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# The genetics of phytotoxin production by plant pathogenic fungi

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*Abstract.* Little is known about the genetic control of phytotoxin production by plant pathogenic fungi. The production of host-selective toxins known to play a role in disease development has been genetically analyzed in three species of *Cochliobolus*. In *C. heterostrophus*, a single genetic locus with two alleles has been identified controlling the production of HMT-toxin. This locus appears to be at or near the breakpoint of a chromosome rearrangement. Single genetic loci have also been identified controlling the production of HC-toxin by *C. carbonum* and HV-toxin by *C. victoriae*. The locus in *C. carbonum* may be a cluster of tightly linked genes.
*Key words.* Host-selective phytotoxins; HC-toxin; HMT-toxin; HV-toxin; *Cochliobolus heterostrophus*; *C. carbonum*; *C. victoriae*.

## *Introduction*

Over fifty fungal metabolites have been reported to be toxic to plants and at least thirty of these are known or suspected to contribute to plant disease[18]. Nothing is known about the genes controlling the production of the vast majority of them. The purpose of this review is to introduce the reader to some of the reasons for wanting to determine the genetics of phytotoxin production and to the progress in the few systems that have been examined. Because the only genes controlling phytotoxin production identified to date are in species of *Cochliobolus*, this work will be emphasized. Prospects for progress in the genetics of phytotoxin production by *Alternaria alternata* will be mentioned briefly. There have been several previous reviews of the field[15, 25, 33, 38].

Historically, one of the prime motivations for the genetic analysis of phytotoxin production has been to test or confirm the roles of the toxins in disease development. Fungi produce numerous substances in culture that are toxic to plants[18]. Yoder[33] and others[15, 18] have argued the merits of genetic analysis as a tool for testing their significance. In essence, if the same gene or genes which control toxin production also control pathogenicity (the ability to cause disease symptoms) or virulence (the severity of the symptoms), a meaningful role of the toxin in disease development can be inferred.

The strength of the genetic test depends on the likelihood that the strains being tested differ only by their ability to produce the toxin. The most convincing tests thus far for the roles of fungal toxins in disease development have involved studies of the cosegregation of toxin production and pathogenicity or virulence in the progeny of a cross or in the progeny of a series of backcrosses. These instances will be reviewed below. Recent years have seen the development of methods for the molecular genetic manipulation of several of the phytotoxin producing fungi[25, 38]. These techniques should permit the comparison of disease induction by strains that are identical except for the precise deletion or inactivation of a structural gene controlling a terminal step in toxin biosynthesis. Modification of genes in earlier steps and regulatory genes could give suggestive information, but would be less convincing because of an increased probability of effects on other metabolic pathways.

Genetic analysis in conjunction with biochemical analysis will allow the study of how phytotoxins are made. The pathways of toxin synthesis should be inferable from mutants by observing differences in levels of intermediates in the pathway and the supplementation needed to overcome specific blocks. Such analyses would be enhanced by the cloning and functional characterization of genes involved in synthesis, regulation or secretion. The utilization of such biosynthetic information and cloned

biosynthesis genes for the rational design of bioherbicides has been proposed [34]. Despite interest in this field, mutational analyses and surveys of naturally occurring variation to identify genes involved in the production of phytotoxins have been limited. Effort underway to collect and characterize mutants of certain *Fusarium* species in order to elucidate the trichothecene biosynthetic pathway has yielded strains that have been used to test the role of these metabolites in disease development [1, 4]. Trichothecenes are well known for their toxicity to animals, but have also been reported to be toxic to plants. [21].

