Fig. 2. Regulation of genes for enzymes of the β -ketoadipate and associated pathways. **A**, Expression of β -ketoadipate pathway genes in response to different phenolics. Transcript levels (fold induction relative to control) are shown; *, **, and *** indicate significance at $P < 0.05$, 0.01, and 0.0001, respectively, by Student's t test. **B**, Regulation of predicted quinic acid (QA) cluster genes by QA, coumaric acid, and protocatechuic acid (PCA). WT = wild type. The cluster was identified by BLAST search of the *Cochliobolus heterostrophus* C5 genome. The functional annotation is from *Neurospora crassa*. Results are presented as log₁₀ values.

inducers to PCA, we analyzed catabolism of different phenolics in WT and in a *cchd1* deletion mutant. We exposed small fungal mats of WT and *cchd1* to 5 mM PCA in complete xylose medium (CMX) and analyzed UV spectra of the medium each day up to 7 days. PCA itself was catabolized by WT much more efficiently than by *cchd1* (Fig. 4A). After 4 days, 5 mM PCA was completely consumed by WT whereas, in *cchd1*, 72% PCA remained, compared with the initial concentration in the medium (Fig. 4B). PCA accumulation in *cchd1* was accompanied by formation of colored products (Supplementary Fig. S2).

In order to study the connection between the metabolism of phenolic compounds and fungal growth with phenolics, we subjected WT and *cchd1* to different concentrations of phenolics and calculated fungal growth rate relative to control. In the same experiment, we also followed catabolism of the phenolic compounds by simple spectroscopic UV analyses of the medium. We were able to use CMX as a growth medium, because β -ketoadipate pathway activity is not inhibited by xylose in *C. heterostrophus*. On the other hand, *C. heterostrophus* did not grow on phenolics as sole carbon source at subtoxic concentrations. In treatments with 5 mM CA, coumaric acid, phloretic acid, 4-HBA, VA, and DHF (Fig. 5C, group A), the supplied phenolic acid disappeared completely after 4 days of growth in both WT and *cchd1*, whereas PCA accumulated in *cchd1* due to the lack of β -ketoadipate pathway activity (Fig. 5B). FA and 3,4-DMC, applied at 5 mM, completely disappeared after 7 days. Cinnamic acid, catechol, 4-MCA, and BA (Fig. 5C, group B) were toxic at 5 mM; therefore, we applied them at lower concentrations. The feeding component was not detected after 7 days and PCA accumulation in *cchd1* was observed (Fig. 5B). As expected, PCA accumulated in the *cchd1* mutant

after exposure to QA (15 mM) whereas, when sinapic acid or catechol was applied, there was no accumulation of the intermediate PCA (Fig. 5).

There are routes for phenolic catabolism in addition to the pathways shown in Figure 1A. Catechol, for example, is predicted to enter the β -ketoadipate pathway at a stage after the reaction catalyzed by carboxymuconate isomerase in Figure 1A. Intradiol dioxygenases other than or in addition to CCHD1 could be important for these routes. Therefore, we compared the induction of all four intradiol dioxygenase genes in *C. heterostrophus* (Shanmugam et al. 2010) by catechol, chlorocatechol, and PCA at 0.16 mM. CCHD3 (ortholog of *C. albicans* 1,2-CTD responsible for phenol degradation through the *ortho*-cleavage pathway) (Tsai and Li 2007) was upregulated only approximately fivefold by catechol. CCHD4 was induced approximately 40-fold by chlorocatechol (Fig. 6). CCHD4 transcript abundance was low (data not shown); however, the enzyme might still be important. CCHD2 was upregulated by PCA (approximately 10-fold) and moderately upregulated (up to fivefold) in response to CA, coumaric acid, phloretic acid, and 4-HBA (Fig. 3). Catechol and chlorocatechol, however, had no effect on CCHD2 expression (Fig. 6). The four dioxygenase genes, most notably CCHD4 compared with CCHD1, show differential induction by compounds predicted to be metabolized by different routes. The *cchd1* mutant did not grow on QA as sole carbon source, indicating that the enzymes encoded by CCHD2,3,4 cannot substitute for CCHD1.

