Host-Selective Toxins: Agents of Compatibility

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INTRODUCTION

Host-selective toxins (HSTs) are, with one exception, low molecular weight compounds with diverse structures that act as positive agents of virulence or pathogenicity. Approximately 20 HSTs have been documented. In general, HSTs are determinants of host range or specificity in that plant species, varieties, or genotypes sensitive to an HST are those that are susceptible to the producing pathogen. HSTs have been critical factors in two major epidemics of crops in the United States in the 20th century, including the Southern corn leaf blight epidemic of 1970 that destroyed ~15% of that year's crop. They are also important factors in several other economically significant diseases throughout the world. Studies of diseases involving HSTs led to the first elucidation of the molecular basis of disease susceptibility in any interaction (Southern corn leaf blight; Dewey et al., 1988) and to the first cloning and functional characterization of a Mendelian disease resistance gene (Northern corn leaf spot; Johal and Briggs, 1992). Some HSTs have highly unusual chemical structures and unusual biological activities. Although some HSTs are extremely toxic, the reaction to them is often controlled by single plant genes. The study of HSTs and the diseases in which they occur continues to contribute fundamental knowledge about the processes and regulation of disease susceptibility and resistance, about basic plant biochemistry through their use as specific metabolic inhibitors, about the structure and organization of secondary metabolite pathways, and about the organization of fungal genomes and the evolution of new pathogen races.

Because cell death is a symptom of many plant diseases, it was hypothesized early on that phytotoxic compounds contribute to the virulence or pathogenicity of plant pathogens. Although it is now well established that many plant pathogenic bacteria and fungi produce phytotoxic compounds, the majority of these compounds are nonselective. That is, these compounds affect a broader range of organisms than the producing organism infects. Some nonselective toxins, such as fusicocin, trichothece, coronatine, phaseolotoxin, syringomycin, and tabtoxin, contribute to virulence or symptom development in the diseases in which they occur (Stoessl, 1981; Ballio and Graniti, 1991; Proctor et al., 1995; see also Knogge, 1996, in this issue), but by definition, nonselective toxins are not primary determinants of host range.

Although bacteria make many nonselective toxins, all known HSTs are made by fungi. (One bacterial pathogen does make a low molecular weight host-selective elicitor that causes necrosis; see below.) Even among the fungi, most known HSTs are produced by species or pathotypes in just two genera, Alternaria (Figure 1) and Cochliobolus (also known by the old and new names for its imperfect stage, Helminthosporium or Bipolaris, respectively; Figure 2). Other well-documented HSTs are PM-toxin, produced by Phyllosticta maydis (Mycosphaerella zeae-maydis), and peritoxin, produced by Periconia circinata (Figure 3). Unlike other known HSTs, Ptr-toxin, made by Pyrenophora tritici-repentis, is a ribosomally synthesized 13.2-kD polypeptide (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995). In addition, there are many preliminary reports of HSTs from other fungal pathogens.

Why most known HSTs are made by just two genera is unknown, but the answer may be related to their particular pathogenicity strategy. Both Cochliobolus and Alternaria compete well as facultative saprophytes, yet they are more adapted to a pathogenic lifestyle than are organisms that can only infect senescent tissue or through wounds (Scheffer, 1991). Another explanation for the overrepresentation of these two genera among known HST producers might simply be the ease of coaxing these particular pathogens to produce their HSTs in culture. HSTs that are made only in the plant or under specific inductive conditions are much less likely to have been discovered. There are a number of diseases in which HSTs are strongly suspected to occur but have not been found (Walton and Panaccione, 1993).