The evolution of phytotoxin production has received increasing attention in recent years. The genotypically uniform crops of modern agriculture create strong selection pressures favoring fungal strains with the ability to produce phytotoxic metabolites which contribute to disease development. It is not generally known whether these new strains arise by mutation, or whether they are selected from previously unknown populations. Nor is the nature of presumed mutations to toxin production known. Of the toxins genetically analyzed to date, only single loci have been found which control their production, yet none of the toxins are primary gene products. Are these loci clusters of closely linked genes? If so, how did they arise? The cloning and characterization of genes controlling phytotoxin production should help to answer such questions. This knowledge, as well as a knowledge of fungal metabolism and selection pressures, may someday permit the prediction of fungi likely to undergo mutations to the production of phytotoxic compounds, and permit a change in agricultural practices before an epidemic occurs.

The genetic analysis of most of the phytotoxin producing fungi has been seriously restricted by a lack of known or easily manipulated sexual cycles. The emphasis of this review will be on three species of *Cochliobolus* for which genetic methods are relatively well developed. These are *C. heterostrophus*, which produces HMT-toxin, *C. carbonum*, which produces HC-toxin, and *C. victoriae*, which produces HV-toxin. All of these toxins are host-selective.

### *HMT-toxin*

*Cochliobolus heterostrophus* (anamorph: *Bipolaris maydis* = *Helminthosporium maydis* = *Drechslera maydis*) is a filamentous ascomycete that causes southern leaf blight of maize. Two races of this fungus have been defined. Race T produces a family of long chain linear polyketols known as HMT-toxin, or simply T-toxin, which conditions high specific virulence on maize with Texas male-sterile cytoplasm (cms-T) [7]. Race T was responsible for a severe epidemic on maize in 1970. Race O, which does not produce this toxin, is moderately virulent on all maize cytoplasms. A possible third race, race C, has been described and has been reported to produce a host-selective toxin [31], but these reports have not been confirmed. Methods for the genetic and molecular analysis of *C. heterostrophus* have been reviewed [35].

The polyketol structure of T-toxin suggests that multiple enzymatic functions may be required for its synthesis. It has been speculated that these functions may be encoded by multiple genes [3]. To date, a single genetic locus with two alleles, *Tox1* has been demonstrated to control T-toxin production. Lim and Hooker [12] were the first to report the genetic analysis of known T-toxin producing strains. In a cross between a race O isolate ($tox^-$) and a race T isolate ($tox^+$), segregation for T-toxin synthesis was 1:1, indicating a single locus difference between the strains. All progeny which produced T-toxin had high specific virulence to cms-T maize. Several apparent exceptions to this correlation were noted, but were attributed to the insensitivity of the toxin bioassay.

The association of T-toxin production and host-specific virulence was confirmed by Tegtmeier et al. [23] by applying an established T-toxin isolation procedure to culture filtrates from strains that were nearly isogenic except for alleles at the locus controlling host-selective virulence, that is, *Tox1*. These strains were prepared by six generations of backcrossing of a race O donor strain to a race T recurrent parent. The isolation procedure yielded T-toxin from the strains with high virulence on cms-T maize, but no comparable fraction from the strains with low virulence on cms-T maize.

Efforts to identify additional loci and further test the correlation between toxin production and high specific virulence were undertaken by Yoder [32] and Yoder and Gracen [36] by the examination of naturally occurring variation in field populations. In approximately one third of the crosses among race T and race O field isolates, progeny segregated 1:1 for T-toxin production, suggesting a single locus difference. In the remaining crosses, segregation more closely approximated 3:1 ($tox^-$ : $tox^+$) or 7:1, suggesting that the parents differed by two, three or more loci controlling T-toxin production. However, none of the asci from the crosses heterozygous for toxin production produced their full complement of eight ascospores; the remaining ascospores had either not formed or had aborted and disintegrated before maturity. The investigators could not conclude whether the ratios observed were due to multiple locus segregation or preferential abortion of $tox^+$ progeny. In all progenies, T-toxin production was absolutely associated with high virulence on cms-T maize.

