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A BIOCHEMICAL BASIS FOR HERITABLE RESISTANCE OF MAIZE TO THE FUNGAL PATHOGEN COCHLIOBOLUS CARBONUM

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A BIOCHEMICAL BASIS FOR HERITABLE RESISTANCE OF MAIZE TO THE FUNGAL PATHOGEN COCHLIOBOLUS CARBONUM

By

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ABSTRACT

A BIOCHEMICAL BASIS FOR HERITABLE RESISTANCE OF MAIZE TO THE FUNGAL PATHOGEN, COCHLIOBOLUS CARBONUM

By

Robert Brendan Meeley

The fungus *Cochliobolus carbonum* Nelson race 1 is a foliar pathogen of maize hosts that are homozygous recessive for the resistance gene locus *hm*. *C. carbonum* race 1 produces HC-toxin, a cyclic tetrapeptide required for successful colonization of *hm/hm* maize. This dissertation addresses questions about the function of the *Hm* allele in providing resistance to *C. carbonum* race 1. Previous workers have determined that near-isogenic maize lines, differing only at the *hm* locus, are differentially sensitive to the effects of HC-toxin. The working hypothesis of this dissertation proposes that the *Hm* allele confers decreased sensitivity to HC-toxin by metabolic inactivation of this compound. An inactive derivative of a radiolabeled form of HC-toxin was recovered from resistant (*Hm/-*) maize and characterized by thin-layer chromatography, NMR, and mass spectrometry. An enzymatic basis for metabolite formation was detected, partially purified, and characterized. The enzyme HC-toxin reductase utilizes NAD(P)H for stereoselective reduction of the 8-keto group of the amino acid 2-amino-9,10-epoxy-8-oxodecanoic acid. A variety of genetic materials were examined for HC-toxin reductase activity. The results establish HC-toxin reductase activity as the biochemical phenotype

for the *Hm* resistance allele. Best evidence suggests that the *Hm* allele encodes the gene for HC-toxin reductase. Purification procedures enrich for three polypeptides of 49, 43.5, and 40 kD; an estimate for the HC-toxin reductase molecular weight is 42 kD. HC-toxin reductase is induced in resistant maize by inoculation of etiolated seedlings with a spore suspension of *C. carbonum* race 1, indicative of a defense-related role for this enzyme. In addition, enzyme activities analogous to HC-toxin reductase were detected in extracts from several other monocot species, but not in dicot extracts. Evidence suggests that the alternate *C. carbonum* race 1 resistance gene of maize, *Hm2*, may provide resistance to *C. carbonum* race 1 by a similar mechanism of HC-toxin inactivation. This dissertation presents a description of the biochemical function of a disease resistance gene from a major crop, and raises difficult but approachable questions about host-pathogen coevolution.

To Ken and Holly

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LIST OF ABBREVIATIONS

Aeo	2-amino-9,10-epoxy-8-oxodecanoic acid
bis-Tris	bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane
δ_{ppm}	chemical shift (parts per million)
DTT	dithiothreitol
HCTR	HC-toxin reductase
p-HMB	*p*-hydroxymercuribenzoate
Mops	3-[N-morpholino]propanesulfonic acid
p-NBP	4-(p-nitrobenzyl)-pyridine
pers. commun.	personal communication
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone

CHAPTER 1

INTRODUCTION

Plant pathogens have evolved presumably because living plants offer a practical niche for the growth, development, and reproduction of certain organisms. As an evolutionary response, the development of specific defenses by plants might be considered an essential step for their own productivity and survival. In the interest of understanding the process of co-evolution between a host and parasite (reviewed by Frank, 1992), and to eventually manipulate genetic traits for disease resistance in higher plants (reviewed by Gasser and Fraley, 1989), scientists have studied the genetic principles that govern plant-microbe interactions in a variety of model systems. These models typically involve major crop species and their perennial microbial pests.

Despite great strides in our knowledge of host-pathogen genetics, very little is known about the gene products that mediate plant-microbe interactions. Current molecular and/or biochemical models largely represent extrapolations of genetic data (Gabriel and Rolfe, 1990), particularly with regard to the biochemical nature of specific genes for disease resistance in plants. For this reason a protracted review of specific models in modern plant pathology will be avoided. A variety of detail and opinion is expressed regularly in review articles (Gabriel and Rolfe 1990; Keen, 1990). This introduction will focus on the fundamentals of host-pathogen genetics, for virtually all of the models expressed in plant pathology today have their roots in a single tenet known as the gene-for-gene hypothesis.

The Gene-for-Gene Hypothesis

H. H. Flor (1900-1987), a plant pathologist from the USDA in North Dakota, is widely acknowledged for his critical contribution to the knowledge of plant-pathogen interactions. Flor examined the genetic principles that govern the interaction between flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*), developing a seminal hypothesis which states:

> "For each gene conditioning resistance in the host there is a specific gene conditioning avirulence in the parasite." (Flor, 1942)

and alternatively:

> "For each gene conditioning resistance in the host there is a specific gene conditioning pathogenicity in the parasite." (Flor, 1956)

As complementary genes, a gene in one organism is defined in context with a gene from the other. The difference between the above hypotheses concerns the type of pathogen being studied. In many interactions, pathogen resistance by the host occurs *via* genes that complement, or recognize, characteristics for avirulence in the pathogen. The product(s) of dominant avirulence genes are thought to interact with the product(s) of dominant plant resistance genes at some molecular level. A physical interaction between gene products is implied, that is somehow transduced into a resistance response in the infected plant tissue (Gabriel and Rolfe, 1990; Keen, 1990). Hypersensitivity is the term

used to describe this response because it involves local necrosis of the plant tissue surrounding the site of infection. This premature death of host cells restricts the growth of pathogens to a few infected cells. Further infection is thus avoided (Gabriel and Rolfe, 1990). Diseases involving this type of complementarity are quite common, and this was the type studied by Flor himself.

Avirulence and hypersensitivity are not central to the disease being studied in this dissertation. Our host-pathogen system involves gene-for-gene complementarity with respect to pathogenicity, so Flor's 1956 wording of the gene-for-gene hypothesis will serve as our starting point. Examples of this type involve the interaction of pathogenicity gene product(s) with the product(s) of a complementary host resistance gene at some molecular level.

Figure 1.1 illustrates a quadratic check for a hypothetical complementary genic system involving host resistance in the context of traits for pathogenicity. In systems where sexual manipulation of a plant pathogen can be accomplished in the laboratory, a single gene for pathogenicity (denoted *P*) can be identified in context with its complement; a gene that conditions resistance (*R*) or susceptibility (*r*) in the host. Host resistance is based on specific "recognition" of the pathogenicity gene product(s). We have little or no evidence to propose a general biochemical definition for "recognition". This remains an ultimate objective in the field. In the pages that follow, this quadratic check will be elaborated to communicate the historical and experimental details of our system, such that a hypothesis about pathogen recognition can be developed for our specific case.

Figure 1.1. A hypothetical complementary genic system. This quadratic check shows a plant gene for resistance (R) or susceptibility (r) defined in context with a complementary pathogen gene pair for pathogenicity (P) or non-pathogenicity (p). (+) indicates a compatible interaction (disease favorable). (-) indicates an incompatible interaction (no disease).

Hypothetical Pathogen/Plant Interaction

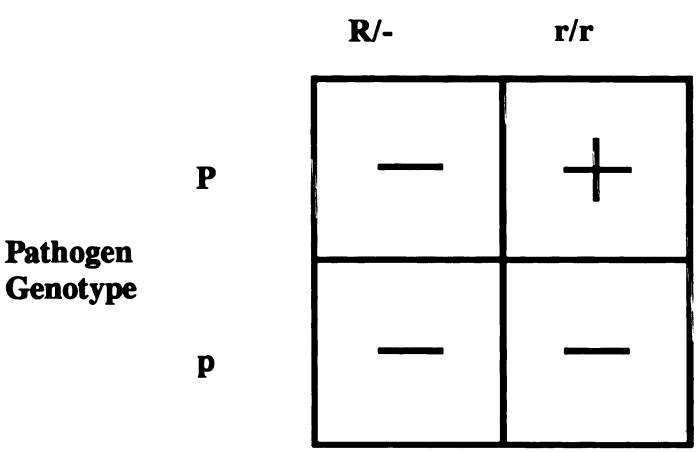


Figure 1.1.

Another distinction to be made is that our model system involves an interaction at the level of *race-cultivar specificity*. Plant pathogens are capable of distinguishing potential hosts at a variety of levels, and *vice versa*. For example, a certain set of traits common to a fungal genus may condition pathogenicity to monocotyledons in general, while another set may be required for pathogenicity of a particular species on maize. The same might be said of traits for avirulence, or even plant characteristics that limit the host range of potential pathogens. These kinds of traits are important because they increase the web of genetic complexity in host-pathogen interactions. They are regarded as polygenic traits that comprise the elements of *basic compatibility* between two organisms (Heath, 1991). Race-cultivar specificity denotes *specific compatibility*, or discrimination between a potential host and potential pathogen at the sub-species level. Like Flor, plant pathologists frequently concentrate on the race-cultivar level of compatibility since it appears to represent a high degree of specialization and can often be resolved into complementary genic systems involving allelic differences at single genes.

The Genetic System

The subject of this dissertation is a complementary genic system involving the fungus *Cochliobolus carbonum* Nelson race 1 and its specific host, *Zea mays* L. A *compatible* or *pathogenic* interaction results when a spore from a pathogenic race of *C. carbonum* encounters a susceptible maize line (cultivar) to produce a disease known as Helminthosporium leaf spot.

The Host

Helminthosporium leaf spot came to the attention of pathologists and plant breeders when it was discovered as a new foliar disease of maize in the midwestern Corn Belt region of the United States. Severe fungal infection of a specific inbred line of corn was noted during field trials in Indiana in 1938-39 (Ullstrup, 1941). In addition to the original susceptible inbred, Pr, other susceptible inbred stocks were soon discovered. The F_1 progeny of single crosses between these susceptible inbreds were infected with equal severity, suggesting that a single recessive gene governed susceptibility to *C. carbonum* race 1 (Ullstrup, 1944). This notion was confirmed when a single Mendelian locus for susceptibility to Helminthosporium leaf spot, called *hm*, was genetically mapped to the long arm of chromosome-1 (Ullstrup and Brunson, 1947). The dominant allele *Hm* conditioned full resistance to *C. carbonum* race 1, even in the heterozygote. It was postulated that this pathogen went undetected in the field due to the cultivation of open-pollinated corn, a largely heterozygous population. The disease's full severity was not discovered until the development and cultivation of the susceptible inbred, Pr (Ullstrup, 1941). Fortunately, full resistance was detected in most other commercial maize lines, and incorporation of *Hm* into new stocks became standard practice in maize breeding programs.

It should be noted that additional genetic elements in maize have been implicated in resistance to *C. carbonum* race 1. A separate locus, non-allelic to *Hm*, conditions developmentally limited resistance to *C. carbonum* race 1. This locus, called *Hm2* is located on chromosome 9L, displays incomplete dominance (heterozygotes have intermediate resistance), and exhibits a dependence on plant age for its expression (Nelson

and Ullstrup, 1964). *Hm*-mediated resistance to *C. carbonum* race 1 is regarded as epistatic to *Hm2*, thus *Hm* is the primary source of heritable resistance to *C. carbonum* race 1 (Nelson and Ullstrup, 1964). This dissertation is focused on the identity and function of *Hm*, although *Hm2* will be discussed later in Chapters 4 and 5.

The Pathogen

Cochliobolus carbonum Nelson race 1, is a filamentous ascomycete of the Loculoascomycetes. This genus largely consists of species associated with grass hosts (Kline and Nelson, 1969). These include *C. heterostrophus*, a maize pathogen, and *C. victoriae*, a pathogen of oats. *Cochliobolus* is the genus name given to a teleomorph (sexual) stage of *Helminthosporium* (Nelson, 1959). The species *Cochliobolus carbonum* Nelson (anamorph, *Helminthosporium carbonum* Ullstrup) is presently distinguished into three or possibly four separate races based upon their host range on maize (Leonard, 1978). This dissertation is concerned with races 1 and 2 which are distinguished by their pathogenicity in context with the *hm* locus of maize. Race 1 of *C. carbonum* is pathogenic solely to *hm/hm* maize, race 2 is a designation for isolates that are non-pathogenic to *hm/hm* maize (Nelson and Ullstrup, 1961).

The genetics of *C. carbonum* pathogenicity were studied after the sexual stage of the fungus' life cycle was demonstrated in culture (Nelson, 1959). Segregation data from crosses between race 1 and race 2 of *C. carbonum* suggested that pathogenicity against *hm/hm* maize was under the control of a single gene (Nelson and Ullstrup, 1961).

In the years to follow, rapid progress was made toward identifying the biochemical nature of race 1 pathogenicity. This progress was due, in part, to the fact that another

member of the genus *Cochliobolus* was known to produce a low-molecular-weight phytotoxin that caused necrosis on host tissues (Meehan and Murphy, 1947). The production of such compounds by pathogens was thought to have a direct implication in disease development (Scheffer and Livingston, 1984).

For example, specific oat breeds carrying the single gene *Vb* are highly susceptible to pathogenic isolates of *Cochliobolus victoriae*. Victoria Blight of oats occurred on a major scale in the early 1930's due to widespread cultivation of *Vb*-containing oats. Researchers revealed the involvement of a phytotoxic molecule called victorin that was produced by pathogenic *C. victoriae* (Meehan and Murphy, 1947; Luke and Wheeler, 1955). Victorin alone could initiate many of the symptoms of blight on susceptible (*Vb*-containing) varieties of oat, with very little effect on the resistant variety (Yoder and Scheffer, 1969). Victorin was one of the premier examples of a host-selective toxin: a pathogen-derived metabolite that influences host-range and pathogenicity (Scheffer and Livingston, 1984). Pathogenicity of *C. victoriae* was linked to the production of victorin (Scheffer *et al.*, 1967), and in oat, susceptibility to pathogenic *C. victoriae* was indistinguishable from sensitivity to victorin (Yoder and Scheffer, 1969).

Given its close relationship to *C. victoriae*, researchers began examining the interaction between maize and *C. carbonum* race 1 for a similar phenomenon. Like the Victoria blight of oats, the symptoms of Helminthosporium leaf spot included yellowing of the leaf tissue well in advance of the actual lesion area; suggestive of an effect caused by a diffusible substance.

Pathogenicity of *C. carbonum*, already shown to be under the control of a single gene, was soon associated with the ability to produce a low-molecular-weight compound

that displayed selective toxicity toward *hm/hm* maize (Scheffer and Ullstrup, 1965). The crude culture filtrate from *C. carbonum* race 1 was shown to differentially inhibit the growth of susceptible (*hm/hm*) corn roots *versus* the growth of nearly isogenic (*Hm/-*) resistant roots (Pringle and Scheffer, 1967). This toxin was given the name HC-toxin for the fungus' anamorph name *Helminthosporium carbonum*. Non-pathogenic, race 2 isolates of the fungus did not produce this toxin (Scheffer and Ullstrup, 1965). Progeny from crosses between race 1 and race 2 isolates of *C. carbonum* segregated 1:1 for production:no production of HC-toxin (Scheffer *et al.*, 1967). All isolates deficient in toxin production, including mutants and non-pathogenic wild-types, fail to initiate disease on *hm/hm* maize.

Matings were also carried out between pathogenic *C. carbonum* and pathogenic *C. victoriae*. To a degree, these species are sexually compatible. The progeny were scored for the production of toxins and evaluated for their disease phenotype on both susceptible corn and oats. Progeny from interspecific crosses segregate 1:1:1:1 for production of HC-toxin or victorin (parental phenotypes), both toxins (fully pathogenic, non-parental recombinant), or neither toxin (non-pathogenic, non-parental recombinant). As sexually compatible anamorphs, these two fungi are thought to differ by two gene pairs, one locus controlling the production of HC-toxin in *C. carbonum*, the other of victorin in *C. victoriae* (Scheffer *et al.*, 1967).

The importance of HC-toxin to the race 1 infection process was demonstrated by supplying purified HC-toxin to race 2 spores inoculated on *hm/hm* maize leaves. In the presence of HC-toxin, race 2 inoculum was able to penetrate and infect *hm/hm* maize as effectively as a race 1 isolate (Comstock and Scheffer, 1973). Similar results were

observed when HC-toxin was provided to inocula of *C. victoriae* on *hm/hm* leaves; exogenously applied HC-toxin was able to extend the host-range of *C. victoriae* to include *hm/hm* maize (Comstock and Scheffer, 1973). Production of HC-toxin is considered the critical required element for pathogenicity against *hm/hm* maize. Genetic and physiological data supported the notion that the single gene controlling pathogenicity of *C. carbonum* race 1 was responsible for the production of HC-toxin. This genetic locus was deemed *TOX2* (Yoder *et al.*, 1989).

With a basis for race 1 pathogenicity established, the susceptibility of specific maize lines to race 1 was purported to involve differential sensitivity to HC-toxin. A survey of maize lines exhibiting different degrees of susceptibility to *C. carbonum* race 1 were analyzed for their sensitivity to HC-toxin. The maize lines most sensitive *C. carbonum* race 1 were also most sensitive to HC-toxin in the root growth bioassay, while lines most resistant to the fungus were most resistant to the toxin (Kuo and Scheffer, 1970a). Thus, HC-toxin represents a chemical entity critical to the infection process, that also displays selective biological activity toward maize depending on the allelic condition at the *hm* locus.

Returning to an updated version of our quadratic check (Figure 1.2), some of the genetic details of Helminthosporium leaf spot can be entered. *C. carbonum* race 1 is pathogenic in the context of *hm/hm* maize, and the genetic trait controlling pathogenicity was associated with the ability to produce a toxin. The designation *P* from our hypothetical interaction can be replaced by *TOX2*, the single locus required for HC-toxin production and pathogenicity against *hm/hm* maize. A single completely dominant gene from maize, denoted *Hm/-*, exists as the complementary gene for resistance to *TOX2*.

Race 2 isolates are designated *tox2* (replacing *p*) because they do not produce HC-toxin and are non-pathogenic against either *Hm/-* or *hm/hm* maize.

A *compatible interaction* between *C. carbonum* and maize is that which results in disease. On susceptible (*hm/hm*) leaf surfaces, *C. carbonum* race 1 spores, which contain *TOX2* and produce HC-toxin, form specialized structures for attachment and penetration called appressoria. Once penetration is established, the fungus grows rapidly throughout the leaf mesophyll resulting in the collapse of host cell walls and the breakdown of organelles. As infectious hyphae advance, the boundaries of necrotic lesions expand, creating large leaf spots (Jennings and Ullstrup, 1957; Comstock and Scheffer, 1973).

The interaction of resistant leaf tissues with *C. carbonum* race 1 is said to be *incompatible*. Germinating race 1 spores form appressoria and penetrate the leaf epidermis, but because of the resistance factor provided by *Hm*, infection is confined to only one or two epidermal cells. The end result of an incompatible (resistant) response is small necrotic flecks on the leaf surface, similar to the hypersensitive response common to other diseases (Jennings and Ullstrup, 1957; Comstock and Scheffer, 1973).

C. carbonum race 2 spores, which are *tox2* and do not produce HC-toxin, are non-pathogenic on both *Hm/-* and *hm/hm* maize. This interaction is *incompatible* in either case; both host genotypes are resistant to race 2 and display the typical small necrotic flecks around the sites of fungal penetration (Jennings and Ullstrup, 1957; Comstock and Scheffer, 1973).

Figure 1.2. A complementary genic interaction between *C. carbonum* race 1 and maize. Gene *P* from Fig. 1.1 has been replaced by the *C. carbonum* race 1 gene *TOX2*, which controls pathogenicity and the production of HC-toxin. The complementary host resistance gene receives the designation *Hm*, for resistance to race 1. *hm* confers susceptibility (+) in context with *TOX2*. Race 2 fungi are *tox2*, and non-pathogenic (-) against either *Hm* or *hm*.

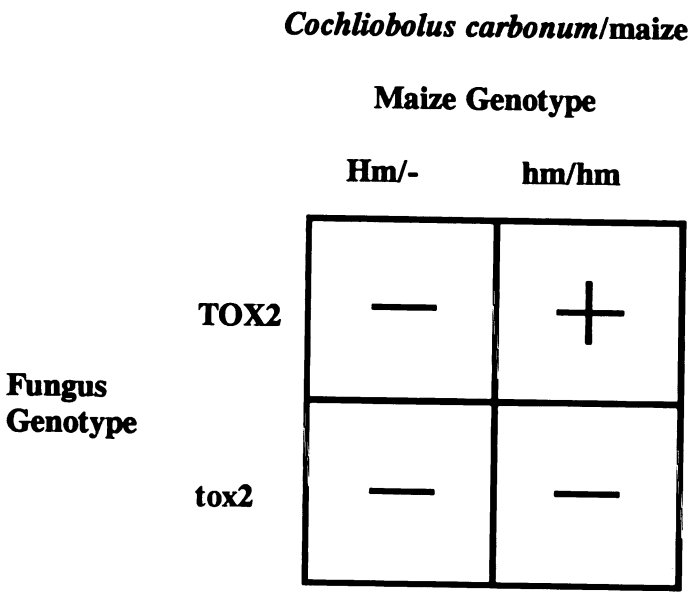

Cochliobolus carbonum/maize
Maize Genotype
Hm/-
hm/hm
TOX2
tox2
Fungus Genotype
—
+
—
—

Figure 1.2.

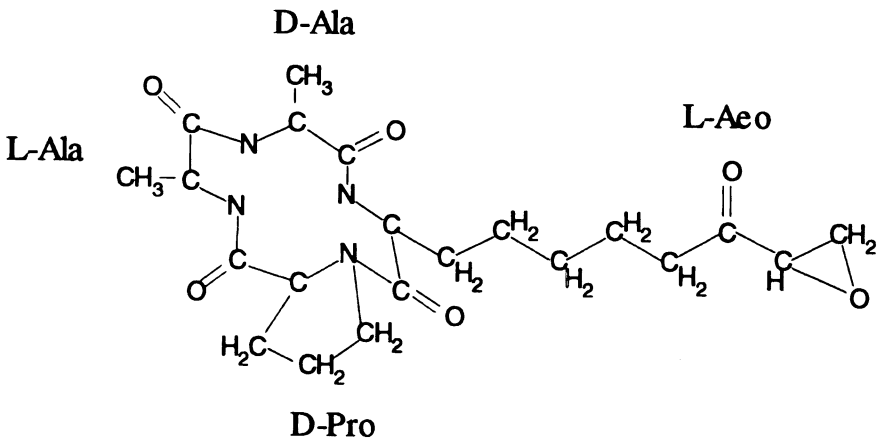
HC-toxin

HC-toxin's role in pathogenicity inspired efforts to identify its structure. Initial efforts to purify HC-toxin from culture filtrates were aided extensively by the development of an effective bioassay that evaluates the inhibition of root growth of susceptible (*hm/hm*) corn seeds as a function of HC-toxin concentration (Pringle and Scheffer, 1967). Qualitatively, toxic fractions will differentially inhibit the growth of susceptible roots when compared to growth of resistant (*Hm/-*) controls. Quantitatively, toxin preparations can be evaluated against a standard curve; typical homogeneous preparations of HC-toxin have an EC_{50} (effective concentration inhibiting susceptible root growth by 50%) between 0.2 and 0.5 μg/ml (Ciuffetti *et al.*, 1983).