CHEMISTRY OF HSTs

All known HSTs except Ptr-toxin are secondary metabolites. That is, they are low molecular weight compounds of diverse structure that are restricted in their taxonomic distribution and are not necessary for normal survival and reproduction. Whereas we are ignorant of the adaptive advantage, if any, that the vast majority of secondary metabolites confer on
organisms that produce them, it is clear that on sensitive host plants, HSTs allow pathogens to colonize more plant tissue and therefore enjoy greater reproductive success. The established role of HSTs in host-pathogen interactions supports the emerging consensus that secondary metabolites have mainly ecological roles, modulating the interaction of an organism with its environment and with other organisms (Stone and Williams, 1992). Secondary metabolites are important to many other aspects of plant-microbe biology, including plant defense, biocontrol, and plant-symbiont interactions (see Handelsman and Stabb, 1996; Knogge, 1996; Long, 1996; Osbourn, 1996, in this issue).
Chemical classes that are represented in the HSTs include polyketides, cyclic peptides, terpenoids, saccharides, ribosomally synthesized polypeptides, and compounds of uncertain biogenesis. Some HSTs are chemically related to other toxic compounds, for example, AAL-toxin to the mycotoxin fumonisin, produced by Fusarium moniliforme (Gilchrist et al., 1995), and HC-toxin to four other cyclic tetrapeptides containing Aeo (2-amino-9,10-epoxy-8-decanoic acid; see Figure 2B), produced by four unrelated filamentous fungi (Walton et al., 1996). Some HSTs are chemically related to each other; for example, T-toxin from C. heterostrophus and PM-toxin from M. zeae-maydis both affect Texas male-sterile cytoplasm (Tcms) maize and are structurally very similar, although the fungi themselves are not closely related. Three pathotypes of A. alternata with different host ranges (Japanese pear, strawberry, and tangerine) make HSTs containing 9,10-epoxy-9-methyldecatenoic acid (AK-, AF-, and ACT-toxins, respectively; Ballio and Graniti, 1991; Otani et al., 1995). At least one pathogen, the tangerine pathotype of A. alternata, makes more than one class of HST (Figures 1B and 1D). Some HSTs, such as victorin and peritoxin, are novel among known secondary metabolites. These two HSTs are particularly unusual because they are halogenated (Figures 2A and 3B).

In common with many other secondary metabolites, most HSTs exist as families of compounds, each member of which is produced in a different amount and has a different potency. Bipolaris sacchari (an asexual relative of Cochliobolus spp), in addition to the HST known as HS-toxin (Figure 2D), also makes related compounds that are antagonists of HS-toxin. These protective compounds, or “toxoids,” lack one or more galactose residues (Livingston and Scheffer, 1984).

BIOCHEMICAL BASES OF HST ACTION AND SPECIFICITY

Not surprisingly, toxins are usually toxic, that is, they kill cells. HSTs are active at concentrations ranging from $\sim 10$ pM to 1 $\mu$M, and their degree of specificity (host selectivity) ranges from 100-fold to $>10^6$-fold. The most common bioassays for both selective and nonselective toxins include stimulation of electrolyte leakage, inhibition of root growth, and chlorosis and necrosis when injected into leaves. Electrolyte leakage most likely reflects the inability of moribund cells to maintain membrane integrity. However, it has also been postulated to be a critical biological effect of HSTs because it would cause low molecular weight nutrients such as sugars and amino acids to diffuse out of plant cells and into the apoplast, where they could be absorbed by the fungal mycelium. Many HST-producing fungi are leaf pathogens, and because low molecular weight toxins are mobile, initial evidence for the involvement of a toxin in a particular disease has often come from the occurrence of symptoms, such as leaf chlorosis, at some distance from the site of infection.

Despite their name, not all toxins are toxic under all conditions. HC-toxin, for example, does not kill nondividing leaf mesophyll protoplasts but actually promotes their survival (Wolf and Earle, 1991), an effect that is perhaps related to its function as a suppressor of defense responses (see below). Light suppresses the symptoms of AM-toxin toxicity without altering the response of the host plant to the fungus (Tabira et al., 1989). Inhibitors of protein and RNA synthesis protect sensitive cells against some HSTs, for example, victorin, peritoxin,
AAL-toxin, AK-toxin, and AM-toxin (Walton and Panaccione, 1993; Dunkle and Macko, 1995; Gilchrist et al., 1995). This finding suggests that HSTs are not simply metabolic poisons but rather that their toxicity requires active participation (transcription and translation) on the part of the plant cell.