Structure activity relationships for nuclear retention of ChAP1 and toxicity.

The compounds that are relatively ineffective in inducing CCHD1 expression (Fig. 3) are also the most inhibitory to fun-

Table 1. Gene names, protein ID, and location in the assembly (Joint Genome Institute *Cochliobolus heterostrophus* database v1.0) and primers used for quantitative polymerase chain reaction

Gene description	Protein ID	Name	Scaffold	Sequence
Actin	31040	Act	12	s-TCAAGATCATCGCTCCTCCC as-GGACCGCTCTCGTCGTACTC
Quinic acid utilization factor	109440	qa-1F	15	s-ACGTCTCAGGGTTAATGCAAG as-ATCAGAGCGTTGTAAGGATCG
3-Dehydroquininate dehydratase	109429	qa-x	15	s-CCAATCGAATCATGAAGGCG as-CCTCGACAAACGGTATATCCAC
Quinate 5 dehydrogenase	109433	qa-3	15	s-CGCACGTAGAAAATGGATGAAAG as-GATTGTGCTTGTGAGACTGTG
Quinate permease	90894	qa-y	15	s-CGGTGTCTACATGTTCTTTGC as-GATGCCATGTGCTTTGTACG
Shikimate quinate dehydrogenase	90900	qa-1S	15	s-TCGTGGATTGTCTGGATGG as-TCCAACACCTCAACACTTCC
β -Ketoadipate succinyl CoA transferase	116701	CoA-T	5	s-CACCTGCTTTCCTACCTGAG as-GTGTGACTGTTTCTTTGCCCTG
β -Ketoadipate CoA thiolase	102076	Thiolase	5	s-TCGTCTTCTATCTGCCCTC as-GTCTATCTTCGCTGGGTCAAG
Aromatic ring monooxygenase	103177	MO	6	s-AGAGCAACTGGGTATCAACAT as-GATAGATCCAGCATCAGAGTGG
Aldehyde dehydrogenase	31373	ALD	14	s-ATCGATGCCTGGGACAATG as-GCGTGGTACTAATGGTCTGAC
4-Carboxymuconolactone decarboxylase	26048	CMDE	16	s-GCTTACTCATAACCTACCCACG as-TCATTTGGAGGTTTCGCTGG
3-Oxoadipate enol-lactonase	118718	OEL	16	s-TCCAGAAGCACAATGCCACG as-AGGCTTATCTTTTGATCCCGG
3-Carboxy-cis,cis-muconate cyclase	91531	CMLE	16	s-GGACATCCCAAAGCAAACGATA as-TGTTGCCATAGCCAGCGTAGT
Intradiol dioxygenase 1	110080	CCHD1	16	s-GTCGCACCGTATCTCTGATGTCC as-CTTTCGTTGGCACTCGCTTG
Intradiol dioxygenase 2	102317	CCHD2	5	s-ACGAATGGATGGCAGGAATC as-TGAATTCTTGTGCGACGTCAC
Intradiol dioxygenase 3	24644	CCHD3	1	s-TTTCGCGAGAGAGAATGAGCTT as-GCGACCTGCTTCATTCATCAG
Intradiol dioxygenase 4	112199	CCHD4	22	s-GATACGATCCCTCATAACTGC as-GCTTGCCTTTCATCCTTAAC

gal growth (Fig. 5B and C). FA and 3,4-DMC, the first maize compounds found to cause nuclear retention of ChAP1 (Lev et al. 2005), belong to this group (Fig. 5B, group B). Therefore, we investigated whether the ability of a given compound to promote green fluorescent protein (GFP)-ChAP1 nuclear retention is correlated with its toxicity. We measured half maximal inhibitory concentration (IC_{50}) of each phenolic acid by exposure of germinating WT spores to various concentrations (0.25 to 30 mM) and measured fungal mat dry weight after 4 days. The correlation between IC_{50} and GFP-ChAP1 nuclear retention is shown in Figure 7. Cinnamic acid and its methoxylated derivatives, while relatively ineffective in induction of *CCHD1* expression, caused strong nuclear GFP-ChAP1 re-

