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Molecules of Interest

HC-toxin

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Abstract

HC-toxin is a cyclic tetrapeptide of structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid. It is a determinant of specificity and virulence in the interaction between the producing fungus, *Cochliobolus carbonum*, and its host, maize. HC-toxin qualifies as one of the few microbial secondary metabolites whose ecological function in nature is understood. Reaction to *C. carbonum* and to HC-toxin is controlled in maize by the *Hm1* and *Hm2* loci. These loci encode HC-toxin reductase, which detoxifies HC-toxin by reducing the 8-carbonyl group of Aeo. HC-toxin is an inhibitor of histone deacetylases (HDACs) in many organisms, including plants, insects, and mammals, but why inhibition of HDACs during infection by *C. carbonum* leads to disease is not understood. The genes for HC-toxin biosynthesis (collectively known as the *TOX2* locus) are loosely clustered over >500 kb in *C. carbonum*. All of the known *TOX2* genes are present in multiple, functional copies and are absent from natural toxin non-producing isolates. The central enzyme in HC-toxin biosynthesis is a 570-kDa non-ribosomal synthetase encoded by a 15.7-kb open reading frame. Other genes known to be required for HC-toxin encode alpha and beta subunits of fatty acid synthase, which are presumed to contribute to the synthesis of Aeo; a pathway-specific transcription factor; an efflux carrier; a predicted branched-chain amino acid aminotransferase; and an alanine racemase.

Keywords: *Cochliobolus*; Maize; Histone deacetylase; Cyclic peptide

1. Introduction

An enduring theme in the study of the interactions between plants and their pathogens is the elucidation of the mechanisms of specificity. Why do only certain pathogens attack certain plants, and, conversely, why are only certain plants susceptible to certain pathogens? A number of fungal secondary metabolites have been shown to have biological specificities that correspond with the specificity (host range) of the producing fungi. These compounds, known as host-selective or host-specific toxins (HSTs), can therefore account for the disease specificity of these host/pathogen interactions. There are now about 20 HSTs that have been biologically and chemically characterized (Walton, 1996). HSTs have to date been found only in plant pathogenic fungi, and they are particularly well-represented in two related genera, *Alternaria* and *Cochliobolus*.

Cochliobolus carbonum causes Northern corn leaf spot disease, which was first noted in the US in 1938 (Ullstrup, 1941). *C. carbonum* is also known by its anamorph (asexual) stage as *Helminthosporium carbonum* or *Bipolaris zeicola*. Originally there were two known races of *C. carbonum*, races 1 and 2. Race 2 is a weak pathogen of many corn lines. Race 1 is also weakly pathogenic on most lines but is highly virulent on maize that is homozygous recessive at the Mendelian loci *Hm1* and *Hm2*. Spontaneous mutation in both genes, due to a transposon insertion and a deletion, combined with inbreeding for hybrid seed production, led to the appearance of the disease (Multani et al., 1998). Pathologists now recognize additional races of *C. carbonum*, but none of them are known to produce HSTs (Walton et al., 1997).

C. carbonum is one of the most virulent plant pathogens ever observed by this author. Young seedlings are killed to

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Fig. 1. Infection of maize by *Cochliobolus carbonum* race 1 (HC-toxin-producing). The plants in the foreground are genotype *hm1*/*hm1 hm2*/*hm2* (susceptible). A few plants were inoculated by spraying conidia and the other plants became infected by natural spread. The plants in the background are genotype *Hm1*/- (resistant). Photograph was taken by Holly Ruess and is used by generous permission of Professor Guri Johal, Purdue University.

the ground within a few days after inoculation with a moderate (10^4/ml) spore concentration, and the fungus can colonize all parts of maize plants, including the stems and cobs of mature plants (e.g., Baidyaroy et al., 2001). In the field, plants are completely killed (Fig. 1).

Scheffer and Ullstrup (1965) reported that the culture filtrate of *C. carbonum* selectively inhibits root growth of susceptible maize. This was conceptually important as one of the first clear demonstrations of host selectivity being conferred by a pathogen metabolite. Interest in HSTs was especially stimulated by the Southern Corn Leaf Blight (SCLB) epidemic of 1970, which destroyed ~15% of that year's crop. The SCLB epidemic was caused by a new race of *Helminthosporium maydis* (perfect stage *Cochliobolus heterostrophus*), called race T, which produces a polyketide HST called T-toxin (Walton, 1996).