Studies on the mode of action of HSTs have been guided by consideration of the genetics of the host response. The HSTs from Alternaria affect a range of dicotyledonous plants, and many of these are not readily amenable to experimental genetic analysis (e.g., apple, pear, strawberry, and citrus). Pedigree reconstruction indicates that sensitivity to Alternaria HSTs and susceptibility to the producing organisms are usually genetically dominant. Susceptibility to A. alternata f sp lycopersici and sensitivity to its HST, AAL-toxin, are controlled by the nuclear gene Asc in tomato. Whereas resistance to the pathogen is fully dominant, sensitivity to AAL-toxin is semidominant (Otani et al., 1995).

HST-producing species of Cochliobolus are pathogens of grass species that can be genetically manipulated (the exception is sugarcane, the host of B. sacchari). The sensitivity of plants to the Cochliobolus HSTs can be genetically dominant (oats to victorin), cytoplasmically inherited (maize to T-toxin), or genetically recessive (maize to HC-toxin). Sensitivity of maize to PM-toxin is, like sensitivity to T-toxin, inherited cytoplasmically. Sensitivity to Ptr-toxin in wheat is genetically dominant and in situ are quickly and specifically perturbed morphologically and biochemically by T-toxin. The basis of sensitivity to T-toxin is a rearrangement of the mitochondrial genome of Tcms plants that results in the production of a chimeric open reading frame, T-urfl3, which encodes a 13-kD protein (URF13) localized in the inner mitochondrial membrane (Forde et al., 1978; Dewey et al., 1986). When expressed in Escherichia coli, yeast mitochondria, or tobacco, URF13 confers T-toxin sensitivity, providing definitive proof of the importance of T-urfl3 in sensitivity to T-toxin (Dewey et al., 1988; Leving et al., 1995).

Physical biochemical studies and modeling indicate that URF13 forms oligomeric pores in membranes in the presence of T-toxin (Korth et al., 1991; Leving et al., 1995).

Fertility is restored to Tcms plants by the combined action of the nuclear genes Rf1 and Rf2. However, fertility-restored plants are still sensitive to T-toxin and susceptible to C. heterostrophus race T. Rf1 by itself suppresses the expression of T-urfl3 by ~80%; the residual 20% is apparently sufficient to give T-toxin sensitivity and hence disease susceptibility. Rf2 has no effect on T-urfl3 expression (Dewey et al., 1987). Rf2 is predicted to encode an aldehyde dehydrogenase, but the biochemical basis of its action in fertility restoration is not yet established (Cui et al., 1996).

There are two major unanswered questions concerning Tcms and sensitivity to T-toxin: How does Tcms cause male sterility? And what is the mechanism(s) by which fertility restoration modulates male sterility and disease susceptibility? In regard to how T-urfl3 causes male sterility, it has been hypothesized that developing anthers make a T-toxin–like compound that destroys the developing pollen, but searches for this proposed endogenous T-toxin have been inconclusive.

C. heterostrophus, however, which does not produce T-toxin, is a minor pathogen of maize regardless of cytoplasm. Research on C. heterostrophus race T and its HST was spurred by the Southern corn leaf blight epidemic of 1970, the event that focused attention on the genetic vulnerability of our major crop plants. This epidemic had its origins in the increasingly widespread use of Tcms, an economical alternative to manual detasseling, in maize hybrid production during the 1960s. By 1970, most of the hybrid seed grown in the United States had Tcms, and in that year, weather conditions were favorable for the development of Southern corn leaf blight.

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Victorin

Victorin was one of the first HSTs discovered, and its remarkable toxicity (effects have been recorded at 10 pM), drastic and rapid effects on cells, and high degree of specificity have made it the archetypal HST. Like C. heterostrophus race T, C. victoriae (cause of Victoria blight of oats) negatively impacted the economy of the United States during the 1930s by causing major epidemics among oats. The origins of the Victoria blight epidemic, parallel to those of the Southern corn leaf blight, were rooted in human modification of a crop genome for reasons unrelated to HST sensitivity. In the 1930s, crown rust caused by Puccinia coronata was a serious disease, and Victoria blight was unknown. The Pc-2 gene, discovered in Uruguay, gives good resistance to P. coronata and was introgressed into the major oat varieties in the United States. Subsequently, the previously unknown Victoria blight appeared, which specifically attacked oat varieties with the Pc-2 gene. The gene that confers susceptibility to C. victoriae, called Hv-1 or Vb, is either the same gene as Pc-2 or very tightly linked.