calization (Fig. 7) (Shanmugam et al. 2010) and were toxic to fungal growth. Cinnamic acid was the strongest inducer, causing complete translocation of GFP-ChAP1 from cytoplasm to nuclei at 0.6 mM within 10 min. For CA, weak nuclear relocalization was observed, and only in the germ tubes. For PCA, no nuclear relocalization was observed. Structural requirements for GFP-ChAP1 nuclear retention were the presence of a conjugated double bond (ferulic >>> DHF; cinnamic >>> benzoic) and the absence of the hydroxyl group on the aromatic ring at the *para* position (4-MCA >>> coumaric; 3,4-DMC > CA). Addition of the methoxy group at the *meta* position decreased nuclear retention (4-MCA > 3,4-DMC). Sinapic acid, which has methoxy groups at *meta* positions and hydroxyl at

Fig. 3. Structure-activity series for induction of *CCHD1* and *CCHD2*. Transcript levels induced by aromatic acids at 0.16 mM were measured by real-time quantitative polymerase chain reaction. The structure of each compound is indicated below the corresponding bars in the graph. The left-hand y-axis indicates *CCHD1* transcript level normalized by actin (calculated using the comparative $\Delta\Delta$ cycle threshold method; DataAssist Software version 2.0; Applied Biosystems). Error bars indicate standard deviation for at least three biological replicates.

Fig. 4. Catabolism of protocatechuic acid (PCA) in wild type (WT) and *cchd1*. **A**, UV absorption of PCA accumulated in WT (solid line) and *cchd1* mutant (dashed) after 3 days (thin lines) and 4 days (thick lines). **B**, Concentration of remaining PCA in WT and *cchd1* compared with the starting concentration, 5 mM (dashed line).

the *para* position, was ineffective in both *CCHD1* induction and GFP-ChAP1 nuclear retention. With the exception of BA (a well-known antimicrobial that might have an additional mechanism of toxicity), more toxic compounds were able to generate stronger ChAP1 nuclear retention signals.

DISCUSSION

C. heterostrophus, as a fungal pathogen of maize, is exposed to a range of host phenolic compounds, of which the two cinnamic acid derivatives coumaric acid and FA are the most abundant (Shanmugam et al. 2010). When *C. heterostrophus* senses the phenolic compounds, it is likely to perceive them in at least two different ways: i) as potential nutrients to be metabolized and ii) as potentially damaging compounds causing stress responses and needing to be detoxified (Shanmugam et al. 2010). The aim of our work was to investigate the interplay between metabolic and stress responses. In and on the host plant, the pathogen is exposed to a number of phenolic compounds in various concentration ranges. The type and level of

phenolics could depend on the specific host phenolic composition as well as on the ability of fungus to release phenolics from the cuticle by the action of fungal cutinases (Kolattukudy 1995) and rapidly metabolize them by the activity of nonspecific enzymes (hydrolases and monooxygenases).

Metabolism of phenolics in the presence of oxygen via the β -ketoadipate pathway has been extensively described in prokaryotes and, to some extent, in eukaryotes (Harwood and Parales 1996). Upper β -ketoadipate pathway genes encode enzymes converting cinnamic acid derivatives to the common intermediate PCA via aromatic ring oxidation and elimination of the allyl side chain through coenzyme A esterified intermediates. The lower β -ketoadipate pathway starts with ring cleavage by incorporating two oxygen atoms into PCA and, subsequently, converts β -carboxymuconate to acetyl CoA and succinyl CoA. Using coumaric acid as an inducer in the micromolar range, we were able to demonstrate the rapid and simultaneous upregulation of most β -ketoadipate pathway genes in *C. heterostrophus* (Figs. 1 and 2). Furthermore, we explored the structure–activity relationships of a series of aromatic compounds and found that