2. Structure of HC-toxin

In the 1970s, Pringle and co-workers purified and partially determined the structure of HC-toxin. They determined that the molecule was a peptide containing Ala and Pro in the ratio of 2:1 (Pringle, 1971). Years later, several groups working independently established the structure of HC-toxin (Liesch et al., 1982; Gross et al., 1982; Walton et al., 1982). Walton et al. (1982) also determined the chiralities of the four amino acids, but mistakenly concluded that the second Ala had the L configuration. In the course of determining the conformation of HC-toxin by NMR, Rich and colleagues deduced that the second Ala has the D configuration, which was subsequently confirmed experimentally and by total synthesis (Kawai et al., 1983; Kawai and Rich, 1983).

The complete structure of HC-toxin is cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Fig. 2). Like other cyclic tetrapeptides, HC-toxin can exist in at least two relatively stable conformations depending on the solvent (Kawai et al., 1983). As a result it is soluble in chloroform as well as water and lower alcohols. There are probably no significant hydrophilic or hydrophobic barriers to its movement through living tissues.

Minor forms of HC-toxin contain hydroxyPro in place of Pro, or Gly in place of D-Ala. The different forms are active in the root growth bioassay at 0.2–20 μg/ml. A fourth minor form has apparently not been chemically characterized (Rasmussen and Scheffer, 1988).

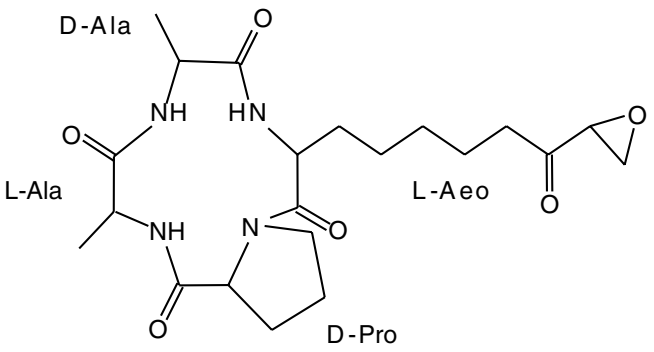


Fig. 2. Structure of the major form of HC-toxin.

HC-toxin loses activity when the epoxide is hydrolyzed by acid (Walton and Earle, 1983; Ciuffetti et al., 1983). Synthetic compounds in which the epoxide is replaced by other alkylating groups such as chloromethyl, diazomethyl ketone, or hydroxamic acid functionalities are biologically active (Furumai et al., 2001; Shute et al., 1987). In a prescient experiment, the 8-carbonyl of Aeo was also shown to be required for activity of HC-toxin (Kim et al., 1987) (see below).

3. Biosynthesis of HC-toxin

Even before HC-toxin was discovered, race 1 and race 2 isolates of *C. carbonum* had been shown to differ by a single genetic locus, which is now called *TOX2* (Nelson and Ullstrup, 1961). Later, it was discovered that *TOX2* controls production of HC-toxin (Scheffer et al., 1967). With the discovery of the structure of HC-toxin, it was a puzzle how a single locus could control the biosynthesis of an obviously complex metabolite. The mystery was heightened by the discovery that the biosynthesis of other HSTs, particularly T-toxin by *C. heterostrophus* and victorin by *C. victoriae*, is also under the control of single-genes (called *TOX1* and *TOX3*, respectively). These three HSTs are not chemically closely related. In the early days of fungal genetics it was not yet known that secondary metabolite genes are often clustered in fungi (and hence co-segregate as a single locus), and also that most fungi have highly polymorphic karyotypes, which reduces crossing over. Both of these processes contribute to the structure and inheritance of *TOX1* and *TOX2* (e.g., Baker et al., 2006).

In the late 1960s, Fritz Lipmann, Horst Kleinkauf, and co-workers established the enzymology of biosynthesis of non-ribosomal peptides such as gramicidin and tyrocidine (for recent reviews, see Walsh, 2004; Walton et al., 2004). Following these bacterial precedents, we identified enzyme activities in *C. carbonum* with the hallmarks of a putative HC-toxin synthetase, notably D-Ala-dependent ATP/PP_i exchange (Walton, 1987; Walton and Holden, 1988). Purification of the enzyme and screening of an expression library with an antibody yielded a partial cDNA for a gene encoding a non-ribosomal peptide synthetase, which is now called *HTS1*. Chromosome walking from the cDNA identified a contiguous 22-kb region of DNA that is present in all toxin-producing isolates but absent in toxin non-producing isolates. Two postdoctoral associates, John Scott-Craig and Dan Panaccione, started at opposite ends of this region and sequenced towards each other. Over 100 gels later (this was before the days of automated sequencing), they concluded that most of the 22-kb toxin-unique region was a single open reading frame of 15.7-kb, encoding a 570-kDa polypeptide (Panaccione et al., 1992; Scott-Craig et al., 1992). The gene is called *HTS1*. At the time, this was the largest known single-chain protein.