Structure

Many years of effort in HC-toxin purification and structure elucidation can be summarized by Figure 1.3 which shows the structure of HC-toxin (Walton *et al.*, 1982; Pope *et al.*, 1983; Leisch *et al.* 1982). The compound is a cyclic tetrapeptide, cyclo[D-prolyl-L-alanyl-D-alanyl-L-Aeo], where Aeo stands for the amino acid component 2-amino-9,10-epoxy-8-oxodecanoic acid. Aeo is a peculiar long chain amino acid originally described as a component of another fungal cyclic tetrapeptide called Cyl-2, produced by the phytopathogenic fungus, *Cylindrocladium scoparium* (Hirota *et al.*, 1973). Confirmation of HC-toxin's structure was obtained when a completely synthetic HC-toxin was shown to exhibit specific toxicity toward susceptible corn in the root growth bioassay (Kawai and Rich, 1983).

Figure 1.3. The structure of HC-toxin. HC-toxin is a cyclic tetrapeptide, cyclo-[D-prolyl-L-alanyl-D-alanyl-L-Aeo], where Aeo stands for 2-amino-9,10-epoxy-8-oxodecanoyl.



HC-toxin

Figure 1.3.

Biosynthesis

HC-toxin's novel structure and relationship to disease spurred interest in its biosynthesis. Cyclic peptides of varying size and character are common as antibiotics or secondary metabolites from bacteria and fungi. Considerable research into the synthesis of so-called non-ribosomal peptides has been conducted. Some of the best examples describe the biosynthesis of the antibiotic gramicidin-S, produced by the bacterium, *Bacillus brevis* (Kleinkauf and von Döhren, 1983), and the enniatins, cyclic depsipeptide ionophores produced by the fungus, *Fusarium oxysporum* (Zocher *et al.*, 1982). In these and all subsequent cases, cyclic peptides were found to be synthesized by large, cytosolic, polyfunctional enzymes. Amino/imino acids are activated by ATP, forming an aminoacyl-AMP and PP_i. The activated amino acids are transferred to enzyme thioesters (4'-phosphopantetheine groups) for condensation into cyclic peptides (Kleinkauf and von Döhren, 1983). Purification and characterization of these synthetase enzymes can be followed by monitoring amino acid-dependent ATP/PP_i exchange; exploiting the reversibility of the activation reaction using radiolabeled pyrophosphate (Zocher *et al.*, 1982).

Based on ATP/PP_i exchange activity, two *C. carbonum* race 1 enzymes were implicated in the synthesis of HC-toxin. These large enzymes, called HC-toxin synthetases (HTS), are found in cytosolic preparations of race 1 of *C. carbonum*, but are lacking in race 2 (Walton, 1987). HTS-1, with an apparent molecular weight of 220 *kD* based on SDS-PAGE, specifically recognizes and activates the L-isomer of proline and epimerizes it to the D-isomer for incorporation into HC-toxin. The second enzyme, HTS-2 (160 *kD* on SDS-PAGE), recognizes and activates both L- and D-alanine, and also

epimerizes L-alanine to D-alanine (Walton and Holden, 1988). Both isomers of alanine are found in HC-toxin, yet HTS-2 catalyzes epimerization only in the L- to D- direction. Both enzyme activities are found only in toxin producing isolates of the fungus, and co-segregate with toxin production in sexual crosses between race 1 and race 2 isolates (Walton, 1987).

The *TOX2* locus of *C. carbonum* race 1 was thought to encode these enzymes. Molecular genetic analyses have identified a 15.7kb ORF that is required for HC-toxin biosynthesis. Interestingly, this massive ORF appears to encode both HTS-1 and -2 as a single gene. Separable HTS-1 and HTS-2 enzymes in race 1 extracts are perhaps products of proteolytic cleavage following translation (Scott-Craig *et al.*, 1992). The function of the *TOX2* locus has been investigated by transformation-mediated gene disruption of *C. carbonum* race 1. Importantly, both HC-toxin biosynthesis and race 1 pathogenicity are nullified by disrupting the *TOX2* gene. When inoculated on *hm/hm* maize, the *TOX2* knockout mutants produce only small necrotic flecks similar to non-pathogenic race 2 isolates. These findings constitute the most substantial proof that HC-toxin is required for pathogenicity against *hm/hm* maize (Panaccione *et al.*, 1992). In turn, the results also support the hypothesis that *Hm*-mediated resistance to *C. carbonum* race 1 is synonymous with resistance to HC-toxin.

To date, the DNA included in *TOX2* does not provide any clues to the biosynthesis or activation of Aeo. An epoxide-containing compound thought to be a precursor of Aeo or HC-toxin has been detected as a by-product in culture filtrates from the *TOX2* knockout mutants (D. Panaccione, personal communication). Its characterization may provide insight into Aeo biosynthesis.

Biological Activity

It is evident from a number of studies that production of HC-toxin must, in some way, give a critical advantage to a germinating fungal spore for successful colonization of susceptible leaf tissues. Many researchers have examined susceptible and resistant maize tissues for a differential response to HC-toxin in attempts to explain the toxin's mode of action and/or the role of the *Hm* allele in disease resistance. Paradoxically, some of the first studies documented stimulatory effects by this "toxin". For example, root growth and general metabolism of both resistant and susceptible maize are actually stimulated at low concentrations of HC-toxin (Kuo and Scheffer, 1970a,b). Evidence of this can be seen in Figure 1.4. At low HC-toxin concentrations in the root growth bioassay, the growth of resistant roots is increased in comparison to untreated controls. In addition, stimulatory effects on leaf respiration, CO_2-fixation (Kuo and Scheffer, 1970b), and solute uptake (Yoder and Scheffer, 1973a,b) have been documented for toxin-treated maize tissues.

Importantly, resistant maize is actually sensitive to HC-toxin when the concentration is increased 100-fold (Kuo and Scheffer, 1970a). This effect is also illustrated in Figure 1.4. HC-toxin administered at 0.2 μg HC-toxin/ml inhibits susceptible root growth by 50%. Root growth of resistant maize is 50% inhibited when HC-toxin is given at 20 ug/ml. These maize lines are near-isogenic, thus the data suggests that a 100-fold differential in HC-toxin sensitivity is attributable to the *Hm* allele. This differential has been observed repeatedly regardless of the biological response being examined.

Figure 1.4. Root growth bioassay of HC-toxin. A repeat of the classic experiment documented by Pringle and Scheffer (1967). Purified HC-toxin is administered to germinated corn seedlings from the near-isogenic hybrids PrxK61 (*hm/hm*, ○) and Pr1xK61 (*Hm/hm*, ●). Dotted vertical lines indicate toxin concentration causing 50% inhibition of root growth (EC_{50}).

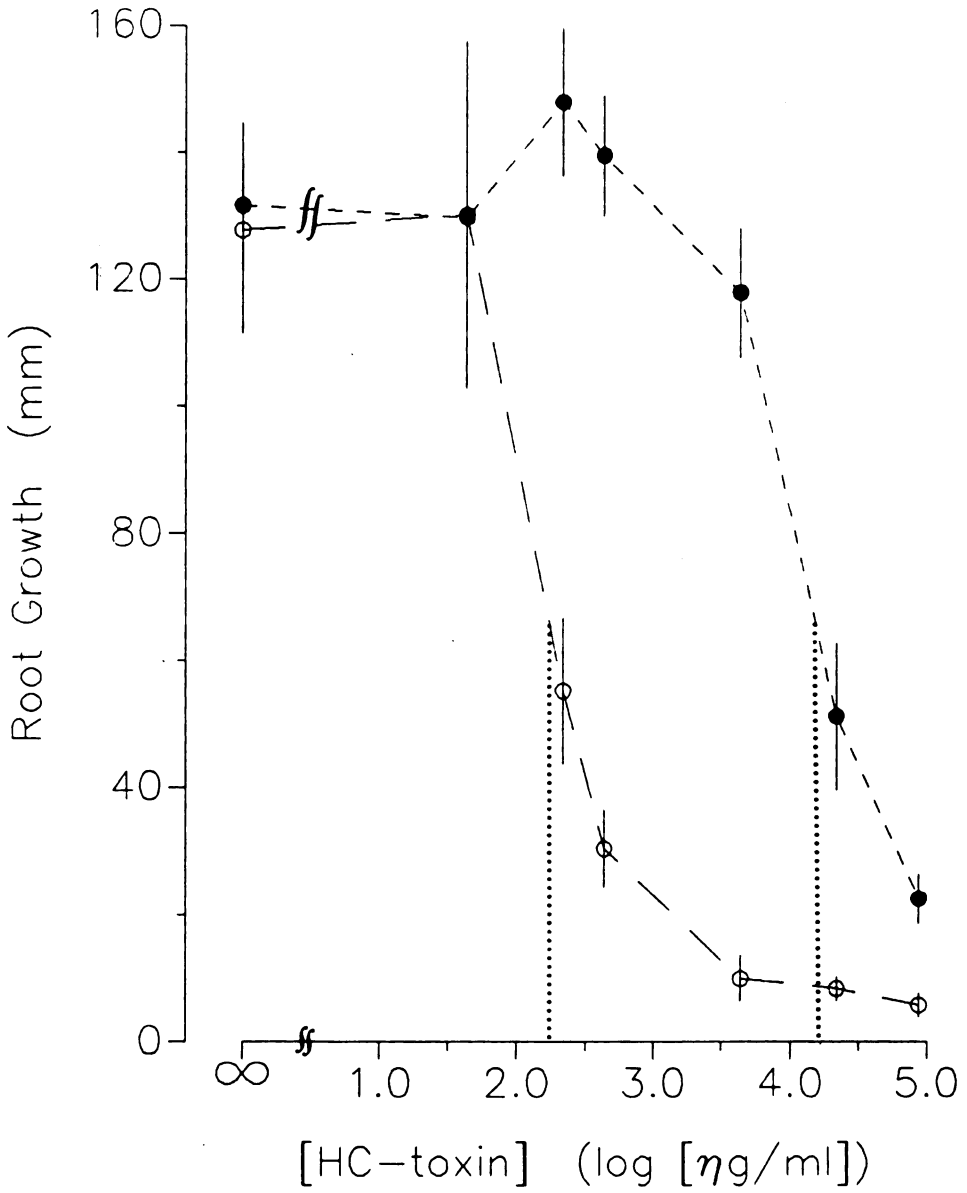

160
120
80
40
0
Root Growth (mm)
ff
ff
∞
1.0
2.0
3.0
4.0
5.0
[HC−toxin] (log [ηg/ml])

Figure 1.4.

The following are documented biological effects of HC-toxin:

Perturbation of the plasma membrane. HC-toxin causes a rapid but transient increase in negative electropotential across the plasma membrane of susceptible coleoptile cells (Gardner *et al.*, 1974). Within 2-3 minutes after toxin exposure, increases from 10 to 40mV were recorded, followed by a gradual return to the original level. An increase in negative electropotential is dramatically different from the effect of other toxins such as victorin (on oats) and PC-toxin (on grain sorghum) which cause significant decreases in negative electropotential across plasma membranes, K^+ ion loss, and electrolyte leakage. The increase in potential induced by HC-toxin is perhaps indicative of a direct effect on the electrogenic ion pumps of the plasma membrane (Gardner *et al.*, 1974).

Effects on solute uptake. Significant increases in nitrate uptake occur 2-3 *h* after toxin treatment of susceptible tissues (Yoder and Scheffer, 1973a). Increases in nitrate reductase activity stem from increased availability of NO_3^-. Uptake of Na^+, Cl^-, leucine, and methylglucose is also stimulated to a lesser extent by treatment of susceptible roots with toxin, while uptake of NO_2^-, K^+, PO_4^{3-}, Ca^{2+}, SO_4^{2-}, and glutamate are unaffected (Yoder and Scheffer, 1973b). In addition, HC-toxin appears to affect solute uptake only on the plasma membrane; no effects on membrane permeability are observed when maize mitochondria and chloroplasts are isolated from toxin-treated cells (Yoder, 1971). Ionophoric compounds can be potent toxins, but the data for HC-toxin are not consistent with the physical properties and ion selectivity of microbial ionophores (Dobler, 1981).

Differential inhibition of chlorophyll synthesis in leaf tissue. Susceptible maize leaves, when exposed to HC-toxin, begin to show inhibition of chlorophyll synthesis after 6 *h* in light. Protection from inhibition can be afforded by supplying leaves with the

chlorophyll precursor, δ-aminolevulinic acid. Resistant leaves show the same inhibition at 100-fold higher HC-toxin concentration (Rasmussen and Scheffer, 1988a).

Effects on chloroplast clustering and differentiation. Maize leaf protoplasts display a tendency to cluster their chloroplasts together after several hours in cell culture. HC-toxin appears to inhibit this phenomenon (Earle and Gracen, 1982). Susceptible protoplasts appear smaller, with uniformly distributed chloroplasts following treatment with HC-toxin. Other observations of protoplasts indicate a cytostatic effect by HC-toxin that actually helps extend the life of susceptible protoplasts in culture (Wolf and Earle, 1991). This effect is quite different from toxins like victorin, which is much more pernicious. Victorin collapses susceptible oat protoplasts after only a few hours of toxin treatment (Earle and Gracen, 1982).

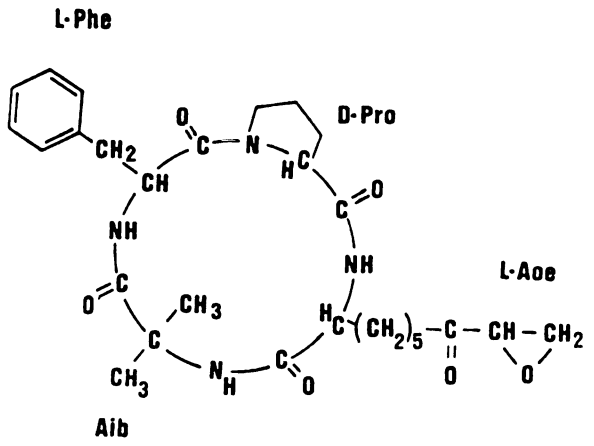
Biological activity in mammalian systems. Other cyclic tetrapeptides produced by fungi contain Aeo (Closse and Huguenin, 1974; Hirota *et al.*, 1973; Kawai *et al.*, 1986). Several of these structures are shown in Figure 1.5. Aeo-containing cyclic tetrapeptides have been shown to be biologically active in mammalian systems (Closse and Huguenin, 1974). Cytostasis and inhibition of both leucine and uridine incorporation in cultured murine P-815 mastocytoma cells was reported in response to HC-toxin, chlamydocin, and Cyl-2 (Walton *et al.*, 1985). HC-toxin and chlamydocin were also found to inhibit mitogenesis of Con A^{-} stimulated lymphocytes (Shute *et al.*, 1987). The discovery of these antimitogenic properties generated interest in Aeo-containing cyclic peptides as chemotherapeutic agents for cancer treatment (Shute *et al.*, 1987). Thus, the biological activity of HC-toxin, or other Aeo-containing cyclic peptides, is not restricted to plants.

Figure 1.5. Other Aeo-containing cyclic tetrapeptides from fungi.

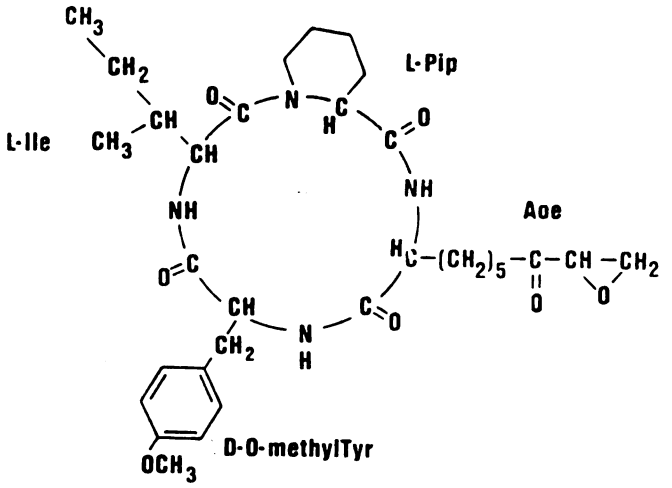
Chlamydocin, cyclo-[aminoisobutyryl-L-phenylalanyl-D-prolyl-L-Aeo], produced by the fungus *Diheterospora chlamydospora*.

Cyl-2, cyclo-[D-O-methyltyrosyl-L-isoleucyl-L-pipecolyl-L-Aeo], produced by the fungus *Cylindrocladium scoparium*.

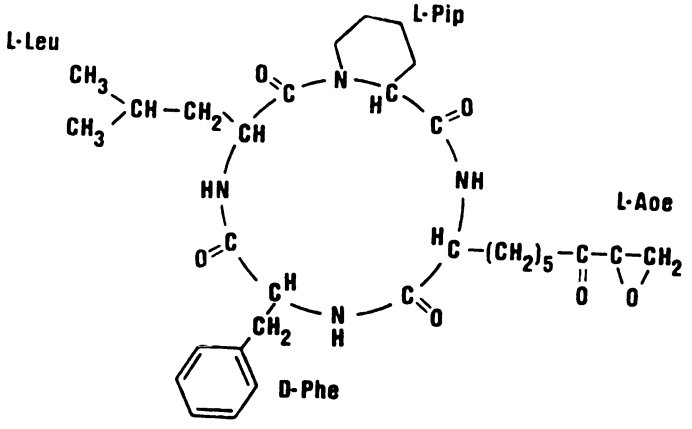
WF3161, cyclo-[D-phenylalanyl-L-leucyl-L-pipecolyl-L-Aeo], produced by the fungus *Petriella guttulata*.



Chlamydocin



Cyl-2



WF-3161

Figure 1.5.

Structural Requirements

Relevant discoveries about HC-toxin's structure were made during the analyses of HC-toxin's biological activity. For example, destruction of the terminal epoxide moiety of Aeo *via* acid hydrolysis results in nearly complete loss of toxicity to susceptible roots (Ciuffetti *et al.*, 1983; Walton and Earle, 1983). In addition, the 8-keto group of Aeo, vicinal to the terminal epoxide, is required for biological activity of HC-toxin. Sodium borohydride selectively reduces this carbonyl group to the corresponding alcohol. Both 8-hydroxy isomeric products of this reaction fail to inhibit susceptible root growth in the bioassay (Kim *et al.*, 1987).

Aeo by itself is not biologically active against root growth or against mammalian cells (Shute *et al.*, 1987). Therefore, in addition to Aeo, other structural elements must be important for biological activity. Evidence suggests that a significant contribution is made by the 12-membered cyclic peptide ring. In culture, *C. carbonum* race 1 produces small amounts of alternate forms of HC-toxin, each of which contain Aeo (Rasmussen and Scheffer, 1988b). The primary form of HC-toxin (toxin I) is produced in the greatest amount, and has the greatest biological activity. The alternate forms have essentially the same host-selectivity in the root growth bioassay (the 100-fold differential is maintained), but the potency of the compounds is reduced. For instance, HC-toxin III, which contains hydroxyproline in place of the proline residue, has an EC_{50} of 2.0 ug/ml against susceptible roots. The addition of one hydroxyl group to the β-carbon of proline lowers the potency of the compound by approximately 90% (Rasmussen and Scheffer, 1988b). However, Aeo-containing cyclic peptides composed of other amino/imino acids have different effects in the root growth bioassay. Chlamydocin contains phenylalanine and

aminoisobutyrate in addition to proline and Aeo (see Figure 1.5). Chlamydocin is not as active as HC-toxin against susceptible root growth, but is more active than HC-toxin against resistant roots (Walton *et al.*, 1985) such that the differential is less than the 100-fold differential recorded for HC-toxin. Therefore, the peptide ring appears to contribute to the potency and/or host-selectivity of Aeo-containing cyclic peptides.

The Working Hypothesis

Since the detection of Helminthosporium leaf spot disease in the field, research has uncovered a complementary genetic relationship between fungal pathogenicity and host resistance. The fungus *Cochliobolus carbonum* Nelson race 1, as part of a group of plant pathogens that produce host-selective toxins, presents to us a level of detail not often available in other host-pathogen systems. Here, a body of evidence suggests a scenario in which a small molecule, specifically a secondary metabolite produced by a pathogenic fungus, is absolutely required for the initiation and development of disease on the susceptible host.

The *TOX2* locus of *C. carbonum* race 1 confers the ability to synthesize this defined substance. A last look at our quadratic check indicates a unique opportunity to characterize resistance to this compound at the biochemical and molecular genetic levels. A tremendous amount of detail can be conveyed by including HC-toxin into the equation as the ultimate gene product of our hypothetical gene *P* (Fig. 1.6). The challenge remains to describe how the *Hm* gene product interacts with HC-toxin. Data on this point will serve to provide a biochemical and/or molecular genetic definition of "recognition" in a host-pathogen interaction.

Figure 1.6. Resistance of maize to *C. carbonum* defined in context with the end-product of the *C. carbonum* pathogenicity gene. The production of HC-toxin is the defining element of *C. carbonum* race 1 pathogenicity against *hm/hm* maize. Resistance to *C. carbonum* race 1, mediated by *Hm*, is defined as resistance to the end product of the pathogenicity gene (*HC-toxin+*). Race 2 isolates (*tox2*) do not make HC-toxin and are non-pathogenic (*HC-toxin-*) against *Hm/-* or *hm/hm*.