Fig. 5. Metabolism of phenolics and fungal growth. **A**, UV absorption of 5 mM authentic applied 4-hydroxybenzoic acid (4-HBA), cinnamic acid, and sinapic acid (solid line). Protocatechuic acid (PCA) accumulated in wild type (WT) (solid line) and *cchd1* mutant (dashed line) after 4 days. Average spectra from three biological replicates are shown, with standard deviation (SD). **B**, Concentration of remaining PCA in WT and *cchd1* after 4 days for group A (ferulic acid, 3,4-dimethoxycinnamic acid [3,4-DMC], and sinapic acid; 5 mM) and after 7 days for cinnamic acid, benzoic acid, catechol (2 mM), 4-methoxycinnamic acid (4-MCA) (1 mM), and quinic acid (QA) (15 mM, noted in the figure). DHF = dihydroferulic. Bars indicate means with SD for three biological replicates. **C**, Fungal growth rate (percentage of dimethyl sulfoxide control) of WT and *cchd1* with different aromatic compounds and QA; conditions as for B. Bars indicate growth rate calculated from pooled dry weight of three biological replicates with similar appearance.

expression of the key junction gene of the pathway (*CCHD1*) could be induced by phenolic acids containing a hydroxyl group at position 4, whereas the side chain does not seem to be important (Fig. 3). These requirements support the classical hypothesis that the substrate (PCA) or a related compound induces expression of genes encoding for enzymes for its catabolism. Fungal aromatic acid metabolism has been studied for more than 50 years, beginning with studies on β -ketoadipate pathway enzymes in *N. crassa* (Gross and Tatum 1955). The complete picture of fungal phenolic degradation, however, is still unavailable. The reason for this may be the diverse strategies evolved by different fungi, presumably according to their specific niche or host. A strain of *Rhodotorula mucilaginosa* isolated from *p*-hydroxybenzoate medium could not metabolize catechol (Cook and Cain 1974). In contrast, in a WT strain of *A. nidulans*, the catechol and PCA branches of the β -ketoadipate pathway are both active (Kuswandi and Roberts 1992).

Based on our previous finding that some *CCHD1*-inducing phenolics also trigger a stress-related response by nuclear re-

Fig. 6. Expression of intradiol dioxygenase genes *CCHD1* to *CCHD4* in response to catechol, chlorocatechol, and protocatechuic acid (PCA). Transcript levels (fold induction relative to control) are shown as $\log_{10}$ values.

tention of the transcription factor ChAP1, we subjected germinating spores to a wide range of phenolics at different concentrations and followed the intensity of GFP-ChAP1 nuclear retention by confocal microscopy. For strong ChAP1 nuclear retention, the unsaturated side chain is important, yet the active compounds for this response do not need the phenolic hydroxyl group, although some structures with the *para* hydroxyl are active (Fig. 7). Oxidative stress promotes nuclear retention of the redox-sensitive transcription factor ChAP1 (ortholog of yeast Yap1), and there is also evidence that plant signals cause nuclear retention in the absence of direct oxidative stress (Lev et al. 2005). Plant phenolic compounds that cause nuclear retention of GFP-ChAP1 (Shanmugam et al. 2010) are, indeed, predicted to act as antioxidants rather than oxidants. Sensing of phenolics (polyphenolic flavonoids) by Yap1 and Pap1 transcription factors was reported in *Saccharomyces cerevisiae* and *S. pombe*, respectively (Maeta et al. 2007). These phenolics directly cause the formation of small amounts of hydrogen peroxide (0.4 mM), particularly at alkaline pH. This H_2O_2 is apparently responsible for Yap1 nuclear retention, because addition of catalase prevents the response (Maeta et al. 2007). In *C. heterostrophus*, however, formation of such a low hydrogen peroxide level is ineffective, and catalase did not inhibit ChAP1 nuclear retention in response to plant extracts (Lev et al. 2005). It is difficult, though, to completely exclude a mechanism based on oxidant generation from available data, because spatially resolved measurements of redox state at the subcellular level are not yet available. Instead, the structural requirements found in this study support direct detection by receptors. Intracellular oxidants might then be generated biochemically. Alternatively, an additional transcription factor could interact with ChAP1 to retain it in the nucleus. Such an interaction might override the lack of activation by oxidants, which cause a conformational change that masks the nuclear export signal of ChAP1.