The protein product of *HTS1*, called Hts1, has four predicted adenylation domains, one for each amino acid in HC-toxin. The order of activation is most likely Pro, Ala, Ala, and Aeo. In addition, Hts1 has one epimerase module between domains 1 and 2, which is responsible for epimerizing L-Pro to D-Pro. The absence of an epimerizing module after domain 3, for producing D-Ala, was initially puzzling, but this was clarified by the later discovery of another gene, *TOXG*. *TOXG* encodes an Ala racemase, which produces D-Ala for incorporation by Hts1 into HC-toxin. A *TOXG* mutant is pathogenic because it can still make the minor form of HC-toxin that contains Gly in place of D-Ala (Cheng and Walton, 2000).

Additional genes that have been found for HC-toxin biosynthesis include *TOXA*, a putative efflux carrier of the Major Facilitator Superfamily. *TOXA* is immediately adjacent to *HTS1* (Pitkin et al., 1996). *TOXC* encodes a fatty acid synthase beta subunit and is probably involved in biosynthesis of the decanoic acid backbone of Aeo (Ahn and Walton, 1997). *TOXF* encodes a putative branched-chain amino acid transaminase (Cheng et al., 1999). Like *HTS1* and *TOXA*, *TOXF* and *TOXG* are also immediately adjacent to each other. *TOXF* encodes a predicted branched-chain amino acid transaminase (Cheng et al., 1999); it is required for HC-toxin biosynthesis but its biochemical function is unknown. *TOXE* encodes an unique pathway-specific transcription factor that binds to the promoters of the known *TOX2* genes (Pedley and Walton, 2001).

TOX2 presents particular experimental challenges compared to other fungal secondary metabolite clusters. The cluster cannot be defined by chromosome walking because the genes are not tightly clustered and are embedded in highly repetitive sequences. Furthermore, all of the known genes are present in all natural toxin-producing isolates in two or three functional copies. No toxin phenotype is seen unless all of the copies of a particular *TOX2* gene are disrupted (e.g., Panaccione et al., 1992; Cheng and Walton, 2000).

TOX2 has been mapped using rare cutting restriction enzymes, the introduction of artificial restriction sites, and pulsed field gel electrophoresis. The cluster extends more than 500 kb, interspersed with much repetitive DNA and probably additional genes involved in HC-toxin biosynthesis (Ahn and Walton, 1996; Ahn et al., 2002). All copies of the known *TOX2* genes are in this region, with the exception of one copy of *TOXE* that is on a smaller chromosome in some isolates (Ahn and Walton, 1996). The *TOX2* region is meiotically unstable; in some crosses between toxin-producing isolates ~5% of the random ascospore progeny lack one or more copies of the *TOX2* genes (Pitkin et al., 2000).

We have recently started to search for new *TOX2* genes based on high throughput sequencing. A bacterial artificial chromosome (BAC) library was screened with known *TOX2* genes and positive BACs shotgun sequenced to ~4× coverage. To date we have identified several new putative *TOX2* genes. One encodes a fatty acid synthase alpha

subunit (which would cooperate with the product of *TOXC*) and several encode P450s, one or more of which could contribute to the synthesis of the epoxide group (K. Ohtani and J.D. Walton, unpublished results).

4. Specificity of HC-toxin

Resistance to HC-toxin and to HC-toxin-producing isolates of *C. carbonum* is conferred by the dominant alleles of *Hm1* and *Hm2*. Either one alone gives resistance, but *Hm2* is weaker and expressed more strongly in older tissues. To test whether detoxification could be the basis of resistance conferred by *Hm1* and *Hm2*, radiolabelled HC-toxin was produced by feeding fungal cultures with [^{3}H]D-alanine. Since D amino acids do not enter primary metabolism, this precursor is more specific than L-Ala or L-Pro. The resulting [^{3}H]HC-toxin, with a specific activity of ~70 mCi/mmol, was fed to maize seedlings or leaf discs, and the single product produced was purified and characterized as HC-toxin with the 8-ketone of Aeo reduced to the 8-alcohol (Meeley and Walton, 1991). This compound had earlier been shown to be much less toxic than native HC-toxin (Kim et al., 1987).