A further analysis of naturally occurring variability in the *C. heterostrophus* population demonstrated that the non-1:1 segregation ratios observed by Yoder [32] and Yoder and Gracen [36] were probably not attributable to additional T-toxin production loci. Bronson et al. [3] determined the genetic control of T-toxin synthesis in twelve race T and eleven race O field isolates, including some that had been examined by Yoder [32] and Yoder and Gracen [36]. All the race T field isolates were shown to have the allele of *Tox1* that specifies T-toxin production; all the

race O isolates were shown to have the alternate allele. However, in crosses involving about half of the race O field isolates, segregation ratios were non-1:1, as had been seen by Yoder[32] and Yoder and Gracen[36]. These ratios were shown to be due, not to additional loci controlling T-toxin synthesis, but to a locus linked to *Tox1* that caused segregation distortion. The allele of this locus in the $tox^-$ field isolates appeared to cause preferential abortion of ascospores containing the opposite allele. This locus could account for most or all of the non-1:1 segregations reported previously. The authors concluded that there was no confirmed evidence for naturally occurring variation at T-toxin loci other than *Tox1*.

Attempts to find additional loci by the induction of mutations have been limited and as yet unsuccessful. Taga and Yoder (personal communication) found no $tox^-$ mutants among 2539 survivors from the mutagenesis of a $tox^+$ strain. There have been no reported attempts to induce $tox^+$ mutants from a $tox^-$ strain. The recent development of a microbiological assay for T-toxin utilizing a cloned sensitivity gene from cms-T maize[5] should facilitate future efforts to look for mutants of both types[3,38].

The dominance relationships of alleles at *Tox1* are not clear. The life cycle of *Cochliobolus* has only a transitory diploid stage, precluding simple tests of dominance. In an attempt to determine dominance relationships at *Tox1*, Leach et al.[9] compared T-toxin production by two $tox^+/tox^-$ heterokaryons to T-toxin production by a $tox^+/tox^+$ heterokaryon. The heterokaryons were forced using complementing auxotrophic markers, but tended to resolve into their homokaryotic components. The investigators estimated that about 75% of the mycelia of the forced heterokaryons were heterokaryotic and about 25% were homokaryotic. The homokaryotic fraction consisted of roughly 50% of each type. They predicted therefore that, if T-toxin synthesis is dominant, the $tox^+/tox^-$ heterokaryons should make about 87% as much toxin as the $tox^+/tox^+$ heterokaryon. If it is recessive, the $tox^+/tox^-$ heterokaryons should make about 13% as much toxin. Culture filtrates of the $tox^+/tox^-$ heterokaryons gave 82% as much inhibition in a seedling root growth bioassay and, in most instances, 60–75% as much inhibition in a dark $CO_2$ fixation bioassay as the culture filtrates of the $tox^+/tox^+$ heterokaryon. The investigators concluded that the simplest interpretation of the results was that T-toxin production was dominant or semidominant. This conclusion was considered tentative by the investigators, in part because of high variability between replicate culture filtrates of the $tox^+/tox^-$ heterokaryons.

The authors' conclusion was based on comparisons of percentage inhibition by the two types of heterokaryons. However, their data and the data of Yoder et al.[37] demonstrate that the relationships between percentage inhibition and T-toxin concentration is not linear. Yoder et al.[37] reported that both the dark $CO_2$ fixation bioassay and the seedling root growth bioassay give sigmoidal dosage response curves when percentage inhibition is plotted versus the logarithm of the toxin concentration. Thus, any given difference in percentage inhibition corresponds to a logarithmically greater difference in toxin concentration. Reanalysis of the data of Leach et al.[9] using standard dosage response curves[23,37] and a dosage response curve prepared from the data of the $tox^+/tox^+$ heterokaryon suggest that the initial calculation may have overestimated the amount of T-toxin produced by the $tox^+/tox^-$ heterokaryons (Bronson and Yoder, unpublished observations). If this is true, then the evidence that T-toxin production is dominant or semidominant is even more tenuous than the authors originally concluded. Because of the difficulty of working with heterokaryons of *C. heterostrophus*[9], it may be necessary to clone and analyze *Tox1* to resolve this issue with certainty. Strategies and technology for cloning *Tox1* and other loci involved in phytotoxin synthesis have been described[38].