GFP-ChAP1 nuclear retention was correlated with toxicity for the most of the phenolic acids (Figs. 7 and 8). Toxic and inhibitory effects of phenolics have been shown in *C. heterostrophus* and other fungi (Bisogno et al. 2007; Sethuraman et al. 1998; Shanmugam et al. 2010). The toxicity of compounds in which the *para* hydroxyl substituent is replaced by a methoxy group was much stronger than for the corresponding hydroxylated compounds (Shuen and Buswell 1992). Monomethoxy-

Fig. 7. Correlation between nuclear retention and toxicity. The reciprocal of the concentration resulting in near-complete nuclear retention of a green fluorescent protein-ChAP1 fusion protein calculated in relative units is shown on x axis, and the phenolics' toxicity is shown on y axis as the reciprocal of IC_{50} concentration (mM).

lated compounds were more toxic than dimethoxylated to white rot fungi (Sethuraman et al. 1998). This is consistent with the requirements for ChAP1 nuclearization as shown here. We note that yeast Yap1 is retained in the nucleus in response to some chemical stressors in addition to oxidative stress (Azevedo et al. 2003; Lee et al. 1999). The higher toxicity of methoxylated phenolics (especially at position 4) compared with hydroxylated phenolics may be attributed to the absence of an effective mechanism of degradation of the former.

It would be interesting to address the question of whether GFP-ChAP1 nuclear retention could be responsible for regulation of genes other than the known antioxidant genes. We are looking for additional ChAP1 targets by microarray analysis (work in progress). However, the strong negative correlation between metabolic responses in terms of *CCHD1* induction and GFP-ChAP1 nuclear retention (Fig. 8) hints at an indirect connection between these two fungal responses to phenolics. For example, ChAP1 nuclear retention might act as a signal to promote a stress response to compounds that cannot be rapidly metabolized to PCA (Fig. 5).

For ChAP1 nuclear retention, the unsaturated side chain is important, yet the active compounds for this response do not need the phenolic hydroxyl group. Nevertheless, some structures with the *para* hydroxyl are active. Thus, the conjugation model (Joubert et al. 2002) does not seem to apply to induction of *CCHD1* expression. It might apply, however, to nuclear retention of ChAP1.

Specific receptors would be of great interest for understanding the signaling pathways and also as potential antifungal targets. The micromolar concentration range and specific structural requirements for phenolics to induce expression of *CCHD1* (Shanmugam et al. 2010; this study) suggest that specific receptors are involved. This prediction can be tested experimentally in *C. heterostrophus*. Based on what is known from bacteria, two-component system sensory histidine kinases (HK) would be candidates for processing the sensory input. Genes encoding HK and their response regulators have been identified in *C. heterostrophus* (Robbertse et al. 2003). However, our tests for induction of *CCHD1* using mutants in the four known response-regulator genes (*SSK1*, *SKN7*, *RIM15*, and *REC1*) (Oide et al. 2010) and one HK gene (*NIK1*) (Catlett et al. 2003b) provided no evidence that HK-RR pathways are involved: WT response was observed in all these mutants. Likewise, loss of the following genes did not affect *CCHD1* induction (Supplementary Fig. S3): the single gene encoding a heterotrimeric G protein G β subunit (*CGB1*) (Ganem et al. 2004), a G α subunit

gene (*CGA1*) (Horwitz et al. 1999), or the three mitogen-activated protein kinase genes of *C. heterostrophus* (*CHK1*, *HOG1*, and *MPS1*) (Igbaria et al. 2008). If a novel pathway is, indeed, involved, an unbiased genetic search will be needed to identify the sensors. A positive, high-throughput screen for loss of function of the pathway could be designed based on the colored products formed in a *cchd1* mutant strain.