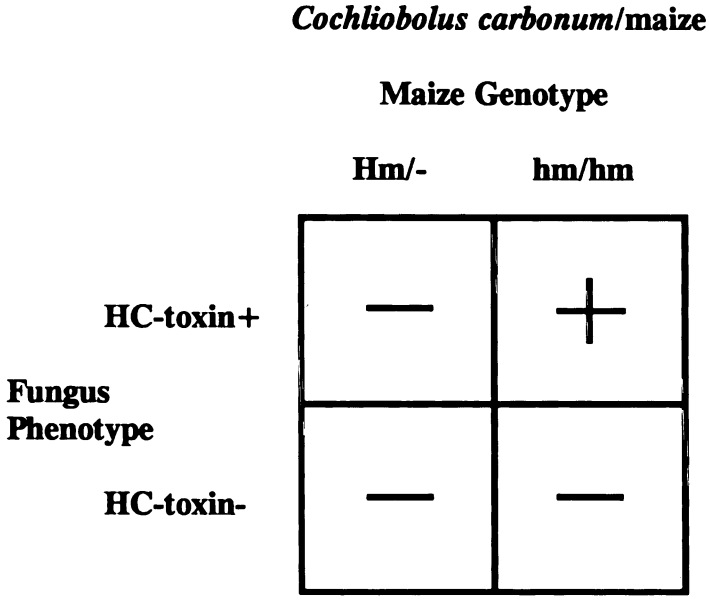

Cochliobolus carbonum/maize
Maize Genotype
Hm/-
hm/hm
HC-toxin+
Fungus Phenotype
HC-toxin-
—
+
—
—

Figure 1.6.

In the past, researchers have addressed *Hm*-mediated resistance to HC-toxin by examining the effects of HC-toxin on maize tissues. The 100-fold differential in sensitivity between *hm* and *Hm* has traditionally been regarded as a critical difference in toxin binding to a receptor, or a difference in transduction of a toxin binding signal to a response (Scheffer and Livingston, 1984). A receptor model for toxin resistance is perhaps the approach most consistent with a theory based on pathogen "recognition", calling to mind images of "lock-and-key" models. But the array of responses to HC-toxin is quite complex, perhaps suggesting that a receptor-mediated mechanism of differential sensitivity is insufficient.

In general, HC-toxin's biological activities are quite diverse and subtle, especially when compared to powerful phytotoxins like victorin (Scheffer and Livingston, 1984). Thus, since a specific mode of action for HC-toxin remains unresolved, the receptor model is open to question. Can a single binding site account for sensitivity to HC-toxin such that it results in differential inhibition of root growth as well as differential inhibition of chlorophyll synthesis? Perhaps, but there remains a great difficulty in delineating the primary from secondary effects of this toxin. As an alternative, it is reasonable to propose that HC-toxin has more than one site of action, or more importantly, that specific toxin resistance controlled by *Hm* occurs by some process independent of toxin action.

Rasmussen and Scheffer (1988b) have concluded that resistant maize has a toxin-sensitive site similar to susceptible maize. While this putative site of action is important to our understanding of the role of toxins in disease, the current hypothesis focuses on the idea that resistant maize may preferentially modify this foreign compound. Given the structural requirements for toxin activity, the question of *Hm*-specific metabolism of the

toxin must be addressed. Differential sensitivity to HC-toxin between *Hm* and *hm* might be explained by a resistance mechanism based on detoxification. With HC-toxin as its substrate, saturation of a detoxicative enzyme in resistant maize might explain the 100-fold differential in toxin sensitivity. HC-toxin above a putative saturation point represents toxin free to act upon its site(s) in the resistant cell.

A fungal secondary metabolite produced in infected maize cells represents a compound foreign to the host. The working hypothesis of this dissertation treats this as a problem of xenobiotic metabolism in plants. Plants, including maize, produce several families of enzymes whose role it is to detoxify foreign compounds (Shimabukuro *et al.*, 1971; Lamoreaux and Rusness, 1986). An enzymatic basis for HC-toxin inactivation is proposed to form the basis of *Hm*-mediated resistance to *C. carbonum* race 1.

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CHAPTER 2*

ENZYMATIC DETOXIFICATION OF HC-TOXIN

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ABSTRACT

Resistance to the fungal plant pathogen *Cochliobolus carbonum* race 1 and to its host-selective toxin, HC-toxin, is determined by *Hm*, a single dominant gene in the host plant *Zea mays* L. Radiolabeled HC-toxin of specific activity 70 mCi/mmol, prepared by feeding tritiated D,L-alanine to the fungus, was used to study its fate in maize leaf tissues. HC-toxin was converted by resistant leaf segments to a single compound, identified by MS and NMR as the 8-hydroxy derivative of HC-toxin formed by reduction of the 8-keto group of 2-amino-9,10-epoxy-8-oxodecanoic acid, one of the amino acids in HC-toxin. Reduction of HC-toxin occurred in cell-free preparations from etiolated (*Hm/hm*) maize shoots, and the activity was sensitive to heat and proteolytic digestion, dependent on NADPH, and inhibited by p-hydroxymercuribenzoate and disulfiram. The enzyme (from the *Hm/hm* genotype) was partially purified by ammonium sulfate precipitation and DEAE-ion exchange chromatography. By gel-filtration chromatography, the enzyme had an M_r of 42,000. NADH was approximately 30% as effective as NADPH as a hydride donor, and flavin-containing co-factors had no effect on activity. When HC-toxin was introduced to maize leaf segments through the transpiration stream, leaf segments from both resistant and susceptible maize inactivated toxin equally well over a time-course of nine hours. Although these data suggest no relationship between toxin metabolism and host-selectivity, we discuss findings in apparent conflict with the current data and describe why the relationship between enzymatic reduction of HC-toxin and *Hm* remains unresolved.

INTRODUCTION

A number of phytopathogenic fungi, especially in the genera *Alternaria* and *Cochliobolus*, produce low-molecular-weight compounds known as host-selective toxins that determine their host range and contribute to their virulence (Scheffer, 1976). Race 1 of *Cochliobolus carbonum* Nelson [*Helminthosporium carbonum* Ullstrup or *Bipolaris zeicola* (Stout) Shoem.] produces a cyclic tetrapeptide, called HC-toxin, that accounts for its exceptional virulence on *Zea mays* L. varieties that are homozygous recessive at the nuclear *hm* locus (Nelson and Ullstrup, 1964; Scheffer and Ullstrup, 1965). HC-toxin has the structure cyclo-[D-prolyl-L-alanyl-D-alanyl-L-Aeo](Kawai and Rich, 1983; Kawai *et al.*, 1983; Leisch *et al.*, 1982; Pope *et al.*, 1983; Walton *et al.*, 1982). Both the terminal epoxide and vicinal ketone of Aeo are required for biological activity of HC-toxin and of its naturally-occuring analogs (Ciuffetti *et al.*, 1983; Closse and Huguenin, 1974; Kim *et al.*, 1987; Walton and Earle, 1983)).

Considerable research has been published on the differential effects of HC-toxin on resistant and susceptible maize (see Scheffer, 1976), but the mode of action of this compound remains unknown. As an alternative to studying the effect of HC-toxin on maize tissues, we have taken an approach that examines the effect of maize tissues on the biological activity of HC-toxin. Given the requirements for the epoxy-ketone moiety of Aeo, we sought to determine if the integrity of these groups is maintained *in planta*. Plants, including maize, are known to contain enzymes capable of inactivating xenobiotic compounds (Lamoreaux and Rusness, 1986). For example, atrazine tolerance in maize is due to elevated levels of glutathione-S-transferase enzymes that inactivate atrazine by

conjugation (Shimabukuro *et al.*, 1971). Knowledge of the biochemical fate of HC-toxin within maize tissues lays the groundwork to ultimately address the hypothesis that host-selectivity in maize to race 1 of *C. carbonum* is due to a difference in ability to detoxify HC-toxin.

We introduce this topic by presenting a method to prepare tritiated HC-toxin for use in metabolic studies. The biochemical fate of HC-toxin is described as we report the conversion of HC-toxin by resistant maize leaves and cell-free extracts to a single non-toxic compound. The enzyme responsible for detoxification is described, and our initial comparison of HC-toxin metabolism between resistant and susceptible maize is critically presented.

MATERIALS AND METHODS

Growth of the Fungus

Maintenance of stock cultures of *Cochliobolus carbonum* Nelson and liquid growth conditions were as described (Walton and Holden, 1988). The toxin-producing isolate SB111 of *C. carbonum* was originally provided by S.P. Briggs, Pioneer Hi-Bred International, Johnston, IA.

Purification and Analysis of HC-toxin

Methods of toxin purification by solvent extraction and reverse-phase HPLC were as given (Walton *et al.*, 1982). HC-toxin was quantified by HPLC and a Spectra-Physics Model 4270 automatic integrator. HC-toxin identity was confirmed by root growth bioassay (Walton *et al.*, 1982), fast-atom bombardment MS, and proton NMR. Mass spectra were collected on a JEOL Model HX110-HF double focusing instrument (accelerating voltage, 10 kV; ionization, 6 keV xenon beam; mass resolution, 1000). NMR analyses were performed in $CDCl_3$ on a Varian VXR-5 500 MHz instrument.

The 8-ketone group of Aeo in HC-toxin was specifically reduced to the corresponding 8-alcohol with sodium borohydride (Kim *et al.*, 1987). The 9,10-epoxide group of Aeo was hydrolyzed with TFA (Walton and Earle, 1983). Derivatives and metabolites of HC-toxin were separated either by reverse-phase HPLC (Walton *et al.*, 1982), by TLC (silica 60, Merck) developed in CH_2Cl_2:acetone (1:1, v/v)(Rasmussen and Scheffer, 1988), or by flash chromatography (Rasmussen and Scheffer, 1988). Epoxides were detected after TLC by spraying plates with p-NBP (Hammock *et al.*, 1974).

In vivo Production of Radiolabeled HC-toxin

Static liquid cultures (125 mL) of *C. carbonum* were grown in 1-L Erlenmeyer flasks. For testing incorporation of various amino acid precursors, 15 μCi of D-[^{14}C]alanine (specific activity 30 to 60 mCi/mmol, ICN); 40 μCi of L-[^{3}H]alanine (30 to 50 Ci/mmol, ICN); or 40 μCi of L-[^{3}H]proline (60 to 100 Ci/mmol, ICN) were added per flask. In experiments conducted to maximize incorporation of radiolabeled alanine, the original growth medium from 8- to 14-day-old cultures was replaced under sterile

conditions by fresh medium containing 5 μCi of [^{14}C]D-alanine (46 mCi/mmol, Amersham). The original medium, which contained any unlabeled HC-toxin, was discarded. The length of incubation in the presence of radioactivity was varied from 24 to 96 h. Once favorable conditions for label incorporation and toxin yield were established, the production of tritiated HC-toxin of high specific activity was initiated by adding 20 mCi of D,L-[2,3-^{3}H]alanine (59 Ci/mmol, Amersham) to a single flask. Radioactivity was monitored during HPLC purification with an in-line scintillation-flow detector (Radiomatic Model CT) at a scintillant:column flow ratio of 3:1 (v/v). Radioactivity was quantified by scintillation counting, corrected for ^{3}H efficiency, and HC-toxin mass was quantified by HPLC with absorbance monitoring at 230 nm.

Growth of Plant Materials

Caryopses of near-isogenic resistant (Pr1 x K61 or K61 x Pr1, genotype *Hm/hm*) or susceptible (Pr x K61 or K61 x Pr, genotype *hm/hm*) maize (*Zea mays* L.) were surface sterilized with 0.5% hypochlorite plus 0.1% Tween-20 for 30 min, rinsed thoroughly with sterile distilled water, and allowed to imbibe water for 2 to 4 h. For the production of green leaves, imbibed seeds from both genotypes were sown in 8-inch diameter clay pots containing a perlite:fine-vermiculite:sphagnum (1:1:1 w/w) mixture. The pots were sub-irrigated with water. Plants were grown in a growth chamber under the following conditions: daylength, 16 h; light intensity, 126 μE/m^2-sec (PAR cool white fluorescent lights); day temperature, 21°C; relative humidity, 72%; night length, 8 h; temperature, 18°C; humidity, 80%.

For production of etiolated shoots for enzyme extraction, imbibed seeds of the

resistant genotype were sown in flats of vermiculite that had been saturated with half-strength Hoagland's solution. The flats were covered with lids and placed in a dark cabinet for 5 to 6 d.

Metabolism of [^{3}H]HC-toxin by Maize Leaf Segments

HC-toxin was administered to mature green leaves through the transpiration stream. Segments of green leaves, ca. 10 cm in length, were cut (approximately 10 cm from the leaf tip) from three- to five-week-old resistant and susceptible plants. The segments were submerged in a beaker of water, evacuated of intercellular air with a laboratory aspirator for 30 min, and then blotted dry with paper towels and placed in 18-mm test tubes containing 5 mL H_2O, with or without 0.25 μCi of [^{3}H]HC-toxin (0.3 μg/mL). The leaf segments were placed in a lighted laboratory fume hood during uptake of [^{3}H]HC-toxin.

For extraction, the leaf segments were rinsed thoroughly in deionized water, frozen in liquid nitrogen, and ground to a powder in a mortar and pestle. Five mL of methanol were added and the leaves were ground again. The methanolic extracts were passed through glass fiber filters (Whatman GF/A) and the methanol was evaporated under vacuum. The aqueous residues were transferred to 1.5-mL polyethylene microfuge tubes and centrifuged at 15,000*g* for 5 min. The supernatants were transferred to fresh microfuge tubes and the pellets discarded. Radioactivity recovered from leaf extracts was analyzed by HPLC coupled to an in-line scintillation-flow detector or by TLC and a scanning beta-detector (Bioscan). As a control, 0.25 μCi of [^{3}H]HC-toxin were added to leaf segments immediately prior to freezing and methanol extraction.

Preparation of Maize Crude Extracts

All steps were carried out at 4°C or on ice. Etiolated plumule tissue (from 5 to 25 g) from freshly germinated resistant (*Hm/-*) maize was cut 1 cm below the coleoptilar node and ground in extraction buffer (0.5 mL per g FW) with a mortar and pestle. The extraction buffer contained 0.1 M Mops (pH 7.4), 0.3 M sucrose, 5% (w/v) PVP, 10% (v/v) glycerol, 5mM DTT, 1mM EDTA, 15 mM ascorbate, and 0.2 mM PMSF. The extract was filtered through four layers of cheesecloth, centrifuged at 3,000*g* for 10 min, and the supernatant saved.

Enzyme Enrichment

The crude extract was initially fractionated with ammonium sulfate. Material precipitating between 30% and 55% saturation (30% saturation equals 17.6 g/100 mL) was collected by centrifugation (10,000*g*, 10 min) and de-salted by gel filtration (PD-10 column, Pharmacia) in 25 mM potassium phosphate (pH 7.5), 2.5 mM DTT, 1 mM EDTA, and 1% (v/v) glycerol. The material was further fractionated on an anion exchange HPLC column (TSK-DEAE-5PW, 7.5 mm x 7.5 cm, Beckman), with a 25-min linear gradient from 0 to 0.5 M NaCl in the same buffer. The flow rate was 1 mL/min and 2 mL fractions were collected.

The molecular weight of the enzyme was estimated by gel filtration HPLC on a TSK-4000 column (30 cm x 7.5 mm, Beckman) equilibrated with 0.15 M potassium phosphate (pH 7.2), 1 mM EDTA, and 5 mM DTT. Approximately 1 mg of protein from an ion exchange fraction containing HCTR activity was loaded onto the column, and 2-mL fractions were collected. Immediately after elution, ascorbate to 10 mM was

added to each fraction. The column was calibrated with the following proteins (Sigma): thyroglobulin (M_r 670,000), IgG (M_r 158,000), ovalbumin (M_r 44,000), and myoglobin (M_r 17,000).

Enzyme Assay Conditions

Typical assay volumes were 125 μL and contained 115 μL enzyme solution, 4 mM NADPH (Sigma), and 0.25 μCi [^{3}H]HC-toxin (23 μM). Reactions were run at 30°C for 15 min and were stopped by the addition of an equal volume of chloroform and rapid mixing. The reactions were extracted twice more with chloroform, and the organic phases were combined and concentrated under vacuum. Concentrated extracts were analyzed by TLC or HPLC. Tritiated substrates and products were quantified with a scanning beta-detector or analyzed by spraying TLC plates with a fluorography enhancer (enHance, DuPont) and exposing the plates to X-ray film (Kodak XAR-5) for several days.

For production of large quantities of the HC-toxin metabolite, unlabeled HC-toxin was used, and the reaction was scaled up to a volume of 4 mL. The HC-toxin metabolite was purified by chloroform extraction, flash chromatography (Rasmussen and Scheffer, 1988), and reverse-phase HPLC (Walton *et al.*, 1982).

RESULTS

Production of Radiolabeled HC-toxin

Several radiolabeled amino acids present in native HC-toxin were evaluated as precursors for *in vivo* production of radiolabeled HC-toxin. As expected from the fact that D-alanine is a substrate for HC-toxin synthetase *in vitro* (Walton and Holden, 1988), D-alanine was incorporated into HC-toxin *in vivo*. D-alanine was incorporated 68-fold more effectively than L-alanine and six-fold more effectively than L-proline (Table I). Radiolabeled D-proline was not tested because it was not commercially available. The racemic mixture of tritiated alanine used in the final experiment was not evaluated in the manner presented in Table I prior to the final labeling experiment.

Although production of HC-toxin can vary from culture to culture, we attempted to optimize the conditions for *in vivo* incorporation of alanine into HC-toxin by evaluating the incorporation of D-[^{14}C]alanine with respect to the time added to culture, and the duration of incubation in the presence of the radioactivity. In our hands, eight days of fungal growth prior to media exchange followed by 48 h incubation with radiolabeled D-[^{14}C]alanine gave the best balance between specific activity and toxin yield (data not shown). Following this protocol, 20 mCi of D,L-[2,3-^{3}H]alanine were added to a single flask, and approximately 2 mg of pure, tritiated HC-toxin were recovered. The chromatographic behavior of tritiated HC-toxin was identical to that of unlabeled toxin during purification. Its biological activity in the root growth bioassay was the same as that of unlabeled HC-toxin (ED_{50} of 0.3 μg/mL). The specific activity of the tritiated HC-toxin was 70.1 $\pm$ 0.2 mCi/mmol.

Table 2.1. Incorporation of radiolabeled amino acid precursors into HC-toxin in vivo. (by J.D. Walton)
The indicated amino acids were added to *C. carbonum* race 1 after 10 d growth, and the culture filtrates were harvested after an additional 2 d.

		Radioactivity Recovered (% of original)		
Amino Acid	Amount Added (μCi)	Crude Culture Filtrate[a]	Chloroform Phase[b]	Final Recovery[c]
D-[^{14}C]alanine	15	10.5	4.4	2.7
L-[^{3}H]alanine	40	51.0	0.06	0.04
L-[^{3}H]proline	40	22.0	0.7	0.45

[a] % of total radioactivity present in crude culture filtrate.
[b] % of total radioactivity extracted from culture filtrate with chloroform.
[c] % of total radioactivity remaining after chloroform was evaporated and sample was redissolved in water.

Alteration of Toxin Structure Within Maize Leaf Segments

Native HC-toxin was altered following its uptake by transpiration into resistant leaf segments (Fig. 2.1). An apparent metabolite (Peak 3) eluted with a polarity intermediate to native HC-toxin (Peak 1) and its 9,10-diol form (Peak 2) (Fig. 2.1B). The diol of HC-toxin is formed by hydrolysis of the epoxide of Aeo, and was present as a minor contaminant in all chromatograms, including controls (Fig. 2.1A). Beyond a minor increase in the amount of diol produced during extraction, no metabolites of HC-toxin apart from **3** were detected. In addition, **3** was the only altered form of HC-toxin observed when leaf extractions were performed in aqueous solvents (not shown), or when leaf extracts were analyzed by TLC. Feeding partially purified **3** (0.05 μCi/mL) back to maize leaves did not result in the formation of any additional tritium-containing compounds (data not shown), suggesting that **3** is an end product of HC-toxin metabolism.

Metabolite Identification

Figure 2.2 shows TLC analysis of native HC-toxin, $NaBH_4$-reduced toxin, and the HC-toxin metabolite recovered from resistant leaf segments (Peak 3). The HC-toxin metabolite (lane 3) had the same R_f as $NaBH_4$-reduced HC-toxin (lane 2). The metabolite reacted with the epoxide indicator p-NBP (lane 7), indicating that the epoxide was still intact. The FAB-mass spectra of chemically reduced HC-toxin and purified toxin metabolite had molecular ions of m/e = 439, consistent with the addition of two atomic mass units to HC-toxin (Fig. 2.3). The NMR spectrum, shown in Figure 2.4, indicates the creation of a new proton signal vicinal to epoxide carbon-9 of the Aeo side chain.

This proton's chemical shift, multiplicity, and influence on spin-spin coupling and chemical shift for carbons 9 and 10 are in agreement with the data for the R-isomer of carbon-8 as described by Kim *et al.* (1987). Rasmussen described a similar spectrum and gave the name HC-toxin IV to this derivative (Rasmussen, 1987; Rasmussen and Scheffer, 1988). Based on these results, we conclude that the metabolite formed in maize leaves is HC-toxin in which the 8-carbonyl group of the Aeo side chain has been reduced to the 8-alcohol. We have confirmed the results of Kim *et al.* (1987) that showed this form of HC-toxin to be non-toxic.

Figure 2.1. Formation of a metabolite of HC-toxin by green maize leaf segments. Following uptake of 0.25 μCi [^{3}H]HC-toxin by transpiration, the leaves were ground and analyzed by reverse-phase HPLC. Radioactivity (shown) eluting from the HPLC column was detected with an in-line scintillation counter. (A) Control: [^{3}H]HC-toxin added to leaf segments immediately prior to extraction, (B) Extract of resistant leaves. Peak 1: native HC-toxin; Peak 2: 9,10-diol-HC-toxin; Peak 3: HC-toxin metabolite.