Shortly after the epidemic incited by race T of *C. heterostrophus* in 1970, cms-T maize was withdrawn from widespread use and race T effectively disappeared[11]. This disappearance has been attributed to a reduced ability of race T to grow on toxin insensitive, male-fertile maize relative to race O. Leonard[10] and Klittich and Bronson[6] demonstrated that a similar difference in ability to grow on male-fertile maize also exists between strains of *C. heterostrophus* bred to be nearly isogenic except for alternate alleles at *Tox1*. No consistent growth differences were seen on artificial media[6,8,10]. They proposed that the reduced fitness associated with *Tox1* could account for the decline of race T after the 1970 epidemic and that the reduced fitness is likely due either to some unknown effect of T-toxin production, a pleiotropic effect of *Tox1*, or a closely linked gene or genes.

Recent research indicates that the chromosome bearing *Tox1* in race T is reciprocally translocated with respect to the chromosome bearing its alternate allele in race O. The first indication that *Tox1* might be associated with a chromosome rearrangement came from studies of ascospore abortion in crosses among *C. heterostrophus* strains. Taga et al.[22] observed nonrandom abortion of ascospores in crosses between $tox^+$ and $tox^-$ strains. Bronson[2] reported that the factor causing the ascospore abortion did not segregate from *Tox1* in up to 10 backcrosses and among 100 progeny, indicating that it was tightly linked to *Tox1*. Based on the patterns of ascospore abortion observed, it was postulated that the factor might be a reciprocal translocation[2]. The existence of the translocation was shown by the creation of a restriction fragment length polymorphism map of *C. heterostrophus* and the mapping of the region around *Tox1*[28]. Among 91 progeny examined, there were no crossovers between *Tox1* and the translocation breakpoint. Studies involving the hybridization of the probes used to make the map to electrophoretically separated chromosomes of field iso-

lates suggest that the difference in chromosome arrangement between race T and race O is common and possibly the rule in field populations (Chang and Bronson, unpublished observations). The failure to find exceptions to the association between *Tox1* and the translocation suggests that *Tox1* may be on the breakpoint.

The significance of the inability to find loci in addition to *Tox1* in field populations and the tight association of *Tox1* with reduced fitness and a translocation breakpoint is not clear. There are several possible explanations for the seeming lack of diversity for T-toxin synthesis loci. *Tox1* may be a cluster of closely linked genes, it may encode a single multifunctional enzyme, loci for other required functions may be essential and therefore invariant, or T-toxin may be a metabolic intermediate which is normally converted by *Tox1* to a nontoxic form [33]. This latter interpretation is consistent with a simple explanation for the tight association of *Tox1* with a translocation breakpoint and reduced fitness. It it possible, for example, that T-toxin production evolved by a chromosome breakage and reunion, that is, a reciprocal translocation, which destroyed the activity of *Tox1*. In this scenario, the reduced fitness associated with *Tox1* could be due to either the loss of normal *Tox1* function or the destruction (or creation) of a gene at the complementary breakage site. Alternatively, the translocation could have created a functional gene at *Tox1*. It is also possible that the association of *Tox1* with a translocation and reduced fitness could be an artifact of the strains studied to date. The ability to produce T-toxin could have evolved in the same strain as, but independently of, reduced fitness and the translocation. Tight linkage may have simply made these traits seem associated. The cloning and characterization of alternate alleles at *Tox1* may shed light on its actual evolution.

### *HC-toxin and HV-toxin*

Phytotoxin production by two additional Cochliobolus species has been studied genetically. These are *C. carbonum* (anamorph: *Helminthosporium carbonum* = Bipolaris zeicola) and *C. victoriae* (anamorph: *H. victoriae*). *C. carbonum* race 1 makes HC-toxin and is highly virulent to maize that is homozygous for recessive alleles at the locus *Hm*. *C. victoriae* makes HV-toxin which confers pathogenicity to oats carrying the dominant allele at the locus *Vb*. This gene is either very closely linked to or the same as the gene *Pc-2*, which confers resistance to *Puccinia coronata* [16].