When fed to seedlings through the transpiration stream, resistant (*Hm1*/-) and susceptible (*hm1*/*hm1*) maize metabolize HC-toxin equally well, which originally suggested that metabolism is not the basis of specificity (Meeley and Walton, 1991). However, when [^{3}H]HC-toxin is incubated with cell-free extracts of etiolated seedlings, in the presence of NADPH, only plants of genotype *Hm1*/- metabolize HC-toxin (Meeley and Walton, 1991; Meeley et al., 1992). This enzyme is called HC-toxin reductase.

A segregating population of maize was analyzed for the ability to metabolize HC-toxin in vitro and simultaneously for susceptibility to *C. carbonum*. There was an absolute genetic co-segregation of ability to metabolize and disease resistance (Meeley et al., 1992). Furthermore, plants that are mutated at *Hm1* by transposon tagging are susceptible, and revertants due to spontaneous transposon excision are resistant (Meeley et al., 1992), consistent with *Hm1* controlling or encoding HC-toxin reductase. Almost at the same time, *Hm1* was isolated by transposon tagging, and it is predicted to encode an NAD(P)H-dependent reductase, i.e., HC-toxin reductase itself. We conclude that the basis of specificity of HC-toxin and hence *C. carbonum* is due to detoxification by the product of *Hm1* (Johal and Briggs, 1992).

Plants of genotype *hm1*/*hm1* and *hm2*/*hm2* have no discernible phenotype other than susceptibility to *C. carbonum*. On this basis, it appears that *Hm1* and *Hm2* apparently evolved and have been maintained in maize solely to protect against fungi that produce HC-toxin and related compounds (see below).

We could detect in vitro HC-toxin reductase activity in other cereals (barley, sorghum, oats, and wheat) but not in several dicots (Meeley and Walton, 1993). Therefore, HC-toxin reductase is not confined to maize, but the basis of insensitivity of dicots is unknown. Orthologs of the *Hm1* gene have been described in other cereals including rice and barley (Han et al., 1997).

5. Mode of action of HC-toxin

The name of this compound is both typologically awkward and a misnomer. "HC" derives from *H. carbonum*, which has been superseded by the taxonomic epithet *C. carbonum*. Journal editors struggle with "HC" as an apparent abbreviation (which it is not) and with the hyphen. Most importantly, HC-toxin is not even a toxin in the strict sense, since it does not kill cells. HC-toxin actually promotes the survival of non-dividing maize leaf mesophyll cells (Wolf and Earle, 1991). One of the most striking effects of compounds related to HC-toxin (see below) is to cause morphological reversion of oncogene-transformed mammalian cells, without cell death (e.g., Yoshida and Sugita, 1992). The low cytotoxicity of HC-toxin is consistent with its mode of action and with the fact that compounds with a similar mode of action are being developed as chemotherapeutic agents (see below).

HC-toxin inhibits root growth of susceptible (genotype *hm1*/*hm1*) maize at 0.5–2 μg/ml. Its selectivity is ~100-fold, so resistant maize (*Hm1*/-) is inhibited at ~100 μg/ml (Walton et al., 1982; Rasmussen and Scheffer, 1988). Although a differential selectivity of 100-fold is less than some other HSTs (for victorin the differential is $>10^6$), it is apparently sufficient to tilt the outcome of the disease interaction from very resistant to very susceptible. HC-toxin inhibits growth of other plants (e.g., in root and seedling assays) at 20–50 μg/ml (J.D. Walton, unpublished results). Inhibition of cell division (cytostasis) and detransformation of mammalian cells is induced by HC-toxin at 10–100 ng/ml (Furumai et al., 2001; Walton et al., 1985).