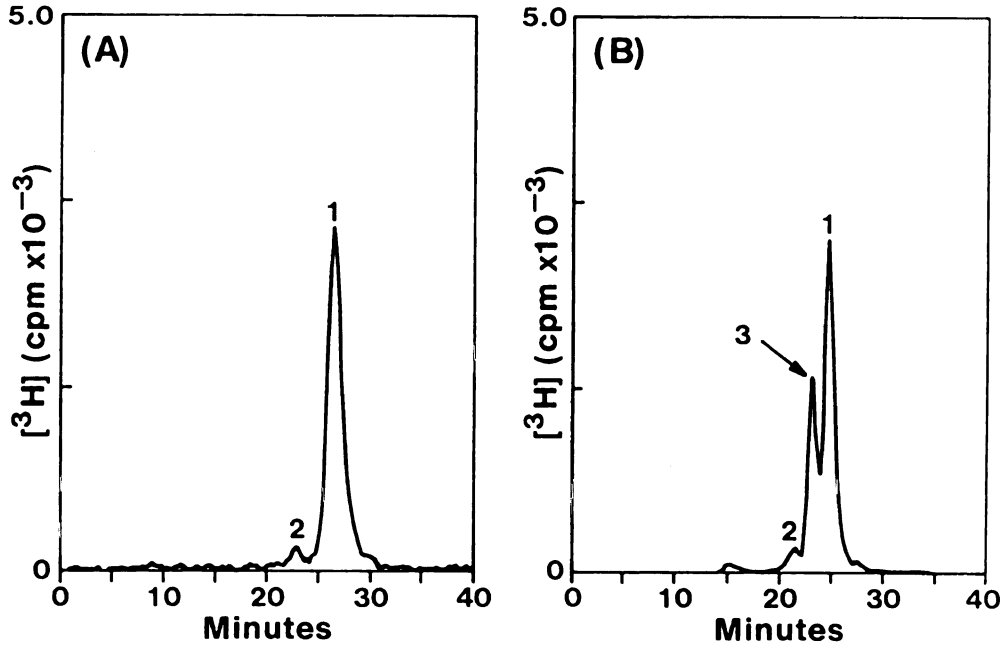
5.0
(A)
[3H] (cpm x10−3)
1
2
0
0
10
20
30
40
Minutes
5.0
(B)
[3H] (cpm x10−3)
1
3
2
0
0
10
20
30
40
Minutes

Figure 2.1.

Figure 2.2. TLC analysis of HC-toxin metabolites produced by green leaf segments and by cell-free extracts of resistant maize. Native [^{3}H]HC-toxin (1), $NaBH_4$-reduced [^{3}H]HC-toxin (2), Partially-purified toxin metabolite recovered from maize leaves (3). Metabolites produced by 10 min (4) and 20 min (5) incubation of cell-free extracts with [^{3}H]HC-toxin, and extract boiled before incubation (6). Lanes 1 through 6 were detected by fluorography. Metabolites produced in cell-free extracts detected with the epoxide indicator p-NBP (7). Both native HC-toxin (R_f 0.55) and the toxin metabolite (R_f 0.35) react with the epoxide indicator.

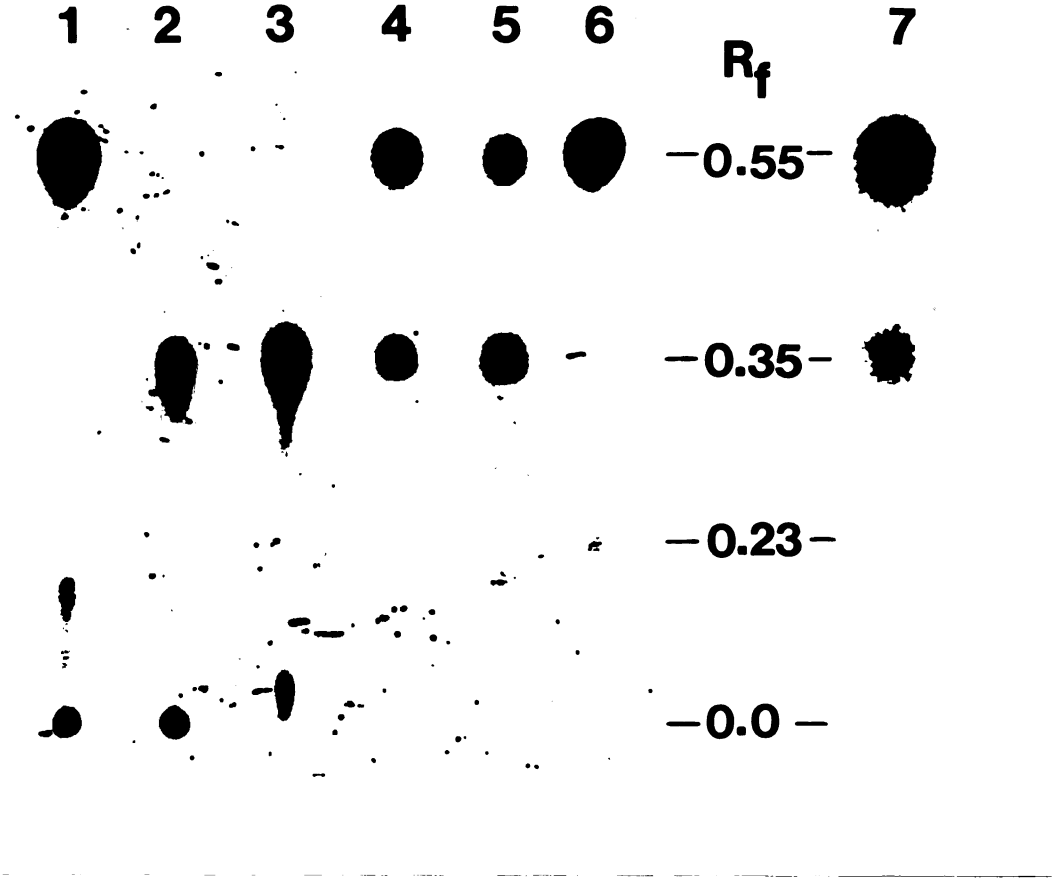

Figure 2.2.

Figure 2.3. Fast-atom bombardment mass spectra of (A) the metabolite of HC-toxin produced by maize cell-free extracts and (B) HC-toxin reduced with $NaBH_4$. Samples were dissolved in a matrix of glycerol and HCl. Both compounds produce molecular ions of m/e 439 $(HC\text{-}toxin + 2H + H)^+$ and the following: m/e 369, $[(glycerol)_4 + H]^+$; m/e 461, $[(glycerol)_5 + H]^+$; m/e 475, $[M + HCl]^+$; m/e 531, $[M + glycerol]^+$.

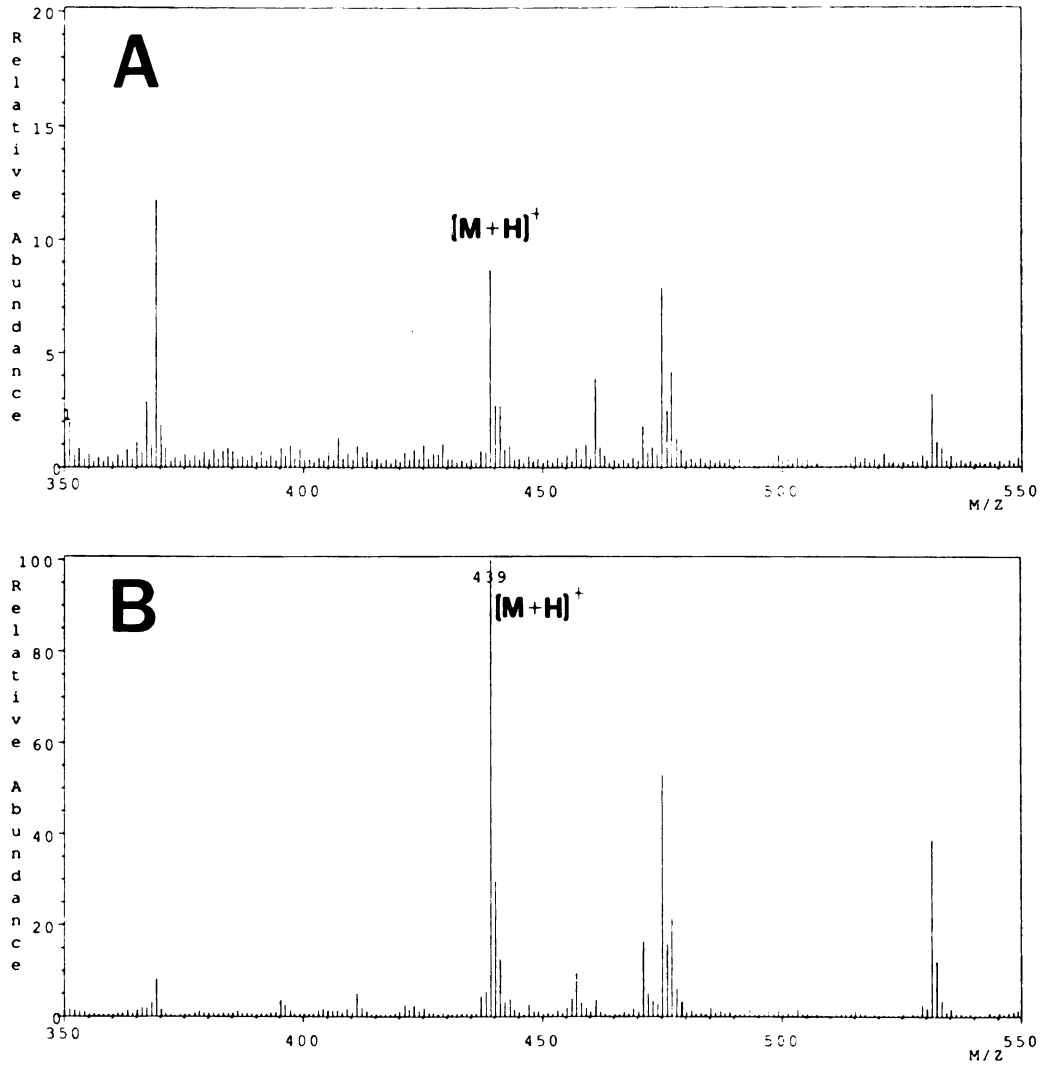
A
Relative Abundance
20
15
10
5
0
[M+H]+
350
400
450
500
550
M/Z
B
Relative Abundance
100
80
60
40
20
0
439
[M+H]+
350
400
450
500
550
M/Z

Figure 2.3.

Figure 2.4. NMR analysis of the HC-toxin metabolite[1]. Shown in A is the relevant region of a 500 MHz proton NMR spectrum for the HC-toxin metabolite purified from a crude extract of resistant maize. The region contains signals for carbons 8-10 of the Aeo side chain. Relative to native HC-toxin, the metabolite contains an additional signal at 3.43 ppm. This signal influences the splitting pattern of C-9 (the signal changes from a sharp doublet-of-doublets to a multiplet due to spin-spin coupling from both C-8 and C-10), and infleunces the chemical shifts for both C-9 and C-10 (δ_{ppm} for C-9 is lowered from 3.38 to 2.96; δ_{ppm} for C-10 lowered from 2.96 to 2.59 (10a), and 2.83 to 2.72 (10b)). A selective pulse at carbon-9 (3.0 ppm) decouples the signal at 3.43, indicating that the signal is attributable to a proton at carbon-8 (shown in panel B). Expected decoupling effects also occur to the protons of carbon-10. The designation *i* indicates a detectable impurity. Selective decoupling of the impurity signal has no effect on any of the HC-toxin resonances (not shown).

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[1]The data in Figure 2.4 were not shown in Meeley and Walton (1991, Plant Physiol. **97**, 1080-1086), but are included here as additional proof.

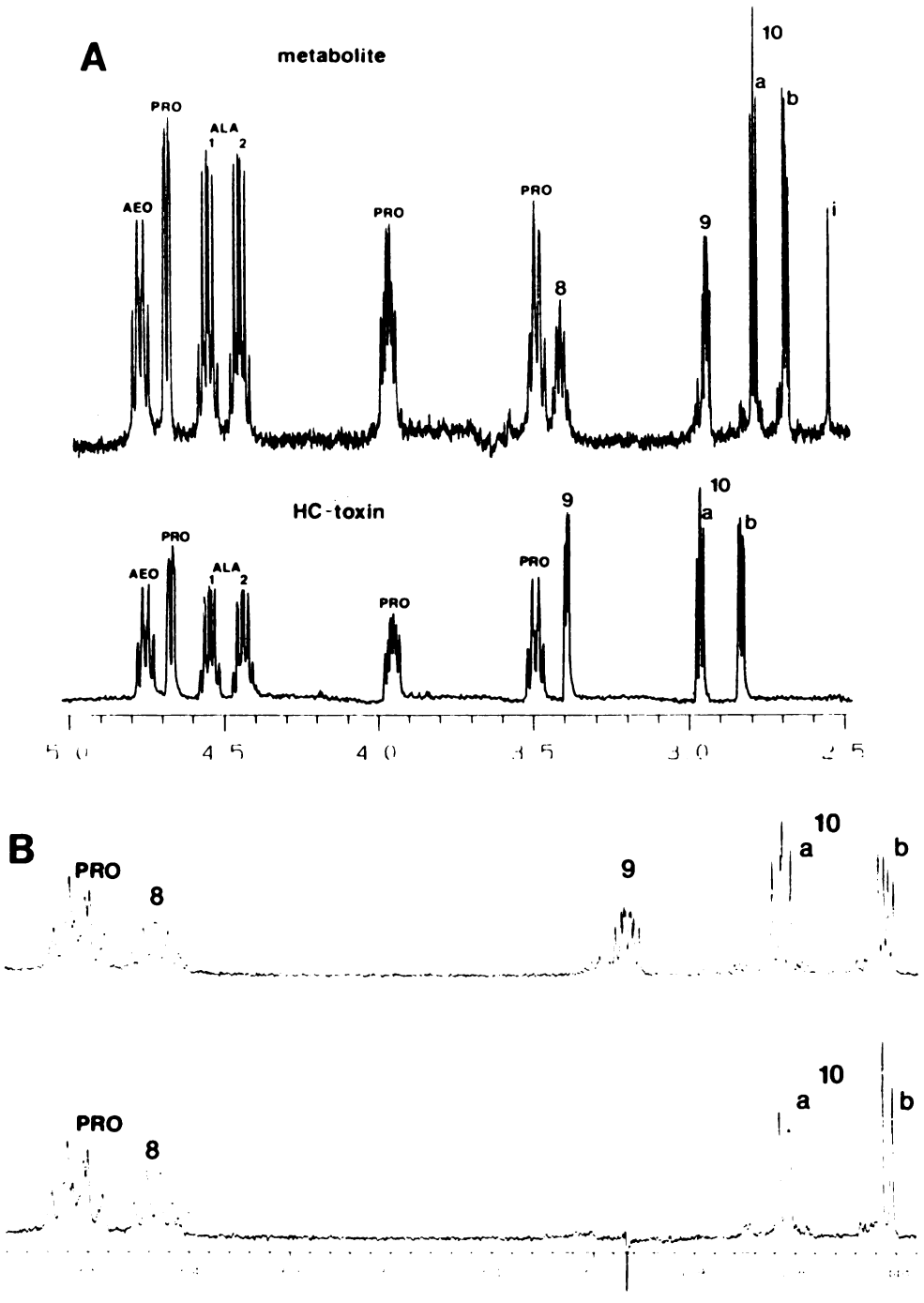
A
metabolite
AEO
PRO
ALA
1
2
PRO
PRO
8
9
10
a
b
i
HC-toxin
AEO
PRO
ALA
1
2
PRO
PRO
9
10
a
b
5.0
4.5
4.0
3.5
3.0
2.5
B
PRO
8
9
10
a
b
PRO
8
10
a
b

Figure 2.4.

HC-toxin Metabolism in vitro

In the presence of NADPH, extracts prepared from the etiolated shoots of resistant maize catalyzed the same metabolic conversion of HC-toxin as intact leaves (Fig. 2.2). The amount of reduced HC-toxin recovered from incubations with cell-free extracts increased with time (Fig. 2.2, lanes 4 and 5), while activity was completely abolished by boiling the extract for 10 min prior to incubation (lane 6).

This enzymatic activity, which we call HC-toxin reductase (HCTR), was partially purified from etiolated resistant shoots. Ammonium sulfate fractionation and anion exchange HPLC resulted in a five-fold enrichment of reductase activity with an 18% recovery. TLC and fluorography of the products formed from [^{3}H]HC-toxin by individual fractions from an anion exchange separation (Fig. 2.5, bottom) is shown below the UV trace (Fig. 2.5, top). HCTR was eluted from the anion exchange column in a single fraction, No. 10; 8-hydroxy HC-toxin is indicated by (a). A second product of greater polarity (labeled b) was formed by Fraction 11. Product b reacted with p-NBP, indicating an intact epoxide, but its formation was partially resistant to boiling and did not require a hydride donor (data not shown). Its formation was never observed in crude HCTR preparations (see Fig. 2.2). When we purged Fraction 11 with nitrogen or included oxygen-scavenging compounds such as GSH, ascorbate, or DTT, in the reaction mixture, the formation of product b was reduced substantially but no formation of 8-hydroxy HC-toxin was observed (not shown). We conclude that product b is a form of HC-toxin produced by a side reaction, perhaps oxygen-dependent, that occurs in solution in Fraction 11. Importantly, this reaction is unrelated to HCTR activity.

Figure 2.5. Anion exchange fractionation of HCTR activity. The top panel represents the elution of protein (A_{280}). Each fraction was assayed for HCTR activity, and the resulting products were separated by TLC and detected by fluorography (bottom panel). (a) the 8-hydroxy derivative of HC-toxin. (b) unknown compound, formed as an artifact of the ion-exchange process (see text).

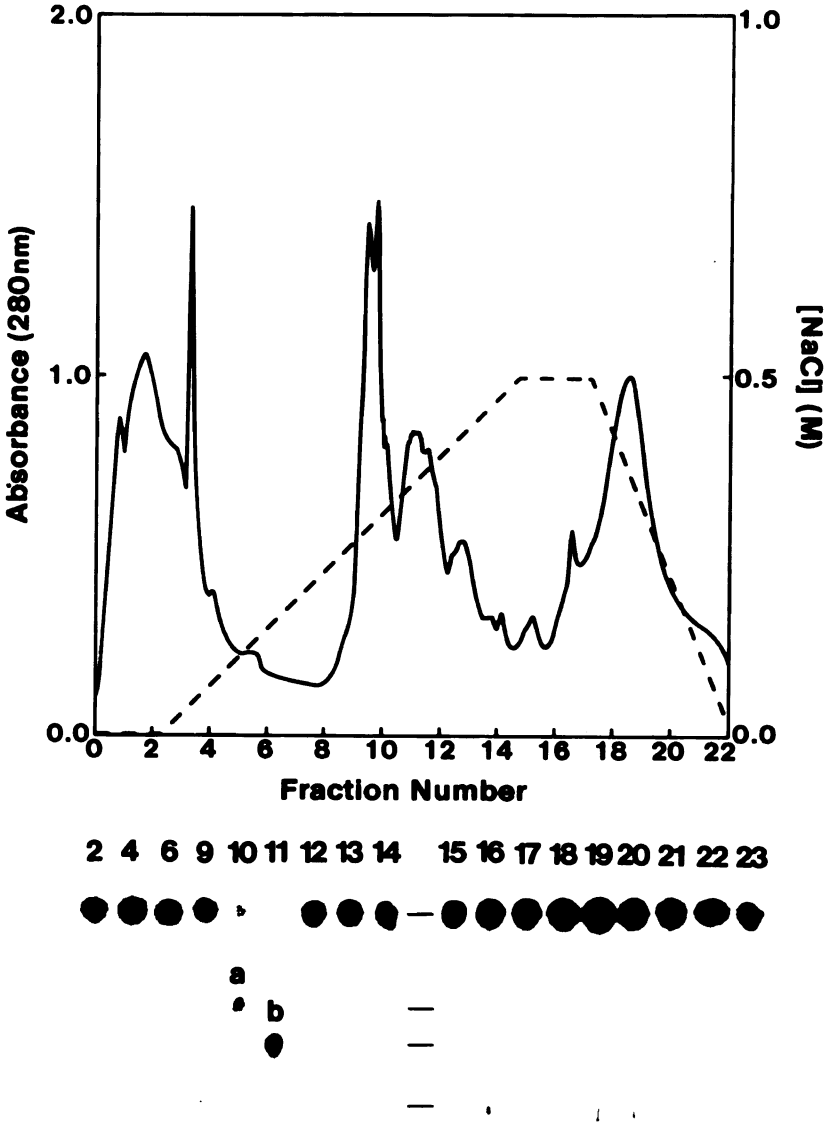
2.0
1.0
0.0
Absorbance (280nm)
1.0
0.5
0.0
[NaCl] (M)
0 2 4 6 8 10 12 14 16 18 20 22
Fraction Number
2 4 6 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
a
b

Figure 2.5.

Characterization of HCTR Activity

Table II shows the effects of various treatments and co-factors on HCTR activity. Activity of partially-purified HCTR was completely abolished by boiling or by pre-treatment with proteinase K. NADPH was a better co-substrate than NADH. A ten-fold excess of $NADP^+$ over NADPH inhibited HCTR activity by approximately 30%, and a ten-fold excess of NAD^+ had no effect. Approximately 35% of the HCTR activity was lost by simply incubating the partially-purified preparation for 60 min at 30°C. Incubation for 60 min in the presence of Zn^{2+} or Fe^{2+}, and to a lesser extent Cu^{2+}, further inhibited (or destabilized) HCTR activity (Table II). Two known inhibitors of carbonyl reductases, p-HMB and disulfiram (Fischer *et al.*, 1988; Iwata *et al.*, 1990), inhibited HCTR by 50% and 70%, respectively (Table II).

On gel-filtration HPLC, HCTR was eluted as a single, symmetrical peak with an M_r of 42,000 (data not shown).

HC-toxin Metabolism by Resistant and Susceptible Leaf Segments

When green leaf segments of equal weight were evacuated of intercellular air and allowed to transpire water containing [^{3}H]HC-toxin, significant production of 8-hydroxy-HC-toxin occurred in both resistant and susceptible leaves (Fig. 2.6). The toxin concentration used (0.3 μg/mL) in these experiments was equivalent to the ED_{50} for HC-toxin in the root growth bioassay. Under these conditions, HC-toxin uptake and metabolism occurred at the same rate in both resistant and susceptible leaves over a 9-h transpiration period (Fig. 2.6). A time course extended over 48 h showed a similar lack of host-selective detoxification (data not shown).

Table 2.2. Characterization of HCTR activity.

Concentration of NADPH was 4 mM unless otherwise indicated. All ions were 2 mM sulfate salts. The concentration of [^{3}H]HC-toxin in each case was 23 μM and assays were run for 15 min at 30°C.