As is the case for T-toxin, the exact nature of the genes for the synthesis of HC-toxin and HV-toxin is not known. To date, single genetic loci have been identified controlling qualitative production of each of these toxins. Scheffer et al. [20] reported that segregation in crosses between *C. carbonum* race 1 and *C. victoriae* was 1:1:1:1 for progeny producing HC-toxin, HV-toxin, both toxins, or neither toxin. Toxin production correlated with the ability to incite characteristic disease symptoms on maize, oats, both maize and oats, and neither host, respectively. Crosses were also performed between race 1 and strains of *C. carbonum* that did not produce HC-toxin. Segregation for toxin production and race 1 type pathogenicity was not significantly different from 1:1. A few exceptions to the correlation were found, but the authors felt these most likely reflected the limitations of the assays. These results confirmed the roles of HC-toxin and HV-toxin in the specific virulence and pathogenicity of *C. carbonum* race 1 and *C. victoriae*, respectively, and demonstrated that at least one Mendelian locus controls qualitative production of HC-toxin and another unlinked locus controls the qualitative production of HV-toxin. These loci have been given the names *Tox2*, for the locus controlling the synthesis of HC-toxin, and *Tox3*, for the locus controlling the synthesis of HV-toxin [38]. The dominance relationships for these loci are not known.

Evidence has been presented in support of the hypothesis that there are also genes controlling the amount of these two toxins that are produced [13, 19, 20]. The titer of HC-toxin and HV-toxin produced in culture has been reported to vary considerably between strains, up to about 1000-fold as measured by the maximum dilution of a culture filtrate that gives reproducible inhibition of plant growth in bioassays, and some progeny of crosses were reported to produce more or less toxin than either of their parents. This was interpreted as being due to transgressive segregation among multiple loci.

Research by Walton [29] and Walton and Holden [30] on the biosynthesis of HC-toxin suggests that *Tox2* may be a cluster of closely linked genes. HC-toxin is a cyclic tetrapeptide. Using strategies developed for the synthetases of cyclic peptide antibiotics, Walton isolated two separable enzymatic activities expected to be required for HC-toxin synthesis. HTS-1 (HC-toxin synthetase 1) catalyzes an L-proline-dependent ATP/inorganic pyrophosphate exchange; HTS-2 catalyzes a D- and L-alanine-dependent ATP/inorganic pyrophosphate exchange. These activities were present in race 1 isolates, but not race 2 and race 3 isolates, which do not produce HC-toxin. These activities also cosegregated with HC-toxin production among 13 random ascospore progeny of a cross between an HC-toxin producing strain and a non-producing sibling. These results suggest that both activities may be the product of *Tox2* and therefore that *Tox2* may be a cluster of tightly linked genes consisting of these activities as well, perhaps, as the remaining activities needed for HC-toxin synthesis. This hypothesis is being tested by cloning *Tox2* using antibodies to purified HTS-1 and oligonucleotide probes based on the amino acid sequence of HTS-1 (J. D. Walton, personal communication).

### *Prospects*

Our understanding of the genetics of phytotoxin production by plant pathogenic fungi is likely to increase dra-

matically in the near future as a result of improvements in techniques for genetic and molecular analysis. Among the phytotoxin producing fungi with sexual cycles, the application of genetic engineering techniques should permit the cloning of genes identified by meiotic analysis. Transformation, cloning by complementation, heterologous gene expression, gene disruption and gene replacement have been demonstrated for *C. heterostrophus*[17,26,27,35] (and O. C. Yoder, personal communication). The vectors developed for *C. heterostrophus* also transform *C. carbonum*, *C. victoriae*, and a variety of other fungi[38].