In 1992, Yoshida and Sugita reported that a compound closely related to HC-toxin, called trapoxin [cyclo(L-Phe-L-Phe-D-Pip-L-Aeo)], causes detransformation of oncogene-transformed mammalian cells. They had earlier shown that trichostatin, which causes the same effect, was an inhibitor of histone deacetylase (HDAC) (Yoshida et al., 1990). The logical deduction was that HC-toxin was also an HDAC inhibitor, and to test this hypothesis my lab began a collaboration with Peter Loidl at the University of Innsbruck, who was at that time one of the few people in the world working on HDACs, and the only one working on plant HDACs. We showed that HC-toxin inhibits the HDACs of yeast, *Physarum*, chicken, and, most importantly for us, maize, both in vitro and in vivo (Brosch et al., 1995). Consistent with our earlier results showing that HC-toxin reductase is the basis of specificity, the HDAC of resistant and susceptible maize are inhibited differentially in vivo (as evidenced by the accumulation of hyperacetylated histones), but equally in vitro (Brosch et al., 1995). While this work was in progress, trapoxin

itself was shown to be an inhibitor of mammalian HDAC (Kijima et al., 1993). In responses to *C. carbonum* infection, histones H3 and H4 become hyperacetylated at early time points, which is consistent with HDAC inhibition by HC-toxin during pathogenesis (Ransom and Walton, 1997).

To the best of my knowledge, all subsequent studies are consistent with HDAC as the sole site of action of HC-toxin and related compounds (see below). HC-toxin seems to be an universal HDAC inhibitor and has been used in many studies on many organisms (e.g., Darkin-Rattray et al., 1996; Joung et al., 2004; Robbins et al., 2005; Witt et al., 2003; Yang et al., 2001). An effect of one of these compounds on a biological process is prima facie evidence for the involvement of HDACs in that process. I am not aware of any counter-evidence on this point. HC-toxin and other HDAC inhibitors, such as trichostatin (TSA) and chlamydocin (see below), are commercially available from several sources, including Sigma and Calbiochem.

A trapoxin affinity column was used to purify the first HDAC from any source (Taunton et al., 1996a,b). This work, together with the isolation of the first histone acetyltransferase (HAT) in the same year (Brownell et al., 1996), generated a huge amount of interest and subsequently thousands of papers on the process of reversible histone acetylation. A discussion of the myriad biological functions of HDACs and HATs is beyond the scope of this review, and readers are referred to some recent reviews (Fischle et al., 2003; Glozak et al., 2005; Jenuwein and Allis, 2001; Kurdistani and Grunstein, 2003). Reversible histone acetylation has been implicated in just about every biological process that involves chromatin, including gene regulation, cancer, development, circadian rhythms, flowering, and, last but not least, plant pathogenesis. Aspects of HDACs and histone acetylation that are relevant to the role of HC-toxin in pathogenesis are discussed below.

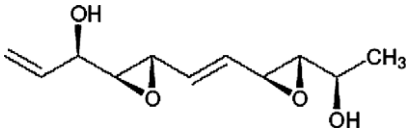
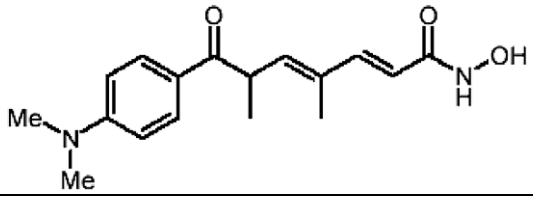
The crystal structures of human HDAC8 complexed with a hydroxamic acid inhibitor and of a bacterial HDAC homolog complexed with TSA and the hydroxamic acid inhibitor SAHA have been solved (Finnin et al., 1999; Vannini et al., 2004). Apparently there is not yet any mechanistic or structural information specifically on the interaction of HDACs with Aeo-containing peptides.

HDAC inhibitors are showing strong promise in the treatment of several types of cancer, and this has led to the development and clinical testing of many natural, synthetic, and semi-synthetic compounds (e.g., Garcia-Manero and Issa, 2005; Miller et al., 2003; Minucci and Pelicci, 2006; Yoshida et al., 2003). A number of derivatives of HC-toxin and other Aeo-containing peptides have been synthesized (e.g., Furumai et al., 2001; Taunton et al., 1996a,b).

6. Structures and activities of compounds related to HC-toxin

Six fungal metabolites related to HC-toxin have been described (Table 1). These are chlamydocin, WF-3161,