Treatment	HCTR Activity (% of Control)
Experiment 1	
+ NADPH	100[a]
boiled (10 min)	0
- NADPH	0
+ proteinase K[c]	0
+ NADH (4 mM)	31
+ NADPH, FAD (0.1 mM)	97
+ NADPH, FMN (0.1 mM)	71
+ NADPH (1 mM), $NADP^+$ (10 mM)	70
+ NADPH (1 mM), NAD^+ (10 mM)	112
+ NADPH + disulfiram (10 μM)	52
+ NADPH + p-HMB (10 μM)	30
Experiment 2	
Preincubation for 60 min with:	
No divalent cations	100[b]
Fe^{2+}	0
Mg^{2+}	95
Zn^{2+}	0
Mn^{2+}	91
Co^{2+}	77
Cu^{2+}	42

[a]100% activity = 659 pmol/min/mg protein.
[b]100% activity = 427 pmol/min/mg protein.
[c]80 μg/mL, 10 min, 30°C.

Figure 2.6. Time course of [^{3}H]HC-toxin metabolism by maize leaf segments during uptake by transpiration. Results are the average of duplicate samples from three independent experiments. Error bars represent $\pm$ 1 SD.

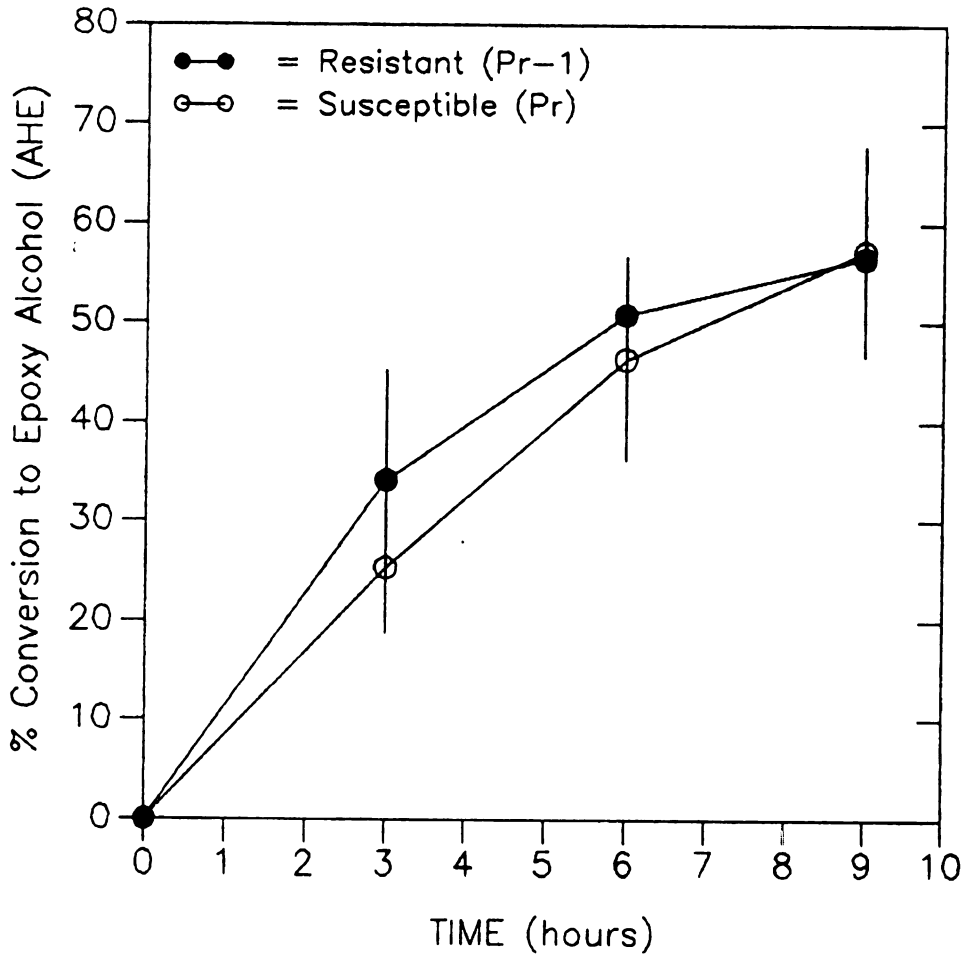
80
70
60
50
40
30
20
10
0
= Resistant (Pr−1)
= Susceptible (Pr)
% Conversion to Epoxy Alcohol (AHE)
0 1 2 3 4 5 6 7 8 9 10
TIME (hours)

Figure 2.6.

DISCUSSION

Previous work from this laboratory has described the purification and characterization of two enzymes involved in HC-toxin biosynthesis (Walton, 1987; Walton and Holden, 1988). One of these enzymes, HTS-2, activates both D- and L-alanine for incorporation into HC-toxin. Perhaps because D-alanine, unlike L-alanine, is not diverted into cellular primary metabolism, its efficiency of incorporation into HC-toxin was relatively high (Table I). Once favorable conditions for radiolabel incorporation and toxin yield were determined, alanine incorporation was exploited to make tritiated HC-toxin *in vivo*. The chromatographic behavior and biological activity of HC-toxin were not affected by the incorporation of tritiated alanine into the peptide ring. The radiolabeled HC-toxin produced by this method was satisfactory for studying its fate in maize tissues.

The 8-hydroxy derivative of HC-toxin was the only metabolite recovered from resistant maize leaves following uptake of [^{3}H]HC-toxin by transpiration (Fig. 2.1). Importantly, 8-hydroxy-HC-toxin is biologically inactive, and was the only toxin metabolite recovered from any the tissues tested, including cut leaves, cell-free preparations from etiolated shoots, excised roots (not shown), and whole leaves (not shown). Notable is the observation that the other critical functional group, the epoxide of Aeo, was not altered *in planta* or *in vitro*.

Reduction of the 8-keto group of HC-toxin is an enzymatic process. An enzyme, referred to as HCTR, that catalyzes this reduction was partially purified from extracts prepared from etiolated resistant shoot tissue. HCTR appears to be similar to other

NADPH-dependent carbonyl reductases found in plants and animals; it is soluble, uses NADPH more effectively than NADH as a hydride donor, and has an M_r in the range of 32,000 to 45,000. Characterized NADPH-dependent carbonyl reductases have biosynthetic functions in anthocyanidin production in plants (Fischer *et al.*, 1988; Welle and Grisebach, 1988) and the interconversion of steroids in mammalian tissues (Iwata *et al.*, 1990; Wolfe *et al.*, 1989). Carbonyl reductases often contain metal ions for catalytic stability and are highly specific for their substrates. In the case of partially-purified HCTR, metal ions did not contribute to enzyme stability, and certain divalent cations inhibited activity (Table II). Whether HCTR has an endogenous substrate is not known at this time.

With the knowledge that HC-toxin is enzymatically metabolized to a single inactive compound in resistant maize tissues, we have sought to determine if this phenomenon is related to the *hm* locus of maize that governs host-selectivity of *C. carbonum*. Our first comparative experiments are summarized by Fig. 2.5, which indicated that both resistant and susceptible maize were capable of reducing and thereby inactivating HC-toxin when toxin was delivered to green excised leaves through the transpiration stream. These data suggest that reduction of HC-toxin is not the basis of host-selectivity and hence resistance to race 1 of *C. carbonum*. However, we feel it necessary to caution that delivery of toxin *via* transpiration is an artificial technique devised to deliver toxin to leaf tissues as quickly as possible.

In maize, host-selective reaction to *C. carbonum* is expressed in etiolated shoots (Heim *et al.*, 1983). Therefore, to address whether kinetic aspects of toxin metabolism are related to host-selectivity, we have initiated a comparative study on HCTR from

etiolated resistant and susceptible maize. Interestingly, these experiments have failed because repeated attempts to detect HCTR activity in extracts from etiolated susceptible maize have been unsuccessful (unpublished results). This result is in apparent conflict with the results of our transpiration experiments. Until we resolve this paradox, we can not base our conclusions solely on data from transpiration experiments. It is possible that HC-toxin encounters a reductase during transpiration that is different from HCTR. Therefore, a relationship between HCTR and the *hm* locus remains an open question. However, whether or not a relationship between HCTR and host-selectivity exists, the discovery that maize tissues contain an enzyme capable of inactivating HC-toxin establishes a novel facet of the interaction between *C. carbonum* and maize. Because toxin production is so critical to the infection process, a completely characterized enzymatic mechanism affecting the biological activity of HC-toxin may in time be considered as an integral part of this host/pathogen interaction.

ACKNOWLEDGEMENTS

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CHAPTER 3*

A BIOCHEMICAL PHENOTYPE FOR A DISEASE RESISTANCE GENE OF MAIZE

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ABSTRACT

In maize, major resistance to the pathogenic fungus *Cochliobolus* (*Helminthosporium*) *carbonum* race 1 is determined by the dominant allele of the nuclear locus *hm*. The interaction between *C. carbonum* race 1 and maize is mediated by a pathogen-produced, low-molecular weight compound called HC-toxin. We recently described an enzyme from maize, called HC-toxin reductase, that inactivates HC-toxin by pyridine nucleotide-dependent reduction of an essential carbonyl group. We now report that this enzyme activity is detectable only in extracts of maize that are resistant to *C. carbonum* race 1 (genotype *Hm*/*Hm* or *Hm*/*hm*). In several genetic analyses, in vitro HC-toxin reductase activity was without exception associated with resistance to *C. carbonum* race 1. The results indicate that detoxification of HC-toxin is the biochemical basis of *Hm*-specific resistance of maize to infection by *C. carbonum* race 1.

INTRODUCTION

Resistance of plants to pathogens is frequently inherited in a simple Mendelian fashion, and typically, resistance is dominant to susceptibility. Although specific resistance has been intensively studied at the genetic level, in no case is the biochemical process controlled by a specific resistance gene known. The fungal pathogen *Cochliobolus carbonum* (*Helminthosporium carbonum*) race 1 causes leaf spot and ear mold of maize. The dominant allele of the Mendelian gene *Hm* gives specific resistance to *C. carbonum* race 1 at all stages of growth (Nelson and Ullstrup, 1964). A number of studies have concluded that specific pathogenicity of *Cochliobolus carbonum* is mediated by a low-molecular-weight compound called HC-toxin, and that *Hm* governs resistance to *C. carbonum* by way of insensitivity to HC-toxin (Yoder, 1980). For example, root growth of susceptible maize (genotype *hm/hm*) is inhibited by HC-toxin whereas root growth of resistant maize (genotype *Hm/Hm* or *Hm/hm*) and of other plants is not inhibited except at much higher concentrations (Scheffer and Ullstrup, 1965). In the fungus, HC-toxin production genetically co-segregates with specific pathogenicity on *hm/hm* maize (Scheffer and Nelson, 1967).

HC-toxin is a cyclic tetrapeptide of structure cyclo(D-prolyl-L-alanyl-D-alanyl-L-Aeo), where Aeo stands for 2-amino-9,10-epoxy-8-oxodecanoic acid (Gross *et al.*, 1982; Walton *et al.*, 1982; Kawai *et al.*, 1983). The epoxide and the 8-carbonyl groups of Aeo are required for biological activity of HC-toxin (Ciuffetti *et al.*, 1983; Walton and Earle, 1983; Kim *et al.*, 1987). During studies on the biochemistry of HC-toxin synthesis (Walton, 1987; Walton and Holden, 1988), it was found that D-alanine is recognized and

activated by one of the biosynthetic enzymes, HTS-2, in vitro, and that radiolabeled D-alanine is incorporated into HC-toxin in vivo (Walton, 1991). We have exploited this fact to prepare [^{3}H]-HC-toxin (Meeley and Walton, 1991).

When fed to green maize leaves through the transpiration stream, [^{3}H]-HC-toxin is metabolized to a single, nontoxic derivative in which the 8-carbonyl group of Aeo is reduced to the corresponding alcohol (Meeley and Walton, 1991). However, leaves of both resistant (genotype *Hm/hm*) and susceptible (genotype *hm/hm*) maize convert HC-toxin equally well. This originally suggested that detoxification by reduction is not the basis of resistance controlled by *Hm* (Meeley and Walton, 1991).

Cell-free extracts of etiolated maize seedlings metabolize HC-toxin to the same compound, 8-hydroxy-HC-toxin, as whole green leaves. The responsible enzyme, called HC-toxin reductase (HCTR), uses NADPH as cosubstrate and has been partially purified and characterized from resistant (*Hm/hm*) maize (Meeley and Walton, 1991). We sought to compare the kinetics of HCTR from susceptible and resistant plants, but as shown in this paper, HCTR activity is detectable only in extracts of resistant maize, genetically segregates with the *Hm* allele, and is severely reduced in transposon-induced mutations of *Hm*. In addition, differential detoxification of HC-toxin occurs in intact tissues when [^{3}H]-HC-toxin is delivered by means other than the transpiration stream. The results argue that HCTR is the critical biochemical function controlled by *Hm* and is responsible for specific resistance of maize to C. carbonum race 1.

MATERIALS AND METHODS

In Vitro HCTR Assay

Tritiated HC-toxin (specific activity 70 mCi/mmol) was prepared as described (Meeley and Walton, 1991). Etiolated maize seedlings were grown as described (Meeley and Walton, 1991). To assay HCTR, single plumules of equal fresh weight (150 mg) were harvested and ground with a pestle in 200 uL extraction buffer [0.1 M Mops, pH 7.4, 0.3 M sucrose, 5% (v/v) polyvinylpyrrolidone, 10% (v/v) glycerol), 5 mM dithiothreitol, 1 mM EDTA, 15 mM ascorbate, and 0.2 mM phenylmethylsulfonate] in 1.5 mL microfuge tubes. NADPH (final concentration 4 mM) and 0.25 uCi [^{3}H]-HC-toxin were added directly to the ground tissue and the mixture incubated for 30 min at 30°C. The reaction mixtures were extracted three times with chloroform, the chloroform evaporated under reduced pressure, and the residue analyzed by thin-layer chromatography (TLC) on silica gel 60 (Meeley and Walton, 1991). Radioactive compounds on the TLC plates were detected either with an automated ß-detector (Bioscan) (Figures 3.1, 3.5, and 3.6) or by fluorography (Figures 3.2, 3.3, and 3.4) as described (Meeley and Walton, 1991).

Pathogenicity Tests

Maize seedlings were grown in darkness as described (Meeley and Walton, 1991). After the plumule was removed for the HCTR assay, the mesocotyl, still attached to the caryopsis, was wounded slightly with a razor blade and inoculated with approximately 100 conidia of *C. carbonum* race 1 in one uL of 0.1% Tween-20. The inoculated seedlings were then returned to darkness for 3 to 6 days before being photographed.

Identification of Revertants

Independent ears (families) were harvested from selfed heterozygotes of mutant *hm*-1040, containing either Spm or dSpm at the *Hm* locus. Batches of 50 kernels from each ear were grown in the dark and the plumules excised, ground in extraction buffer as described above, and frozen at -80°C. The mesocotyls were inoculated with *C. carbonum* race 1, and placed in the dark until disease symptoms developed. When candidates for germinal revertants were identified by resistance to the fungus, the appropriate extracts were thawed and assayed for HCTR activity as described above. Extracts from susceptible progeny were chosen at random as negative controls.

In Vivo Metabolism Assays

For the in vivo green leaf disc assay (Figure 3.5), plants were grown in a growth chamber as described (Meeley and Walton, 1991). Fourth true leaves were abraded on both sides with carborundum using a watercolor brush and 1-cm discs cut with a cork borer. The discs were floated lower side down in 24-well microtiter plates in 200 uL of 5 mM $CaCl_2$, 5 mM KCl, 20 mM sucrose, 0.1% Tween-20, and 2 ug/mL [^{3}H]-HC-toxin (143,000 dpm). The plates were incubated 24 h under room lights. To calculate uptake of [^{3}H]-HC-toxin, the leaf discs were removed, rinsed, frozen, and extracted with methanol. Radioactivity in the methanol extract was measured by scintillation counting. To measure metabolism of [^{3}H]-HC-toxin, the liquid remaining in the wells was extracted three times with $CHCl_3$ and the extracts analyzed by TLC as described above.

For the in vivo plumule assay (Figure 3.6), etiolated plants were grown as described (Meeley and Walton, 1991). Five day old seedlings were excised just above

the coleoptilar node and injected with a 22G hypodermic needle through the base into the space between the coleoptile and leaves with [^{3}H]-HC-toxin (concentration 200 ug/mL) in a volume of 10 uL. The plumules were incubated horizontally on moist filter paper in covered petri plates in the dark for 3 or 6 hr. They were then rinsed with distilled water, ground in Eppendorf tubes in 0.5 mL distilled water, centrifuged to remove cellular debris, and the supernatants extracted three times with $CHCl_3$ and analyzed by TLC as described above.

RESULTS

As shown in Figure 3.1, HCTR is detectable in several maize lines of genotype *Hm/Hm* or *Hm/hm* (inbred Pr1 and hybrids K61 x Pr1 and Pr1 x K61) but not in maize lines of genotype *hm/hm* (inbreds Pr, K61, Mo21a, and K44, and hybrid Pr x K61). To test genetic cosegregation of *Hm* with in vitro HCTR activity, a method was developed to simultaneously assay HCTR activity and disease reaction in single seedlings. In the testcross *Hm/hm* x *hm/hm*, resistant and susceptible plants segregate 1:1. In Figure 3.2, a representative sample of progeny from such a cross is shown. From a total of 50 progeny, only the resistant plants (29 of 50) had HCTR activity, converting from 40% to 70% of HC-toxin to the 8-hydroxy derivative. The susceptible plants (21 of 50) lacked detectable enzyme activity. No recombination between the resistant disease phenotype and presence of HCTR activity was observed. Statistical analysis of these data

Figure 3.1. HC-toxin reductase (HCTR) activity in extracts of etiolated shoots of maize lines susceptible or resistant to *C. carbonum* race 1. Extracts of single plumules (leaves plus coleoptile) were assayed for in vitro HCTR activity and the reaction products separated by TLC and detected with a Bioscan ß-detector. HCTR activity is evidenced by conversion of native HC-toxin (R_f 0.5) to 8-hydroxy-HC-toxin (R_f 0.3). Inbred lines are indicated by ⊗. Pr1, Pr1 x K61, and K61 x Pr1 are resistant; Pr, K61, Mo21a, K44, and Pr x K61 are susceptible. Inbreds Pr and Pr1 are near-isogenic, differing only at the *hm* locus (Scheffer and Ullstrup, 1965).

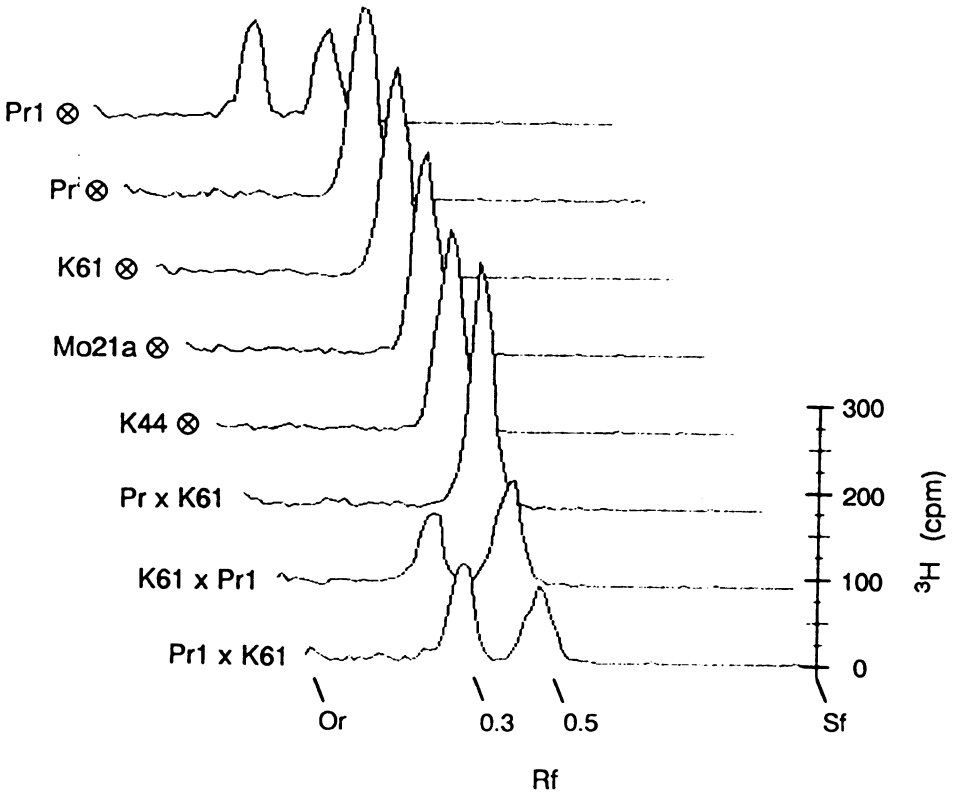
Pr1 ⊗
Pr ⊗
K61 ⊗
Mo21a ⊗
K44 ⊗
Pr x K61
K61 x Pr1
Pr1 x K61
300
200
100
0
^{3}H (cpm)
Or
0.3
0.5
Sf
Rf

Figure 3.1.

Figure 3.2. Cosegregation of HCTR activity and disease reaction to *C. carbonum* race 1. Shown is a representative sample of progeny from a cross between maize of genotype *Hm/hm* and genotype *hm/hm*. Each plant was assayed for in vitro HCTR activity and disease reaction.
(Top) Metabolites of [^{3}H]-HC-toxin were separated by TLC and detected by fluorography. The upper spot is native HC-toxin and the lower spot, when present, is 8-hydroxy-HC-toxin.
(Bottom) In a resistant reaction (lanes 4, 5, 7, and 9-13) the fungus does not spread from the site of inoculation and the mesocotyl remains white. In a susceptible reaction (lanes 1-3, 6, 8, 14, and 15) the fungus colonizes the tissue and the mesocotyl becomes water-soaked and necrotic.

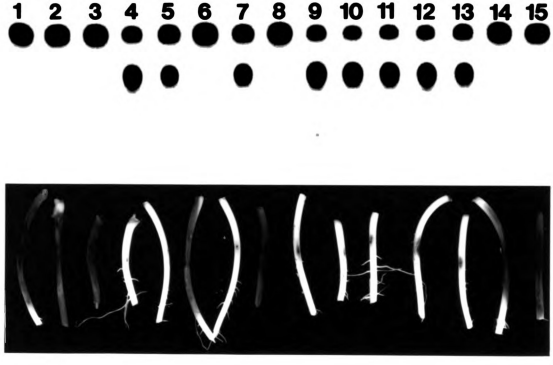

Figure 3.2.

places the locus which is responsible for HCTR activity within 3 cM of *Hm*, at a 95% confidence level (Allard, 1956).