Genetic studies of phytotoxin production by fungi which lack sexual cycles should be advanced by parasexual analysis and molecular genetics. *Alternaria alternata* produces a variety of well-characterized host-selective phytotoxins[14], but the lack of a sexual cycle has hampered genetic analysis of their production and no genes have been identified as yet. However, techniques for mutagenesis and heterokaryon formation have been demonstrated for both the Japanese pear and the apple pathotypes of *A. alternata*[24] and methods of molecular analysis including transformation and cloning by complementation have been demonstrated for the Japanese pear pathotype[25]. These techniques should permit the identification and analysis of genes involved in phytotoxin production by this species. The application of molecular techniques to studies of AK-toxin production by the Japanese pear pathotype of *A. alternata* has been reviewed[25].

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# Bacterial phytotoxins: Mechanisms of action

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*Abstract.* Many species of phytopathogenic procaryotes produce toxins that appear to function in disease development. They affect the plant in different ways, the end result of which is the elicitation of chlorosis, necrosis, watersoaking, growth abnormalities or wilting. The most extensively studied toxins cause chlorosis. They specifically inhibit diverse enzymes, all critical to the plant cell. This inhibition results in a complex series of metabolic dysfunctions ultimately resulting in symptom expression. Substances causing growth abnormalities consist of known phytohormones and other compounds with plant hormone-like activities, but which have no structural relationship to the known hormones. The former act in the usual manner but, because of their elevated levels and imbalances, the host's regulatory mechanisms are overwhelmed and abnormal growth results (hyperplasia, shoot or root formation); the mechanisms of action of the latter group are unknown. High molecular weight, carbohydrate-containing substances, also acting in unknown ways, cause tissue watersoaking or wilting. Likewise, we know little about toxins causing necrosis except for syringomycin which affects ion transport across the plasmalemma.
*Key words.* Bacterial phytotoxins; tabtoxinine-β-lactam; phaseolotoxin; syringomycin; syringotoxin; rhizobitoxine; coronatine; tagetitoxin; IAA, cytokinins, ethylene; *Pseudomonas*; *Bradyrhizobium*; *Corynebacterium.*

## *Introduction*

Members within all genera of phytopathogenic bacteria have been reported to produce phytotoxins [10]. Currently, however, only a few of these toxins have well-defined mechanisms of action. For a larger number of toxins there is physiological information as to which metabolic process(es) is disrupted and/or organelle affected, but their exact molecular targets have not yet been identified; nor, obviously, have their mechanisms of action been elucidated. For a third – and by far the largest – group of toxins, the information available on their mode of action is still too gross to make a meaningful evaluation, e.g., inhibit respiration or photosynthesis, and in many cases their structures have not been completely determined.

The coverage for this article depends, in a major way, on how one defines the term phytotoxin. Like most definitions it can either be inclusive or exclusive. I have chosen more to follow the former path and to define phytotoxins in terms of their biological interaction. Thus, molecular weight, concentration in the plant, chemical class, symptom expression, or the nature of the toxin-target interaction are not considerations for defining phytotoxins; also, enzymes are excluded from consideration [18, 54]. Phytotoxins are defined as compounds synthesized by the pathogen during pathogenesis that are deleterious to the host. Note that this definition does not exclude compounds also produced by higher plants like auxins or cytokinins. Just because at some point in time we realize that a compound which acts in the sense of a toxin also has a function in healthy plants, is not in itself ground for excluding it from consideration. What is important is that we consider only that portion contributed by the pathogen. The classical example of this situation was the discovery that *Gibberella fujikuroi* produced a mixture of gibberellins (up to 1987, 72 free gibberellins had been identified) and the demonstration that they could cause abnormal shoot elongation resembling the 'bakanae', or foolish, disease of rice seedlings incited by *G. fujikuroi*. From this work came the revelation that the gibberellins were a new class of plant growth hormones. Also included under my definition are compounds synthesized by so-called exopathogens [83].