Table 1
HC-toxin and biologically and chemically related compounds

Compound	Structure	Producing organism (and synonyms)	Ecological niche
HC-toxin	cyclo(D-Pro-L-Ala-D-Ala-L-Aeo)	*Cochliobolus carbonum* (*Helminthosporium carbonum*) (*Bipolaris zeicola*)	Plant pathogen
Chlamydocin	cyclo(Aib-L-Phe-D-Pro-L-Aeo)	*Diheterospora chlamydosporia* (*Pochonia chlamydosporia*)	Nematode pathogen
Cyl-2	cyclo(D-*O*-MeTyr-L-Ile-L-Pip-L-Aeo)	*Cylindrocladium scoparium* (*Calonectria morganii*)	Plant pathogen
WF-3161	cyclo(D-Phe-L-Leu-L-Pip-L-Aeo)	*Petriella guttulata*	Coprophyte
Trapoxin	cyclo(D-Pro-L-Phe-L-Phe-L-Aeo)	*Helicoma ambiens*	Saprophyte
Apicidin	cyclo(N-*O*-Me-L-Trp-L-Ile-D-Pip-L-Aod)	*Fusarium pallidoroseum*	Possible nematode pathogen
JM47	cyclo(L-Ala-D-Ala-L-Aoh-D-Pro)	Marine *Fusarium* species	Unknown
Depudecin		*Alternaria brassicicola*	Plant pathogen
Trichostatin		*Streptomyces platensis*	Unknown

All, except perhaps JM47, are known or presumed inhibitors of HDAC. All are produced by fungi except trichostatin. All of the cyclic peptides also exist as minor forms.

Abbreviations: Aeo, 2-amino-9,10-epoxi-8-oxodecanoic acid; Aod, 2-amino-8-oxo-decanoic acid; Aoh, 2-amino-8-oxo-9-hydroxydecanoic acid; Aib, aminoisobutyric acid.

trapoxin, Cyl-2, JM47, and apicidin. All are cyclic tetrapeptides containing Pro or pipecolic acid and all except apicidin and JM47 contain Aeo (for references, see Walton et al., 1997). Apicidin, from *Fusarium pallidoroseum*, contains 2-amino-8-oxo-decanoic acid in place of Aeo. It is active against apicomplexan parasites and mammalian cells (Darkin-Rattray et al., 1996). The fact that this compound is still an active HDAC inhibitor indicates that the epoxide group is not absolutely essential for the biological activity of this family of compounds. JM47, also from a species of *Fusarium*, contains 2-amino-8-oxo-9-hydroxydecanoic acid in place of Aeo (Jiang et al., 2002).

These compounds have been tested in a variety of assays and a variety of organisms. Although not all have been tested in all assays, there is no reason to suspect that they have sites of action other than HDAC.

7. Role of HC-toxin and HDACs in plant pathogenesis

All of the available chemical, physiological, genetic, and molecular data indicate that HC-toxin is a primary determinant of disease in the interaction between maize and *C. carbonum*. Considering the high virulence of toxin-producing isolates of *C. carbonum*, HDACs therefore must have an important role in defense, at least in this pathosystem. However, at this point we do not know what that role is.

Relevant considerations to this question include the following. First, only HDACs of the Rpd3/Hda1 class are sensitive to HC-toxin; HDACs of the Sir2 class are not. One of the Rpd3/Hda1 class of HDACs that is present in mammals, but apparently not plants, is cytosolic and deacetylates tubulin. This particular HDAC is sensitive to TSA but not trapoxin (Matsuyama et al., 2002). Second, plants have a class of nucleolus-localized HDAC, HD2, which is not found in mammals or fungi. It is inhibited by HC-toxin and therefore could be critical during pathogenesis (Brosch et al., 1995; Lusser et al., 1997). Third, all eukaryotes have multiple genes encoding sensitive HDACs. Mammals and plants each have at least ten of the Rpd3/Hda1 class and maize has four of the plant-specific HD2 class (Verdin et al., 2003; www.chromdb.org/). Fourth, HDACs deacetylate proteins other than histones; in particular, many transcription factors are regulated by reversible acetylation (Glozak et al., 2005; Minucci and Pelicci, 2006; Yoshida et al., 2003). Fifth, although histone deacetylation is globally associated with gene repression, expression of some genes is promoted by deacetylation of their associated histones (Baidyaroy et al., 2001; Wang et al., 2002). Therefore, models of the role of HC-toxin in disease must consider the possibility of both enhanced gene repression and enhanced gene expression (Brosch et al., 1995).

The upshot of this complex picture is that we cannot at this point in time deduce either the critical HDAC or HDACs affected by HC-toxin during pathogenesis, nor the protein substrate(s) of that (critical) HDAC. Because HDACs are a rather large gene family, and because plant HDACs also have functions in many aspects of normal growth and development, and may well overlap in those functions, it is difficult to envision a reasonable genetic approach to address the role of maize HDACs in defense against *C. carbonum*.

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