These results were extended by examining specific mutants of *Hm*. Several independent susceptible mutants, due to insertions at *Hm* by members of the Mutator (Mu) and Spm/En families of transposable elements (Gierl *et al.*, 1989; Walbot, 1991), have been identified by cosegregation with flanking markers that detect restriction-fragment-length polymorphisms (G.S. Johal and S.P. Briggs, unpublished, Maize Genetics Newsletter **64**, 36-37). A complete description of the isolation and structure of these alleles will be published elsewhere. These mutants were tested for both HCTR activity and reaction to the pathogen, as shown in Figure 3.3. Two mutants due to insertion of Mu elements (*hm*-1369 and *hm*-656), one Spm/dSpm mutant (*hm*-1040), and one mutant due to insertion of an uncharacterized element (*hm*-1062) had little or no HCTR activity and were susceptible. The low levels of HCTR activity seen in *hm*-1040 and *hm*-656 may be the result of positional effects and/or somatic reversions (Gierl *et al.*, 1989; Walbot, 1991), but these levels of HCTR activity appear insufficient to confer resistance to the fungus (Figure 3.3, bottom). Clearly, transposon-induced mutations of *Hm* have striking effects on the levels of HCTR activity detectable in etiolated seedlings.

Germinal excision of a transposable element can restore the wild-type phenotype (Gierl *et al.*, 1989). A screen to detect reversion events in a population containing the *hm*-1040 mutant allele was initiated by inoculating the progeny of selfed *hm*-1040 (genotype *Hm*::Spm/*hm*) with the fungus. In a population of 300, two seedlings were identified that had reverted from susceptibility to resistance. Figure 3.4 shows that

Figure 3.3. HCTR activity and disease phenotype in extracts of transposon-induced mutants of *Hm*.
(Top) HCTR assays of two plumules are shown for each independent transposon-induced mutant of *Hm* (*hm*-1369, *hm*-656, *hm*-1062, and *hm*-1040), and resistant (K61 x Pr1; *Hm*/*hm*) and susceptible (Pr x K61; *hm*/*hm*) controls.
(Bottom) The disease phenotype of each mutant as compared to resistant and susceptible controls.

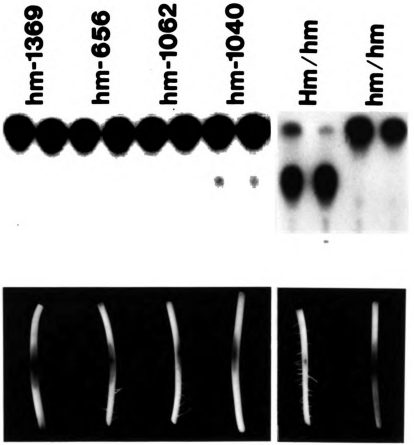

Figure 3.3.

Figure 3.4. HCTR activity and disease phenotype of revertants due to germinal excision of Spm (or dSpm) from *hm*-1040.
(Top) HCTR activity is detectable in extracts from two resistant plants (lanes 3 and 6) identified as revertants from a susceptible *hm*-1040 background. The level of HCTR activity in these progeny is comparable to that of a resistant (*Hm*/*hm*) control (lane 1). Three sibling progeny (lanes 2,4,5) are included as representatives of the mutant HCTR phenotype.
(Bottom) The disease phenotypes of the resistant (*Hm*/*hm*) control (lane 1), and two resistant revertants (lanes 3 and 6) as compared to susceptible mutant siblings (lanes 2,4,5).

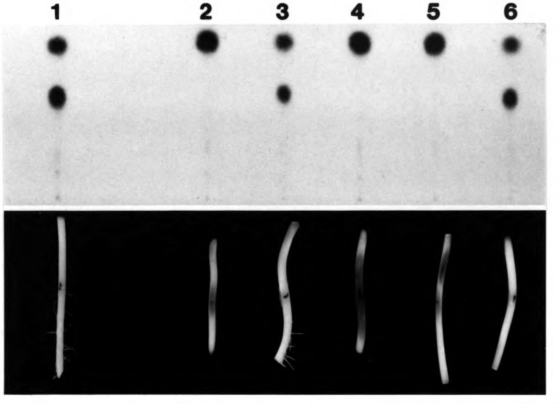

Figure 3.4.

extracts of these revertants contained HCTR activity comparable to that of a resistant control, but HCTR was not detected in susceptible siblings.

There remains the paradox of why HC-toxin delivered through the transpiration stream to green leaves of susceptible plants is metabolized, whereas HC-toxin incubated with extracts of etiolated susceptible shoots is not. Several attempts to extract HCTR from green leaves of either susceptible or resistant plants were unsuccessful. To deliver [^{3}H]-HC-toxin to green leaf tissues by means other than the transpiration stream, discs were cut from green leaves and floated on a solution of [^{3}H]-HC-toxin for 24 hr. Since the recovery of [^{3}H]-HC-toxin from the leaf discs was too low (approximately 10%) to assay directly, we assayed HC-toxin conversion in the solution remaining in the wells. Figure 3.5 shows that susceptible (Pr x K61) leaf discs did not produce significant levels of 8-hydroxy-HC-toxin above background, whereas resistant (K61 x Pr1) leaf discs produced 8-hydroxy-HC-toxin at a minimum of 2 to 3 times the background signal (Figure 3.5).

Figure 3.6 shows that intact plumules from etiolated plants metabolized HC-toxin in a host-selective manner. After 3 hr, resistant plumules had metabolized over 50% of total [^{3}H]-HC-toxin (lane 1) while susceptible plumules failed to produce a detectable amount of the toxin metabolite (lane 2). After 6 hr, etiolated resistant shoots had metabolized nearly all of the supplied HC-toxin (lanes 3 and 4). In one case, etiolated susceptible shoots afer 6 hr showed some metabolism of HC-toxin to a compound with the same R_f as 8-hydroxy-HC-toxin (Figure 3.6, lane 5).

Figure 3.5. Host-selective metabolism of HC-toxin by green leaf discs. Green leaf discs were floated on a solution containing [^{3}H]-HC-toxin for 24 hr, after which the discs were analyzed for radioactivity (as a measure of uptake of 3H-HC-toxin), and the remaining solutions were analyzed for metabolism. Lanes 1-3, genotype *Hm/hm* (resistant); lanes 4-6, genotype *hm/hm* (susceptible). The numbers above the traces indicate integration of the radioactivity in the peak of 8-hydroxy-HC-toxin, centered at R_f 0.25, normalized to the same area in lane 6. Each lane represents one leaf disc. Uptake for the three discs (given as the mean + 1 SD, n = 3, with percent uptake in parentheses) from susceptible plants was 12,200 + 1660 dpm (8.5%) and from the resistant plants was 18,700 + 1570 dpm (13.1%).

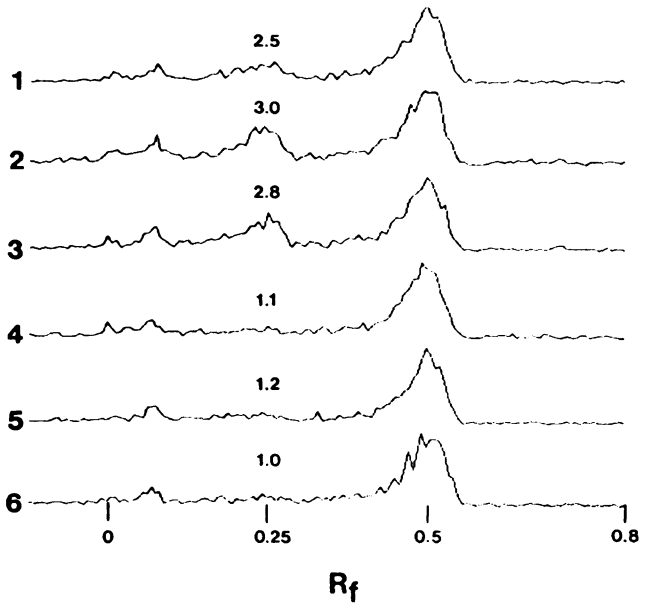

Figure 3.5.

Figure 3.6. Host-selective metabolism of HC-toxin by intact plumules. Etiolated seedlings with unbroken coleoptiles were excised above the coleoptilar node and injected with [^{3}H]-HC-toxin. The plumules were then incubated for 3 or 6 hr, after which they were ground, extracted with chloroform, and analyzed by TLC. Lane 1, genotype *Hm/hm*, 3 hr; lane 2, genotype *hm/hm*, 3 hr; lanes 3 and 4, genotype *Hm/hm*, 6 hr; lanes 5 and 6, genotype *hm/hm*, 6 hr. Each lane represents one plumule.

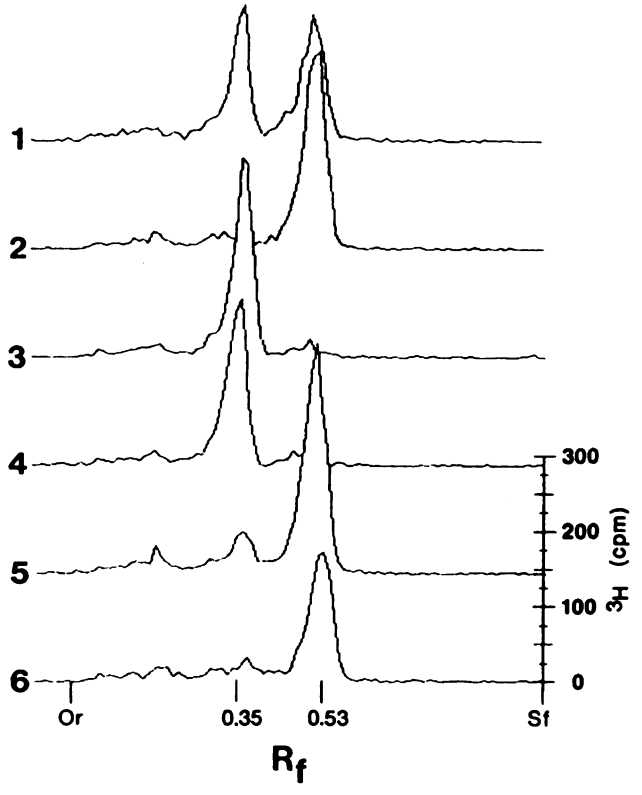

Figure 3.6.

DISCUSSION

The cyclic tetrapeptide HC-toxin has the central role in the interaction between *C. carbonum* race 1 and maize. The results in this paper argue that the *Hm* gene confers resistance to *C. carbonum* race 1 by controlling the presence of HCTR, an enzyme that detoxifies HC-toxin (Meeley and Walton, 1991).

The results with green leaf discs (Figure 3.5) and intact etiolated shoots (Figure 3.6) demonstrate that differential metabolism of HC-toxin due to *Hm* occurs not just in extracts but also in intact green and etiolated tissues. The differential metabolism seen in vitro (Figures 3.1-3.4) therefore is not due to differential extractability or differential in vitro stability of HCTR. The in vitro studies show that HCTR activity in resistant plants is constitutive, and that the lack of comparable toxin metabolism in susceptible intact tissues (Figures 3.5 and 3.6) is not a secondary effect of HC-toxin treatment.

Our results raise three interesting questions. First, is *Hm* the structural gene for HCTR or does *Hm* regulate the expression of HCTR? Work is in progress to answer this question through the cloning of *Hm* and the purification of HCTR.

Second, what is the nature of the apparent HCTR activity seen when HC-toxin is delivered through the transpiration stream to green leaves of susceptible plants (Meeley and Walton, 1991) and at low levels in intact etiolated shoots (Figure 3.6, lane 5)? Clearly, under at least some conditions, maize of genotype *hm/hm* has some HCTR-like activity. One possibility is an unrelated enzyme with capacity to metabolize HC-toxin, for example, one of the various NADPH-dependent reductases involved in cellular metabolism. The transpiration/green leaf results could also be due to an HCTR-like

activity restricted to the vascular tissue. Dinucleotide-dependent reductases have been found in xylem exudates (Biles *et al.*, 1989). A xylem-localized reductase could metabolize HC-toxin in the transpiration stream but would be irrelevant during infection by *C. carbonum* race 1, which does not, at least initially, infect the vascular tissue.

Third, does HCTR have a function in the plant in the absence of *C. carbonum* race 1? *hm* has no known phenotype other than reaction to *C. carbonum* race 1. We have detected HCTR activity in etiolated shoots of barley, wheat, oats, and sorghum, all potential hosts of *C. carbonum*, but not in Arabidopsis, peas, or cucumber (R.B. Meeley and J.D. Walton, unpublished results). Conceivably, HC-toxin or the five related cyclic peptides made by other fungi (Walton *et al.*, 1985; Walton, 1990, Itazaki *et al.*, 1990) have exerted a significant selective pressure on the evolution of the Poaceae. Relevant to this question is whether the recessive alleles of *hm* are nulls or whether they result in the production of HCTR at lower levels or with altered enzymatic properties. For example, if HCTR is the product of the *Hm* gene, *hm* could encode an enzyme with normal affinity for its putative endogenous substrate but reduced or no affinity for HC-toxin.

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CHAPTER 4

STUDIES ON THE PURIFICATION AND INDUCTION OF HC-TOXIN REDUCTASE, AND ITS DISTRIBUTION AMONG PLANT SPECIES

ABSTRACT

The maize enzyme HC-toxin reductase represents a known biochemical function controlled by a plant disease resistance gene. The resistance gene *Hm* of maize confers full resistance to the fungal pathogen *Cochliobolus carbonum* Nelson race 1. Biochemical/genetic evidence indicates that the *Hm* gene of maize either encodes or controls the expression of HC-toxin reductase, an enzyme responsible for inactivation of HC-toxin, the cyclic peptide required for pathogenicity of *C. carbonum* race 1. This report demonstrates further purification of HC-toxin reductase beyond initial enrichment procedures. Three candidate polypeptides from 40-50 kD are visualized by SDS-PAGE when active HPLC fractions from a number of chromatographic steps are compared. HC-toxin reductase activity is inducible. Significant increases in enzyme activity occur in response to inoculation of resistant maize seedlings with race 1 of *C. carbonum*. Enzyme activities analogous to HC-toxin reductase from maize are found in other plant species. These activities are detected in extracts from other monocotyledonous crops, but not in dicot extracts. Other evidence suggests that HC-toxin metabolism is associated with the *C. carbonum* resistance allele *Hm2*. HC-toxin reductase activity is induced following pathogen inoculation, appears specific for monocot species, and possibly shares the same detoxification mechanism with an independent *C. carbonum* resistance gene in maize. Collectively, these observations argue that the *Hm* gene may have a dedicated function in maize for resistance to HC-toxin or similar natural products.

INTRODUCTION

The cyclic tetrapeptide HC-toxin has the central role in the interaction between maize and the phytopathogenic fungus *Cochliobolus carbonum* Nelson race 1. Production of HC-toxin is the principal requirement for pathogenicity of *C. carbonum* race 1 against maize (Panaccione *et al.*, 1992). In the plant, the resistance gene *Hm* confers race-specific resistance to the fungus as well as decreased sensitivity to HC-toxin (Kuo and Scheffer, 1970). A biochemical phenotype for the *Hm* gene has been established by comparing HC-toxin metabolism in resistant (*Hm/-*) and susceptible (*hm/hm*) maize. The *Hm* allele is thought to either encode or control the expression of an enzyme called HC-toxin reductase (HCTR), which inactivates the toxin by carbonyl reduction. Susceptible maize (*hm/hm*) are more sensitive to HC-toxin presumably because they are deficient in this capacity (Meeley *et al.*, 1992).

That *Hm* is the structural gene for HCTR is the simplest interpretation of the genetic data. To investigate this hypothesis, purification of the enzyme is sought in order to obtain amino acid sequence data. Such data could be compared with deduced amino acid sequences generated by others working to clone the *Hm* gene. HPLC procedures presented in this paper detail HCTR purification beyond the initial enrichment procedures reported previously (Meeley and Walton, 1991).

An important issue to be addressed is whether the gene for HCTR is maintained in the maize genome specifically for defense against *C. carbonum* race 1. In this paper, HCTR activity is examined in response to fungal inoculation, and its distribution among other plants is tested.

Resistance to *C. carbonum* is functionally duplicated in the maize genome (Nelson and Ullstrup, 1964). The *Hm* gene for *C. carbonum* race 1 resistance is epistatic to *Hm2*, a gene that confers developmentally limited, and incompletely dominant resistance to *C. carbonum* race 1 (Nelson and Ullstrup, 1964). Experiments examining HC-toxin metabolism associated with the *Hm2* allele are presented. The discussion of these results pertains to the origin and specificity of HC-toxin reductase and to questions about the process of co-evolution between a pathogen and its host.

MATERIALS AND METHODS

Plant Materials and Extraction Procedures

The growth, harvest, and initial extraction procedures for HCTR purification were as described (Meeley and Walton, 1991). Etiolated seedling tissue (100 g) was homogenized in extraction buffer, and HCTR was enriched by an ammonium sulfate fractionation (30-55%). Enzyme preparations were made from the resistant (*Hm/-*) maize hybrid GL-582 (Great Lakes Hybrids Inc., Ovid, MI). For fungal induction experiments, seedlings of the resistant homozygote Pr1 (*Hm/Hm*) were used. For the distribution survey, extracts were prepared from etiolated seedlings of *Avena sativa* (oat), *Hordeum vulgare* (barley), *Glycine max* (soybean), *Sorghum bicolor*, *Triticum aestivum* (wheat), *Pisum sativum* (pea), *Cucumis sativus* (cucumber), and *Arabidopsis thaliana*. Tissues (150 mg) were extracted and assayed for HCTR activity as described (Meeley and Walton, 1991).

Chromatography Columns and Buffers

HPLC separations were performed on a Waters system equipped with two Model 501 pumps, a Model 440 absorbance detector (280 nm), and an automated fraction collector (Pharmacia Frac-100). Columns used for anion exchange chromatography included a Beckman TSK-DEAE-5-PW (10 μm, 7.5 x 75 mm), or a Rainin Dynamax-Hydropore-5-AX (5 μm, 10 x 100 mm), in buffer A, eluted with buffer B. Hydrophobic interaction chromatography was performed with a BioRad Bio-Gel TSK-phenyl-5-PW column (10 μm, 7.5 x 75 mm), in buffer C, eluted with buffer D. Gel filtration chromatography was performed on a tandem coupling of two Beckman Ultraspherogel SEC 3000 (5 μm, 7.5 x 300 mm), in buffer E. Chromatofocusing was performed on a Pharmacia FPLC system with Pharmacia Mono-P HR-5/20 column (10 μm, 5 x 200 mm), in buffer F, eluted with buffer G.

Buffers: (A) 25 mM bis-Tris-HCl (pH 7.0 or pH 6.25), 10% glycerol, 2.5 mM DTT. (B) same as A plus 1M NaCl. (C) 1M $(NH_4)_2SO_4$, 25 mM bis-Tris-HCl (pH 7.0), 10% glycerol, 2.5 mM DTT. (D) 10% glycerol, 2.5 mM DTT. (E) 100 mM bis-Tris-HCl (pH 7.0), 250 mM Na_2SO_4, 10% glycerol, 2.5 mM DTT. (F) 25 mM bis-Tris (pH 7.1), 10% glycerol, 2.5 mM DTT. (G) Polybuffer 74 (pH 4.0, Pharmacia), 10% glycerol, 2.5 mM DTT. Between steps, buffer exchange was performed on samples with a Sephadex G-25 column (Pharmacia PD-10). To all HPLC fractions, ascorbate (to 10 mM) was added for protection against dissolved oxygen. HCTR assays were 30 min long.

Gel Electrophoresis

Aliquots of HCTR-containing HPLC fractions were analyzed by SDS-PAGE on 10% acrylamide gels. Silver staining of gels was performed using a Bio-Rad silver staining kit according to the manufacturer's directions.

Fungal Induction Experiments

Etiolated resistant (Pr1, *Hm/Hm*) seedlings were inoculated with a heavy spore suspension (approximately 50 conidia/μL) of *C. carbonum* race 1 in 0.1% Tween-20. Cotton swabbing was used to "paint" seedlings with the spore suspension. Control seedlings were treated with 0.1% Tween-20. Seedlings were placed back in the dark for 24 h prior to harvest. Crude extracts of seedling tissues (5 g) were prepared and assayed for HCTR activity as described (Meeley and Walton, 1991). Product formation was quantitated by TLC as described (Meeley and Walton, 1991). As starting material for HCTR purification, large batches of etiolated seedlings were induced with *C. carbonum* spores to maximize HCTR levels prior to extraction.

HC-toxin Metabolism and Hm2

Etiolated seedling materials containing the *Hm2* resistance allele (*Hm2/Hm2*) were assayed for disease phenotype and HC-toxin metabolism as described (Meeley *et al.*, 1992). Susceptible (*hm2/hm2*, and *hm/hm*) and resistant (*Hm/-*) seedlings were analyzed as controls. Assay times were 30 min.

RESULTS

HPLC Fractionation of HCTR Activity

Figure 4.1 illustrates enzyme assay data typical of all purification procedures. This example shows fractionation of HCTR activity by anion exchange (pH 7.0), followed by hydrophobic interaction chromatography (pH 7.0). The bulk of HCTR activity eluted in fractions 16 and 17 during anion exchange. No artifactual product (reported previously in Meeley and Walton, 1991) was formed during anion exchange when precautions were taken to minimize oxygen in solutions. Fractions 16 and 17 were combined and exchanged into high-salt buffer for hydrophobic interaction HPLC. HCTR activity was eluted in fractions 14-16 from the hydrophobic interaction column. Without an effective means to stabilize HCTR *in vitro*, the key to success in these procedures is to run the columns as quickly as possible, but even when fraction HI-15 from hydrophobic interaction was immediately taken on to another column, HCTR activity was not recovered (data not shown).

Figure 4.2 shows the enrichment obtained from a series of column procedures. Etiolated tissue (70 g, *C. carbonum*-induced) was extracted, taken through ammonium sulfate fractionation, and a primary round of anion exchange (pH 7.0). Following additional separations, HCTR-containing fractions were analyzed by SDS-PAGE. The active fractions from hydrophobic interaction (lane 1), gel filtration (lanes 2 and 3), and anion exchange (pH 6.25, lanes 4 and 5) are shown. The latter two steps produced an enrichment for three polypeptides of 49, 43.5, and 40 kD. A previous estimate by gel filtration determined HCTR to be M_r 42,000 (Meeley and Walton, 1991).

Figure 4.1. Fractionation of HCTR activity. Each lane represents the assay of individual HPLC fractions from anion exchange, pH 7.0 (IEX fractions 15-18) and hydrophobic interaction, pH 7.0 (HI fractions 11-20). Tritiated HC-toxin was used as substrate; HCTR activity is denoted by conversion of labeled HC-toxin (5.2 cm) to its 8-hydroxy derivative (3.5 cm).