The *Neurospora* ortholog of *CCHD1* is induced by QA and has a *qa* response element in its promoter (Tang et al. 2011). We found that *N. crassa CCHD1* is induced by CA, as in *C. heterostrophus* (data not shown). Therefore, we hypothesized that the sensory pathway in *C. heterostrophus* might be similar to that in *Neurospora* spp. Loss of the ortholog of QA utilization factor *qa-1F* prevented upregulation of a member of the *C. heterostrophus qa* cluster (Fig. 2B). Induction of *CCHD1*, however, was unaffected in $\Delta qa1$ (data not shown). Thus, the sensory permease mechanism known from *Neurospora* spp. is not responsible for regulation of *CCHD1* in *C. heterostrophus*. It is still possible, though, that a similar permease-dependent molecular mechanism is involved, so this would be one model to pursue and test.

We expect that the structural features identified here will guide identification of the receptors. The fungal receptors could provide targets for antifungal strategies designed to interfere with perception of host signals. Inhibiting the sensory pathways that bacteria use to detect plant phenolics could provide new ways to fight plant disease (Charkowski 2009). Indeed, it is possible to interfere with signaling for VIR gene expression in *Agrobacterium* spp. (Hess et al. 1991; Maresh et al. 2007). The sensory pathways of eukaryotic pathogens may be different but the bacterial model could, nevertheless, apply in a general sense to fungal pathogens. Phenolics are being studied for their potential as antifungals, targeting the fungal antioxidant response (Kim et al. 2006, 2007). Flavonoids may act directly to modulate cellular signaling pathways, in addition to their roles as antioxidants (Williams et al. 2004). Knowledge of the structural requirements for phenolic and aromatic signaling molecules to be detected by a fungal pathogen may facilitate identification of specific receptors for these compounds.

MATERIALS AND METHODS

Fungal strains and growth media.

WT C4 (*MAT1-2 Tox1+*) and GFP-ChAP1 strains of *C. heterostrophus* were described previously (Lev et al. 2005; Turgeon et al. 1987). Standard growth medium was CMX. Basal medium

Fig. 8. Structure-activity series for *CCHD1* expression and for nuclear localization of ChAP1. Data are compared graphically to show co-dependence of toxicity and nuclear retention of green fluorescent protein-ChAP1 on the structure of the inducing compounds, while *CCHD1* expression has different structural requirements.

(BM) was prepared as CMX except that xylose, yeast extract, and casein were replaced with peptone at 20 g/liter. Minimal medium (MMX) was the same as CMX except without yeast extract and casein, and 0.3% instead of 1% xylose. MM-QA was the same as MMX except that xylose was replaced by 0.3% QA.

The *Δcchd1* and *Δqaf* integration DNA was constructed using the split-marker method to amplify a linear DNA construct consisting of the hygromycin-selectable marker flanked on both sides with *CCHD1* untranslated regions targeting the integration to the genome (Catlett et al. 2003a). Reactions with primer pairs 1 and 2 (Supplementary Table S1) were carried out using *C. heterostrophus* genomic DNA as template. Reactions with pairs 3 and 4 were carried out using pBlueScript-HyG (derived from pUCATPH) (Lu et al. 2003) plasmid DNA as template. To construct the 5' side of the final sequence, round two used the products of pairs 1 and 3 as template and FP1,NLC37 as primers; for the second half, the products of pairs 2 and 4 as template and NLC38,RP2 as primers were used. The two final products were integrated into the *C. heterostrophus* genome by double-crossover recombination, resulting in reconstruction of the complete hygromycin resistance cassette replacing the entire predicted coding region of the gene of interest. Fungal protoplasts were prepared and transformants selected for hygromycin resistance as described (Turgeon et al. 1987; Wirsal et al. 1996).