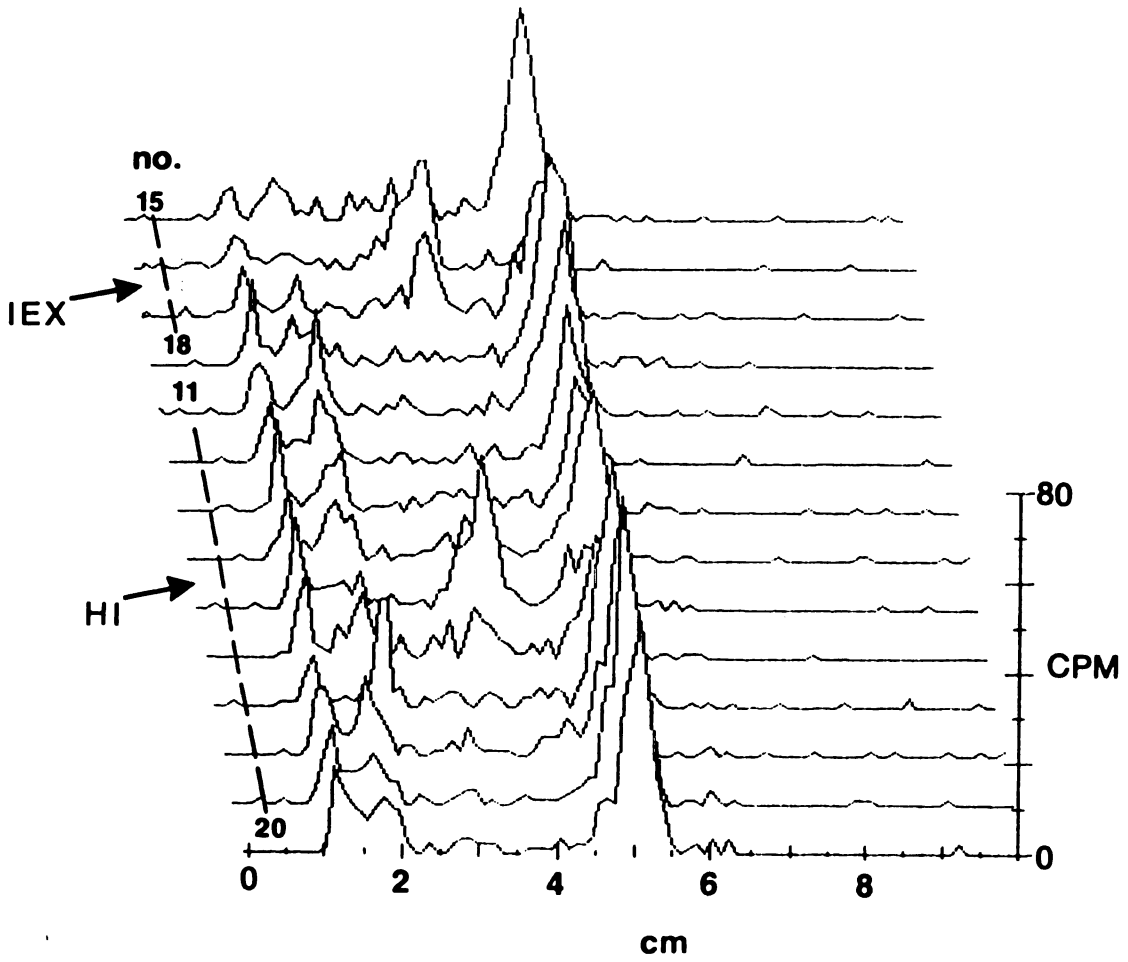

Figure 4.1.

Figure 4.2. SDS-PAGE analysis of HCTR-containing fractions. In this example, the fractionation involved preparative anion exchange (Dynamax, pH 7.0), hydrophobic interaction (pH 7.0) (lane 1), gel filtration (pH 7.0) (lanes 2 and 3), and another round of anion exchange at pH 6.25 (lanes 4 and 5). Molecular weight standards are indicated by the dots at the left, and correspond to 97, 66, 45, 31, and 21.5 kD. These procedures enrich for three polypeptides with molecular weights of 49, 43.5, and 40 kD, as indicated by the dots.

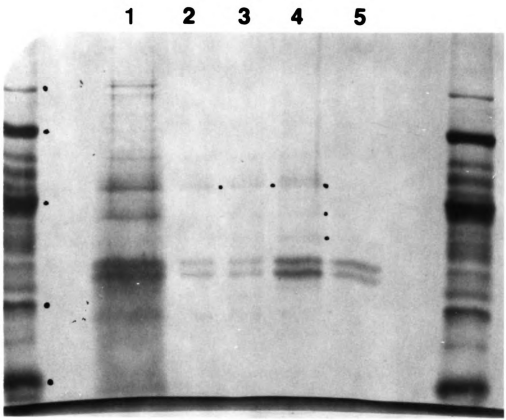

Figure 4.2.

To date, the development of an HCTR purification protocol has involved trial separations on a number of HPLC columns. Matrices found to retain or resolve HCTR include anion exchange, hydrophobic interaction, gel filtration, and chromatofocusing. To identify a polypeptide by SDS-PAGE that corresponds to HCTR, the order of these columns was varied. HCTR-active fractions were then compared by SDS-PAGE. An example of this analysis is shown in Figure 4.3. By examining HCTR-containing fractions from a number of purification attempts, certain polypeptides can be eliminated from consideration. For example, two smaller bands that comprise a major portion of the protein in lanes 5 and 6 are not present in lanes 1-4. HCTR thus does not appear to be a dimer between these two polypeptides. Likewise, many of the larger polypeptides (seen in lanes 2 and 3) are not present in other HCTR preparations. A definitive designation for the HCTR polypeptide can not be made from this analysis, but one of the strongest candidates (indicated by arrows) corresponds to the 49 kD protein from Fig. 4.2.

Fungal Induction of HCTR

As shown in Figure 4.4, a twenty-four hour treatment of etiolated resistant seedlings with a spore suspension of *C. carbonum* race 1 results in an approximate 2.5-fold induction of HCTR specific activity. Comstock and Scheffer (1973) have shown that growth of *C. carbonum* race 1 is arrested during the first 24-48 h post-inoculation on resistant (*Hm/-*) maize tissues. Induction of HCTR activity within this time period is consistent with the enzyme's role in disease resistance.

Figure 4.3. Comparative SDS-PAGE analysis of HCTR-containing fractions. The column methods from a number of purification attempts were as follows:

Lane: (1) Dynamax anion exchange (pH 7.0), gel-filtration (pH 7.0)
(2) Dynamax anion exchange (pH 7.0), gel-filtration (pH 7.0)
(3) Dynamax anion exchange (pH 7.0), hydrophobic interaction (pH 7.0)
(4) DEAE anion exchange (pH 7.5), Mono-P chromatofocusing
(5) Dynamax anion exchange (pH 7.0), hydrophobic interaction (pH 7.0), gel filtration (pH 7.0)
(6) Dynamax anion exchange (pH 6.25) of HCTR from lane 5.

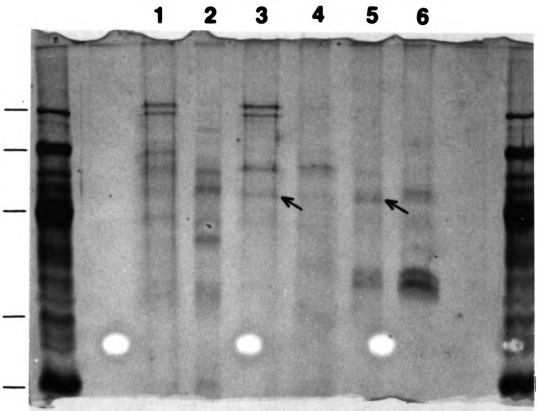

Figure 4.3.

Figure 4.4. Induction of HCTR activity in response to fungal inoculation. Pathogenic fungal spores (*C. carbonum* race 1) were painted onto etiolated resistant (inbred Pr1) seedlings. Seedlings were harvested and extracted for HCTR activity 24 h post-inoculation. Tween-20-treated seedlings served as the non-inoculated control. The specific activity of HCTR was determined in crude extracts prepared from 5 g of inoculated and control tissues.

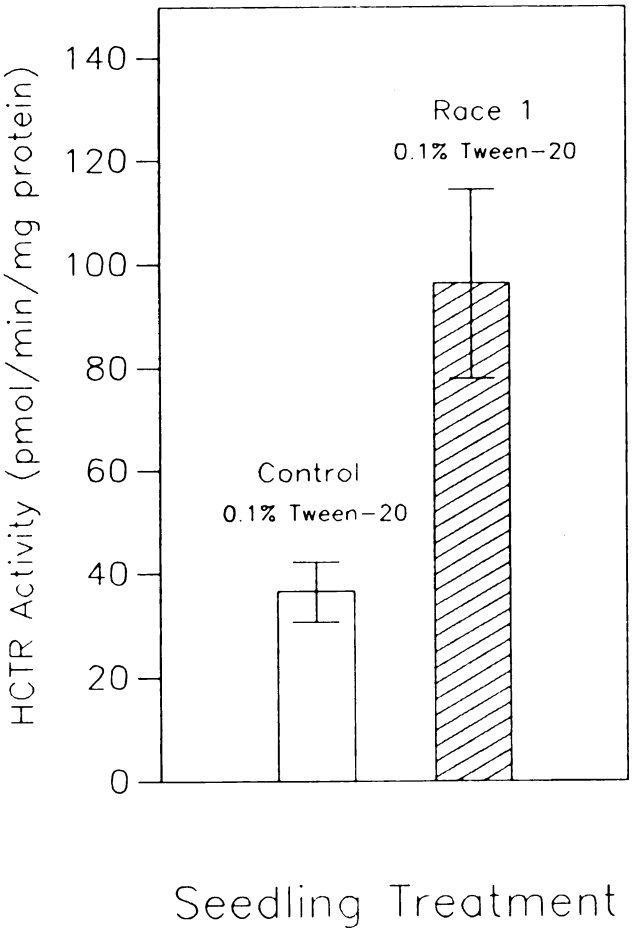
HCTR Activity (pmol/min/mg protein)
140
120
100
80
60
40
20
0
Race 1
0.1% Tween-20
Control
0.1% Tween-20
Seedling Treatment

Figure 4.4.

Distributio

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to *C.*

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metab

hm2/

8-hyd

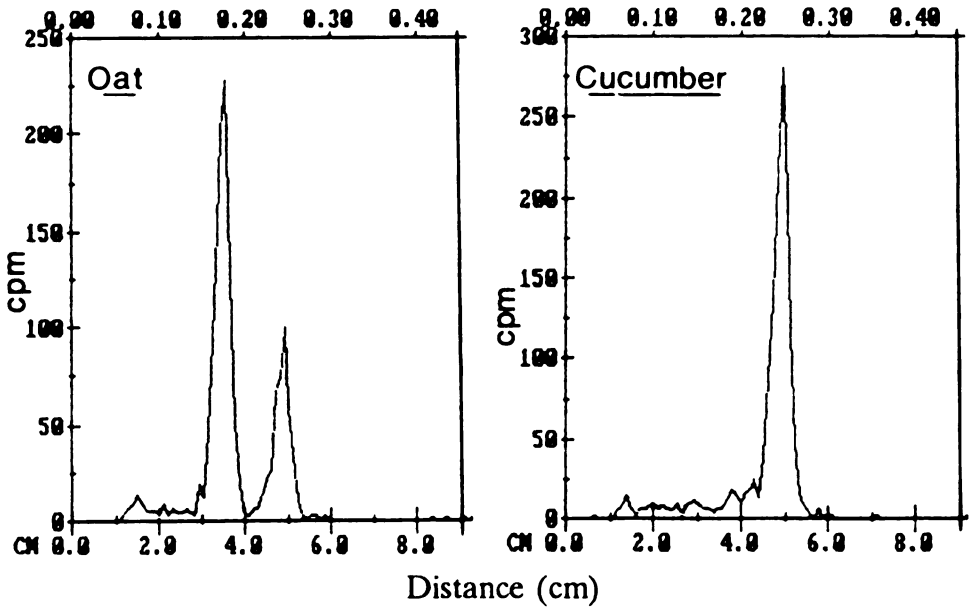
Distribution of HC-toxin Metabolism

Figure 4.5 summarizes the results of a test for HC-toxin metabolism among a number of different plant species. In these experiments, equal weights of etiolated seedlings were extracted and assayed (30 min) for HCTR activity. Extracts from oat, wheat, and barley converted more than 50% of the total [^{3}H]-HC-toxin (0.25 μCi) to its 8-hydroxy derivative. Sorghum extract was also quite active, though less so than the other monocot species. An interesting result was that HC-toxin metabolism was not observed in any of the dicot extracts.

HC-toxin metabolism and Hm2

The locus *hm2* (located on chromosome 9-L) is an alternate source of resistance and susceptibility to *C. carbonum* race 1 (Nelson and Ullstrup, 1964). *Hm* is epistatic to *Hm2*, thus the effects of *Hm2* can be seen only in an *hm/hm* background. Resistance to *C. carbonum* race 1 conferred by *Hm2* is developmentally limited, and is expressed very weakly in the seedling stage (Nelson and Ullstrup, 1964). Evidence of this can be observed in Figure 4.6, which shows an essentially susceptible disease phenotype for both *Hm2/Hm2* and *hm2/hm2* seedlings. However, a small amount of an HC-toxin metabolite is formed in extracts of *Hm2/Hm2* seedlings in comparison with either *hm2/hm2* or *hm/hm* susceptible controls. This metabolite has a polarity identical to the 8-hydroxy derivative of HC-toxin which is formed by *Hm/-* resistant maize.

Figure 4.5. Distribution of HCTR-like activity among several different plant species. Etiolated seedling tissue (150 mg) from each species was extracted and assayed for HCTR activity. The entire extract, including insoluble material, was included in the assay. Results are expressed simply as the percentage of HC-toxin converted. The top panels compare TLC analysis of extracts from oat and cucumber.



Plant	% HC-toxin converted
cucumber	n.d.*
soybean	n.d.
barley	75.4
sorghum	17.2
wheat	81.6
oat	67.2
pea	n.d
Arabidopsis	n.d.

*n.d. - none detected

Figure 4.5.

Figure 4.6. HC-toxin metabolism and *Hm2*. These assays were performed as described (Meeley *et al.*, 1992). Resistance to *C. carbonum* race 1 by *Hm2* is expressed poorly at the seedling stage (Nelson and Ullstrup, 1964). The disease phenotype (bottom) is indicative of this, but a low level of HCTR activity is evident in comparison with *hm2/hm2* plants (top).

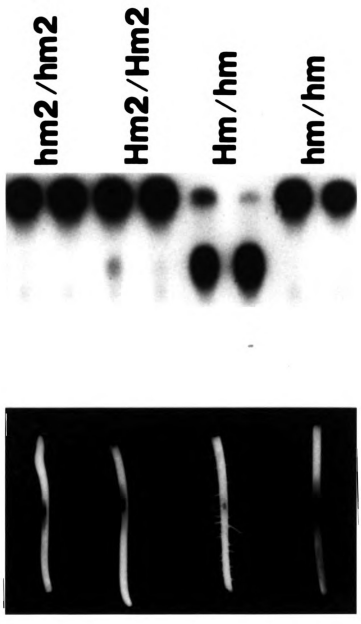


Figure 4.6.

DISCUSSION

A purified form of HC-toxin reductase is important for study of its substrate specificity, and for other useful purposes such as antibody production. The data presented in Figures 4.2 and 4.3 represent the best results obtained to date for purification of HCTR. The purification procedures presented here enrich for three polypeptides of 49, 43.5, and 40 kD. From earlier gel filtration data (Meeley and Walton, 1991), HCTR has an estimated molecular weight of 42 kD. Despite this success, a definite polypeptide assignment can not be made at this time. The chromatography steps presented here must be further developed.

HC-toxin reductase activity is quite unstable following extraction. Several methods meant to stabilize *in vitro* HCTR activity (some described previously in Meeley and Walton, 1991) have met with very little success. For example, storage at 4°C or -20°C appear to have no advantage over storage at -80°C. Storage with NADPH has been recommended for other NADPH-dependent oxidoreductases (De-Eknamkul and Zenk, 1992), but this does not appear to improve HCTR's stability. To date, the most effective precaution is to prevent oxygen contamination of buffers and samples. Ascorbate is added routinely to extraction buffer and to HPLC fractions immediately after collection. HPLC buffers are degassed thoroughly prior to use. Still, the enzyme has a half life of only 24-48 h under routine conditions.

HCTR activity is induced by fungal inoculation in a manner temporally consistent with the resistance response of maize against *C. carbonum* race 1. Yet it remains important to know how other fungi, pathogens and non-pathogens of maize alike, affect

HCTR activity. In particular, race 2 isolates of the fungus and perhaps HC-toxin itself should be examined for any effect on HCTR activity.

In a survey of several monocot and dicot species, the evidence suggests that HCTR, or at least an analogous activity, is present in monocots, but not in dicots. Importantly, results from molecular genetic analyses of *Hm* are consistent with both HCTR induction and its specificity to monocots. Johal and Briggs (pers. commun.), have described transcriptional activation of the *Hm* gene within 24 h (post-inoculation) of resistant leaf tissue with *C. carbonum* race 1. They also report that DNA sequences in the monocots sorghum and *Coix* cross-hybridize with a probe specific for the maize *Hm* gene. No hybridization with *Arabidopsis* DNA was observed (Johal and Briggs, 1992).

Although HCTR activity is the critical biochemical factor involved in resistance to *C. carbonum*, a separate issue is whether the gene for HCTR is intended solely for defense against this (or other) pathogen(s). HCTR operates so slowly with HC-toxin as a substrate that an effective spectrophotometric assay based on NADPH oxidation has never been developed. In addition, barriers to enzyme purification have precluded a meaningful analysis of HCTR's substrate specificity. With these difficulties in mind, it is possible that HC-toxin represents a fortuitous substrate for an enzyme with some other function in the cell. The enzyme activity, "HC-toxin reductase" might be an anomaly, but HCTR activity is induced by fungal inoculation, and appears exclusive to monocots; attributes that place limits on the type of oxidoreductase that could fortuitously metabolize HC-toxin. HC-toxin is certainly not a promiscuous substrate; a number of commercially available oxidoreductase enzymes were unable to reduce HC-toxin (data

not shown), and there is no evidence of HCTR-like activity in a number of non-plant systems. For example, enzyme extracts from yeast, and even from *C. carbonum* race 1 fail to catabolize HC-toxin (Meeley, unpublished observations). In mammalian blood, HC-toxin appears to be inactivated, but by a mechanism very different from the reduction described in maize (Meeley, unpublished data). These details deserve attention because they indicate that there is something rather exclusive about HC-toxin metabolism in maize and other monocots.

Consider the mechanism of toxin inactivation. Reduction of the 8-keto moiety of Aeo is perchance the most sophisticated route to toxin inactivation. If HC-toxin were a fortuitous substrate, and no selection pressure to evolve resistance to it ever existed, it is reasonable to expect that toxin metabolism would involve a more non-specific mechanism, such as inactivation of the epoxide group of Aeo. Enzymes like glutathione-S-transferases, that are able to conjugate epoxides, occur in maize (Shimabukuro *et al.*, 1971). There are also a number of other non-specific enzymatic mechanisms for xenobiotic detoxification in plants (Lamoreaux and Rusness, 1986).

Evidence in favor of HCTR as a dedicated function includes the fact that the *hm/hm* genotype has no known phenotype other than susceptibility to *C. carbonum* race 1. The attribute of fungal induction reinforces a defense role for HCTR. The attribute of monocot specificity raises the prospect that HCTR's distribution among plants is biased because of selective pressures among monocots to evolve resistance to this type of compound.

Given that other fungi have been shown to produce Aeo-containing cyclic peptides (see Walton, 1990, or Figure 1.5), ancestral selection pressure to evolve resistance to these compounds may have been provided by a pathogen other than *C. carbonum*, and/or in a monocotyledonous host other than maize. Thus, if *C. carbonum* race 1 acquired the genes necessary for HC-toxin biosynthesis relatively recently, as has been proposed (Panaccione *et al.*, 1992), susceptible hosts may represent recent inbreeds that carry homozygous mutation(s) in the gene for HCTR (*Hm*). The natural history of Helminthosporium leaf spot is at least consistent with this interpretation, since only a few genetic sources of the *hm/hm* genotype exist. DNA sequence analysis of the *hm* allele(s) is in progress to search for evidence of mutation (S.P. Briggs, pers. comm.). Evidence already suggests that the *hm* alleles are transcriptionally defective (Johal and Briggs, 1992). Therefore, this allele does not appear to produce an enzyme with a function unrelated to HC-toxin metabolism (Johal and Briggs, 1992).

How is metabolism by *Hm2* relevant to these discussions? Maize contains two independent sources of heritable resistance to *C. carbonum* race 1. The *Hm* locus is the focus of this dissertation because it is the major source of resistance, being epistatic to *Hm2* (of chromosome 9L). *Hm2*-mediated resistance to *C. carbonum* race 1 exhibits incomplete dominance and elements of developmental regulation. This paper presents some preliminary evidence that *Hm2*-mediated resistance to *C. carbonum* race 1 involves a mechanism of HC-toxin inactivation similar to that controlled by *Hm*. Since *Hm2* expression increases with plant age (Nelson and Ullstrup, 1964), a reliable method to assay HCTR activity in mature tissues is required. Attempts to extract and detect HCTR activity in the post-anthesis tissue of corn silks have been unsuccessful (data not shown).

HC-toxin metabolism influenced by *Hm2* may be relevant to some of our earlier findings. In previous data showing low levels of HCTR activity in transposon-induced mutants of *Hm* (Meeley *et al.*, 1992, or Figure 3.3), our interpretation was that these low levels were due to positional effects of the inserted elements, or somatic excision of the element. However, most maize lines that are naturally resistant to *C. carbonum* race 1 due to *Hm/-* also have the genotype *Hm2/-* (Nelson and Ullstrup, 1964). It is possible that *Hm* mutant lines contained low-levels of HCTR activity because they were present in an *Hm2/-* background (G.S. Johal, pers. comm.). In turn, this may support a mechanism of HC-toxin metabolism controlled by *Hm2*. Genetic manipulations to resolve *Hm* mutant alleles from *Hm2* are in progress (G.S. Johal, pers. comm.).