Chemicals.

Trans-cinnamic acid, CA, FA, *para*-coumaric acid, 3,4-DMC, chlorogenic acid, sinapic acid, 4-HBA, 3-(4-hydroxyphenyl)-propionic (phloretic) acid, BA, 4-MCA, VA, catechol, and chlorocatechol were purchased from Sigma-Aldrich. 3-(4-Hydroxy-3-methoxyphenyl) propionic acid (DHF), QA, and 3,4-dihydrobenzoic (protocatechuic) acid were purchased from Alfa Aesar (Haysham, Lancashire, England). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Buchs, Switzerland).

Expression of the dioxigenase and other genes.

To assay gene expression, cultures were grown on stationary liquid CMX for 4 to 5 days, and fungal mats were transferred to BM or to CMX with phenolic compound and incubated at room temperature for 30 min. Phenolics were added from stock solutions of 60 or 120 mg/ml in DMSO to reach the required final concentrations. RNA isolation was done as described (Shanmugam et al. 2010). For cDNA synthesis, 1 µg of RNA was used for reverse transcription with random primers following the protocol supplied with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, U.S.A.). Abundance of transcripts was measured by quantitative reverse-transcription PCR. The *C. heterostrophus* actin gene (protein ID 31040) was used as a "housekeeping" gene to balance the amount of cDNA.

Measurement of nuclear retention.

For a semiquantitative measure of nuclear retention, spores of a reporter line expressing GFP-ChAP1 (Lev et al. 2005; Shanmugam et al. 2010) were inoculated in water on a glass slide and allowed to germinate. After 3 to 4 h, the compounds were added to the desired final concentration of 0.1 to 5 mM for each phenolic compound, and the minimal active concentration was established for each aromatic acid (Supplementary Fig. S4A). Considering the effect of the exposure time, we used, for all experiments, 10 min of exposure at room temperature. Images were scanned using laser excitation at 488 nm, and the fluorescence emission was detected at 500 to 550 nm with a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany). The images were analyzed using LSM 5 software, version 4.0.

Relative GFP-ChAP1 nuclear retention (R) was estimated as $R = (\text{mean fluorescence intensity in the area of 10 nuclei [20 to 30 } \mu\text{m}^2]) / ([\text{mean fluorescence intensity in the cytoplasm of the same area (20 to 30 } \mu\text{m by } \mu\text{m})] \times [\text{minimal active concentration applied (mM)}])$.

In order to compare between different biological experiments, we always set the R value for cinnamic acid equal to 1, and normalized R values for other components.

Metabolism, fungal growth, and toxicity of phenolics.

To assay fungal growth on phenolics, 4.5-mm disks from 6- to 7-day-old fungal cultures were transferred to stationary liquid CMX with phenolic compounds and incubated at room temperature for 3 to 4 days. Phenolics were added to the required final concentration from stock solutions of 60 or 120 mg/ml in DMSO. To follow metabolism of phenolics by the fungal culture, 20 µl of growth medium was taken at zero time and each day for up to 7 days of growth. UV and visible absorption spectra of culture media were measured with a Nanodrop ND-1000 spectrophotometer. For quantitative estimation of PCA accumulated in the medium, the concentration was calculated from the spectrum using a calibration curve. Fungal dry weight from each treatment with phenolics (Fig. 5C) was divided by the control (DMSO only) dry weight to calculate growth percentage. To obtain the correlation shown in Figure 7, we calculated the IC₅₀ using growth from fungal spores at a concentration of 400 spores/ml for 4 days on different concentrations of each phenolic acid.

Statistical analyses were performed with GraphPad Prism software (version 5.00; GraphPad Software, San Diego, CA, U.S.A.). Unless otherwise indicated, the threshold level chosen for comparison of means was $P < 0.05$ by Student's *t* test (one-tailed, non-paired, equal variance).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Cochliobolus heterostrophus genome v1.0 database:
genome.jgi-psf.org/CocheC5_1/CocheC5_1.home.html