Further discussion of a common mechanism of toxin metabolism between *Hm* and *Hm2* considers the evidence for "large scale interchromosomal homology" throughout the maize genome (Helentjaris *et al.*, 1988). Even though the origins of modern maize remain nebulous and controversial, evidence in favor of ancestral duplication, as well as a number of possible explanations for its occurrence have been forwarded (Helentjaris *et al.*, 1988). This may explain how two sources of resistance to *C. carbonum* race 1 share the same mechanism. These two genes could be of common origin, and perhaps *Hm* is epistatic to *Hm2* because developmental regulations were either imposed on *Hm2*, or relaxed on *Hm*. If this is the case, heritable resistance to HC-toxin could have its origins in the ancestry of maize. A biased distribution of HCTR-like activity among the monocots may support this notion.

As yet, no model system stands out as one amenable to tracing the biochemical and molecular events associated with host/pathogen co-evolution, but a detailed picture

of HCTR's origin, substrate specificity, and distribution among the plant kingdom is tenable. In time, studies on the interaction of *C. carbonum* race 1 and maize may contribute to our understanding of a particular evolutionary history between a host and its pathogen.

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CHAPTER 5

SUMMARY AND RECOMMENDATIONS

SUMMARY

The fungal pathogen *Cochliobolus carbonum* Nelson race 1 exists in a complementary genic interaction with its host, maize. At the biochemical level, the cyclic tetrapeptide HC-toxin has the central role in this interaction. Race 1 isolates of the fungus are the only isolates pathogenic to *hm/hm* maize, and they are the only isolates that produce HC-toxin. Production of HC-toxin is essential for race 1 pathogenicity as demonstrated by Panaccione *et al.* (1992). In maize, resistance to *C. carbonum* race 1 occurs *via* the *Hm* gene. This allele confers decreased sensitivity to HC-toxin (Kuo and Scheffer, 1970).

This dissertation is aimed at determining the function of the *Hm* gene of maize as it has been defined; a gene for resistance to *C. carbonum* race 1, and decreased sensitivity to HC-toxin. Associated with the resistance allele *Hm* is an enzymatic activity called HC-toxin reductase, that detoxifies HC-toxin by metabolism of a critical functional group (Meeley and Walton, 1991). Analyses of HCTR activity among a variety of resistant and susceptible inbreds, segregating resistant and susceptible progeny, and transposon-induced mutants, and revertants, establish that HCTR is the biochemical phenotype for the *Hm* allele (Meeley *et al.*, 1992). This work, together with the work of Drs. Guri Johal and Steve Briggs (Pioneer Hi-Bred Intl.), represent the premier descriptions of the molecular identity and biochemical function of a plant disease resistance gene.

Purification of HCTR was attempted principally for the purpose of confirming whether *Hm* actually encodes HCTR. By SDS-PAGE, three polypeptide candidates were

observed in HCTR-containing fractions from a number of HPLC steps. A definite assignment of a polypeptide as HCTR can not be made without further experimentation.

A variety of experimental and historical information raises the question whether the *Hm* gene is specifically intended to provide resistance to HC-toxin or similar natural products. The *hm/hm* genotype has no phenotype other than susceptibility to *C. carbonum* race 1, sensitivity to HC-toxin, and a characteristic lack of HCTR activity. Mutations in *Hm* are defective in HCTR, but have no observable pleiotropic effects. The *hm/hm* host appears to be transcriptionally defective, implying that no alternative enzyme product without HC-toxin metabolic capability exists for this allele. The product of *Hm* is HCTR. Both *Hm* transcription and HCTR activity are induced by fungal inoculation which supports a disease resistance/defense role for this gene and its product. This mechanism appears specific to monocots and may share a similar mechanism with *Hm2*. Together, these points imply the existence of an evolutionary history involving HC-toxin production by the fungus, and plant defense against it.

To return to the quadratic check that was used to illustrate this Introduction, our final variation includes what we have learned about the function of the *Hm* gene. Figure 5.1 conveys the same concept of gene-for-gene complementarity, with new information pertaining to the function of *Hm*. Our current model proposes that the *Hm* gene encodes the enzyme HC-toxin reductase, a soluble, NAD(P)H-dependent oxidoreductase, that inactivates HC-toxin by specific reduction of the 8-keto moiety of Aeo. This is the biochemical definition of pathogen "recognition" for this particular host-pathogen system. Resistant maize is generally 100-fold less-sensitive to HC-toxin than susceptible (*hm/hm*)

Figure 5.1. The *C. carbonum* race 1/maize interaction in biochemical terms. This quadratic check has been updated with the recent information gained about the function of the *Hm* resistance gene. The incompatible interaction between a race 1 (HC-toxin$^+$) fungus and a resistant (*Hm*/-) plant can be explained by the action of HC-toxin reductase (HCTR). The compatible interaction results because susceptible (*hm/hm*) plants do not produce HCTR to counteract the toxin.

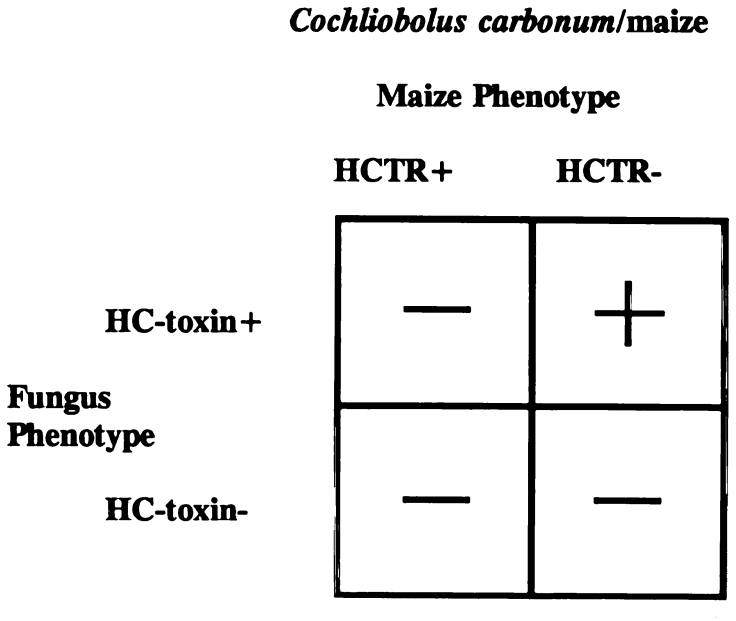
Cochliobolus carbonum/maize
Maize Phenotype
HCTR+
HCTR-
HC-toxin+
Fungus Phenotype
HC-toxin-
—
+
—
—

Figure 5.1.

maize (Kuo and Scheffer, 1970). This dissertation proposes that this differential can be explained, at least in part, by the activity of HC-toxin reductase. Since these data do not concern the mode of action of HC-toxin, differential sensitivity may also reside at the site of toxin action.

The experimental work presented here tells nearly a complete story about the function of the *Hm* gene in disease resistance. One question left unresolved is whether *Hm* actually encodes HCTR. Purification of HCTR was intended to address this question by generating amino acid sequence data that could serve as an independent source of confirmation for our collaborators working to clone the *Hm* gene. HCTR appears reticent to purification to the level necessary for amino acid analysis. Despite this shortfall, many of the procedures were successful, and substantial progress was made. Further development of HCTR purification is feasible and highly recommended.

Presently, the best evidence that *Hm* encodes HCTR is provided by our collaborators at Pioneer (Johal and Briggs, 1992). Figure 5.2 shows a comparison of the deduced amino acid sequence data for the *Hm* gene with that determined for the *A1* gene of maize. These two genes are over 34% identical and 56% similar by this analysis. Importantly, the *A1* gene encodes the enzyme dihydro-4-flavonol reductase (DFR), an NADPH-dependent oxidoreductase involved in anthocyanin biosynthesis (Reddy *et al.*, 1987). The similarity between the enzymes, and their corresponding genes, indicates that *Hm*, like *A1*, encodes an oxidoreductase enzyme. The enzyme encoded by *Hm* is the resistance gene product, HC-toxin reductase.

Figure 5.2. Comparison of the deduced amino acid sequence of the *Hm* gene with the *A1* gene of maize. The maize *A1* gene encodes the enzyme dihydro-4-flavonol reductase (or dihydroquercetin reductase). When compared with the deduced amino acid sequence for *Hm*, the level of amino acid identity (indicated by |), is 34%, and the level of similarity is 56% (indicated by .:|). The highest degree of identity occurs in the amino terminal end of the primary sequence, which contains the consensus sequences for the dinucleotide fold (Johal and Briggs, 1992). The dinucleotide fold is required for coordination of the nicotinamide cosubstrate.

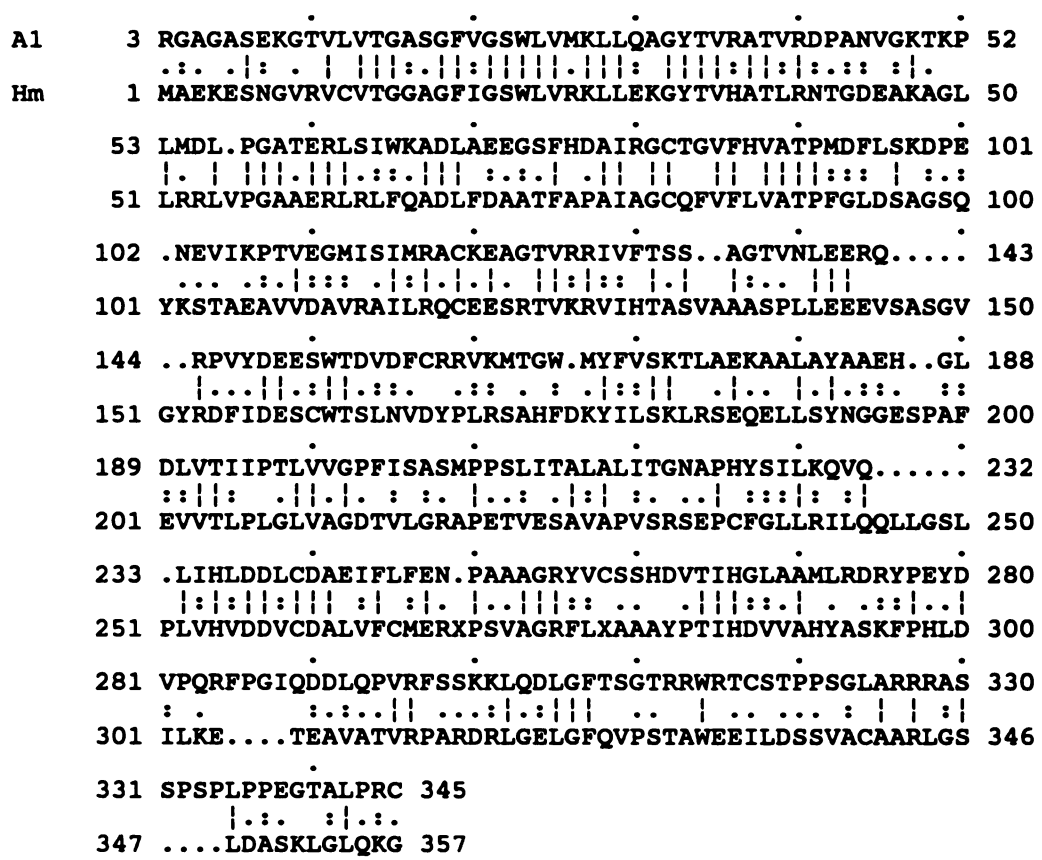
A1 3 RGAGASEKGTVLVTGASGFVGSWLVMKLLQAGYTVRATVRDPANVGKTKP 52
Hm 1 MAEKESNGVRVCVTGGAGFIGSWLVRKLLEKGYTVHATLRNTGDEAKAGL 50
53 LMDL.PGATERLSIWKADLAEEGSFHDAIRGCTGVFHVATPMDFLSKDPE 101
51 LRRLVPGAAERLRLFQADLFDAATFAPAIAGCQFVFLVATPFGLDSAGSQ 100
102 .NEVIKPTVEGMISIMRACKEAGTVRRIVFTSS..AGTVNLEERQ..... 143
101 YKSTAEAVVDAVRAILRQCEESRTVKRVIHTASVAAASPLLEEEVSASGV 150
144 ..RPVYDEESWTDVDFCRRVKMTGW.MYFVSKTLAEKAALAYAAEH..GL 188
151 GYRDFIDESCWTSLNVDYPLRSAHFDKYILSKLRSEQELLSYNGGESPAF 200
189 DLVTIIPTLVVGPFISASMPPSLITALALITGNAPHYSILKQVQ..... 232
201 EVVTLPLGLVAGDTVLGRAPETVESAVAPVSRSEPCFGLLRILQQLLGSL 250
233 .LIHLDDLCDAEIFLFEN.PAAAGRYVCSSHDVTIHGLAAMLRDRYPEYD 280
251 PLVHVDDVCDALVFCMERXPSVAGRFLXAAAYPTIHDVVAHYASKFPHLD 300
281 VPQRFPGIQDDLQPVRFSSKKLQDLGFTSGTRRWRTCSTPPSGLARRRAS 330
301 ILKE....TEAVATVRPARDRLGELGFQVPSTAWEEILDSSVACAARLGS 346
331 SPSPLPPEGTALPRC 345
347 ....LDASKLGLQKG 357

Figure 5.2.

The prospect that the HCTR mechanism is stably inherited in maize for specific defense against pathogens has very exciting evolutionary implications. The DNA for both fungal pathogenicity (TOX2 for HC-toxin biosynthesis) and host resistance (*Hm* for HC-toxin reductase) have been isolated, and perhaps can be used as molecular markers for studying the co-evolution of a host with its parasite. Studies of the natural history of Helminthosporium leaf spot, extended maize lineages, monocot evolution, and other fungi that produce Aeo-containing cyclic peptides present new opportunities for inquiry into the evolution of this particular host-pathogen interaction.

The prospect that HC-toxin is a fortuitous substrate for an enzyme with another function has other implications. One can assume, probably with certainty now, that *hm/hm* maize lines represent inbreeds with homozygous mutations at *Hm*. So *C. carbonum* race 1 appears to have capitalized on this mutation. A race endowed with HC-toxin production has created a niche for itself. But how? A credible answer to this question requires an understanding of HC-toxin's mode of action.

RECOMMENDATIONS

The mode of action of HC-toxin is perhaps best addressed by pursuing new alternatives to obtain a radiolabeled toxin analog. High specific activity is desired for binding studies with maize cell components. Information about HC-toxin biosynthesis has already fostered a method for radiolabeling HC-toxin (Meeley and Walton, 1991). A variation on *in vivo* feeding could be attempted, substituting aminoacrylic acid as an alanine analog. Aminoacrylic acid is an unsaturated analog of alanine which serves as the direct precursor to alanine in catalytic tritiation. The HC-toxin synthetase enzyme HTS-2, which activates both D- and L-alanine could be examined for an ability to activate aminoacrylic acid. Incorporation of this analog into the peptide ring of HC-toxin may provide one with an unsaturated substrate that could be purified and catalytically tritiated to high specific activity.

To assist with binding studies, a maize cDNA expression library in a suitable vector could be probed with radiolabeled HC-toxin to screen for specific sites of toxin action. Such pursuits are planned for the future (G.S. Johal, pers. commun.).

For studies of HCTR's localization and substrate specificity, heterologous expression of the *Hm* gene may present some new opportunities. A considerable amount of time and effort was recently expended trying to express an *Hm* cDNA in a heterologous system such as yeast or *E. coli*. I received a cDNA clone from our collaborators at Pioneer. While this project gave me some needed exposure to molecular biology, the cDNA supplied, called Hm21-1, was quite problematic due to the presence

of an unprocessed, 286 bp intron. Several methods were designed to remove this intron, and are summarized in Figure 5.3.

Attempts to isolate an intron-free *Hm* construct in the proper frame in the *E. coli* expression plasmids pET-3a and pET-9a (Novagen), or the yeast vector pYES2.0 (Invitrogen) have not been successful. Alternative *Hm* constructs and expression systems, such as bacculovirus expression, are being considered for the future (S.P. Briggs, pers. commun.). Heterologous expression could be a very important alternative to traditional protein purification methods. HCTR-specific antibodies and an amenable source of active HCTR are feasible rewards to expect from these pursuits. Meaningful substrate specificity studies will rely on a purified form of HCTR.

In the short term, the issue of whether HCTR activity is encoded by *Hm* can be addressed by *in vitro* RNA transcription-translation experiments. *In vitro* transcription-translation of plasmid encoded cDNA was the method used to link DFR activity with the maize *A1* gene (Reddy *et al.*, 1987). Similar experiments can be performed in pBluescript-II (Stratagene), which contains the T3 and T7 promoters for transcription. pHm21-1 is an *Hm* cDNA clone that contains 5' and 3' untranslated regions, a poly A^+ tail, and a 286 bp intron (Figure 5.3). The NcoI fragment from pHm21-1 can be repaired in a vector that contains no XhoI or NotI restriction sites. The vector pET-3d is suitable to sub-clone the NcoI fragment from pHm21-1, and insert the 408 bp, intron-free, XhoI/NotI fragment from pHM-XN (see Figure 5.3). The repaired NcoI fragment can be cloned back into pBluescript, creating a full-length cDNA construct for transcription-translation experiments.

Figure 5.3. Methods for intron removal and sequence adaptation for expression of the *Hm* cDNA in heterologous systems. PCR primers (Hm1R,F) for adaptation of the sequence are indicated by arrows. The *Hm* cDNA (Hm21-1) consists of a 1.66kb EcoRI/XhoI fragment cloned into pBluescript (provided by G. Johal, Pioneer Hi-Bred Int'l). For cloning in the *E. coli* and yeast expression vectors, Hm1R and F were designed to: 1.) amplify a 1.52kb fragment from Hm21-1, 2.) create BamHI sites on each end, and 3.) create an internal NdeI site for directional cloning into pET-3a or pET-9a. Hm21-B represents the 1.52kb Hm1R,F PCR product (B). The 286 bp intron was removed by amplification of the 5' and 3' halves of the gene using Hm1R and F, and new PCR primers flanking the intron sequence. These primers contribute a novel PvuII site to each half for blunt-end ligation of the PCR products, creating Hm21-P (C). The 408 bp XhoI/NotI fragment from pHM-XN, was also used to replace the intron-containing region, creating Hm21-X/N (D). The BamHI fragments of clones Hm21-P (C), and Hm21-X/N (D) are intended for the yeast vector pYES2.0. The NdeI/BamHI fragments from C and D are intended for cloning into pET vectors .

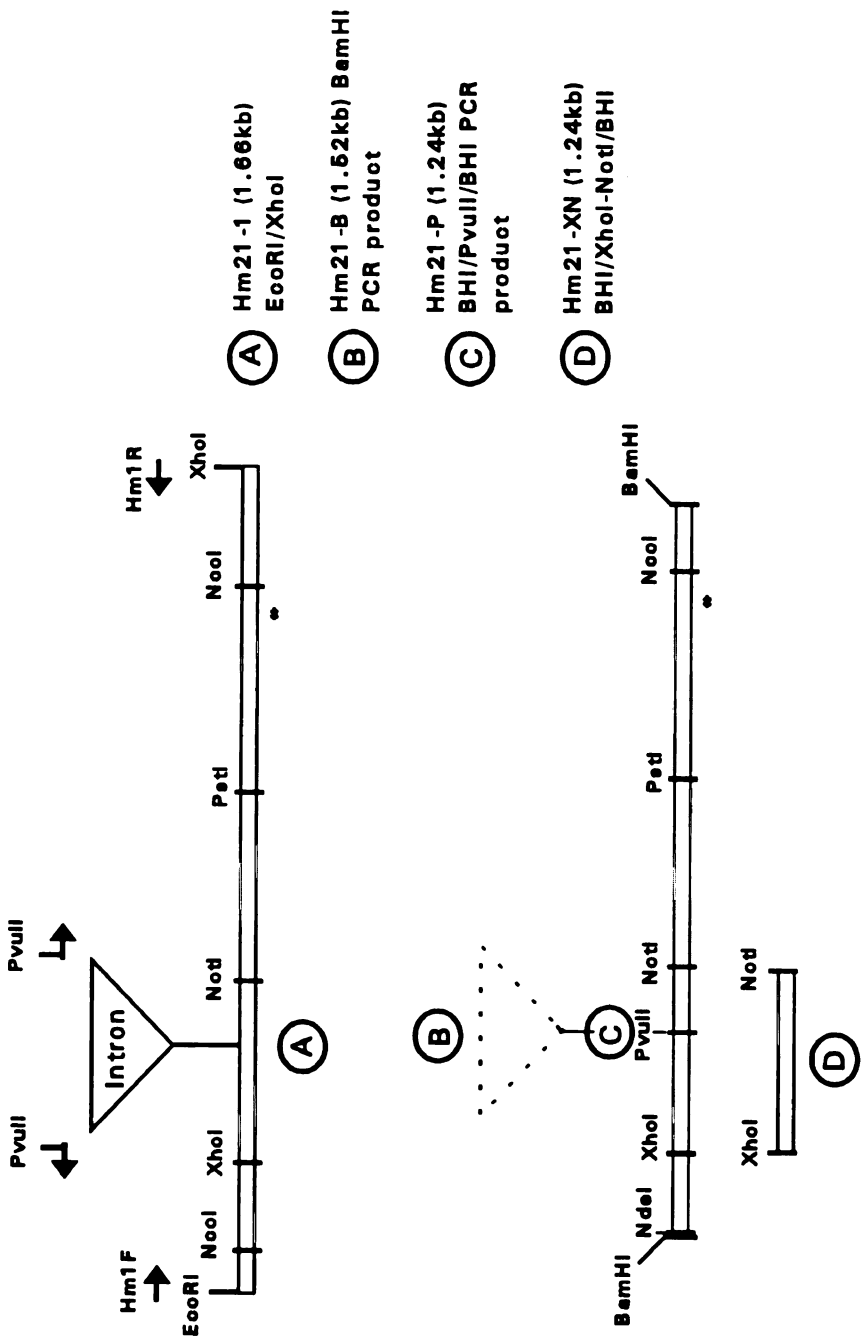
Hm21-1 (1.66kb) EcoRI/Xhol
Hm21-B (1.52kb) BamHI PCR produot
Hm21-P (1.24kb) BHI/PvuII/BHI PCR produot
Hm21-XN (1.24kb) BHI/Xhol-Notl/BHI
Hm1R
Hm1F
EcoRI
NcoI
XhoI
PvuII
Intron
NotI
PstI
NcoI
XhoI
BamHI
NdeI
BamHI

Figure 5.3.

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