A compelling reason to study the mode of action of victorin is the possibility that it will lead to an understanding of specificity in the important but experimentally intractable rust diseases. Radiolabeled victorin binds irreversibly to a 100-kD protein in oats. The binding is host selective in intact leaves, but in cell-free extracts victorin binds equally well to proteins from sensitive and insensitive oats (Wolpert and Macco, 1989). However, another group using antibodies against biotinylated victorin reported that binding was nonspecific in the absence of intact tissues and in cell-free extracts (Akimitsu et al., 1992). The major 100-kD victorin binding protein was purified, and its gene was cloned and identified as encoding the pyridoxal phosphate-containing P subunit of glycine decarboxylase (GD)—a complex enzyme with 42 subunits of four types. It is located in the mitochondrion and has a central role in the photosynthetic cycle. It is also an essential enzyme for nonphotosynthetic plant tissues such as etiolated leaves and roots, which are fully susceptible to victorin. Victorin also binds to the 15-kD lipoamide-containing H subunit of GD but in a nonspecific manner, even in vivo (Navarre and Wolpert, 1995). Victorin inhibits GD in a host-selective manner in intact tissues (EC_{50} 81 pM), but it has no effect on GD in isolated, intact mitochondria because of lack of uptake (Navarre and Wolpert, 1995). In intact tissues, victorin accumulates in the mitochondria of sensitive cells but not in that of resistant cells, which is consistent with its specific binding in vivo (T.J. Wolpert, personal communication).

The relationship between the Hv-1 gene and GD is currently unknown. Hv-1 could encode one of the subunits of GD (most likely either the H protein or the P protein), or it could be involved in the uptake or modification of victorin (Navarre and Wolpert, 1995). In oat, which is an allohexaploid, all of the subunits of GD are encoded by nuclear multigene families. Therefore, it will not be trivial to determine whether the Hv-1 gene encodes one of the members of one of these families.

Although the evidence is strong that GD is the site of action of victorin, it does not yet make sense to exclude the possibility that the basis of victorin's selectivity, that is, the process controlled by Hv-1, resides elsewhere. For example, the Hv-1 gene product could control mitochondrial uptake or metabolism of victorin. Sensitivity to victorin is genetically dominant; therefore, if Hv-1 controls host-selective uptake or metabolism of victorin, it would probably affect an activation reaction that produces a biologically active product from a "protoxin." This effect would contrast with the mode of action of HC-toxin (see below).

HC-Toxin

Susceptibility to C. carbonum race 1 and sensitivity to its toxin, HC-toxin, are controlled by a single Mendelian locus in maize, Hmt1. Resistance is dominant to susceptibility. Radiolabeled HC-toxin was used to demonstrate that resistant maize (genotype Hmt1Hmt1 or Hmt1Hm1) has an enzymatic activity, HC-toxin reductase, that detoxifies HC-toxin by reducing the 8-carbonyl group of the side chain of Aeo (Figure 2B; Meeley et al., 1992). This enzymatic activity is also present in extracts of other grasses (oats, wheat, and barley) but is not detectable in extracts of several dicotyledonous plants. The basis of the insensitivity of dicots to HC-toxin is not known (Meeley and Walton, 1993).

HC-toxin inhibits root growth of hm/hm maize but is not toxic to cells. It has cytostatic and not cytotoxic activity against mammalian cells. Current evidence indicates that the site of action of HC-toxin is histone deacetylase (HD), an enzyme that reversibly deacetylates the core histones (H3 and H4) while they are assembled in chromatin. Acetylation and deacetylation of the core histones alter the inducibility and suppressibility of certain classes of genes, but the details of how this occurs have not been elucidated (Taunton et al., 1996). Trapoxin, an HC-toxin analog in which the two alanines are replaced by two phenylalanines, inhibits mammalian HD in vivo and in vitro (Kijima et al., 1993), and HC-toxin inhibits maize HD in vitro, causing the accumulation of acetylated histones in vivo. HC-toxin also inhibits HD from yeast, Phyrrusium, and chicken (Brosch et al., 1995).

Current research is aimed at understanding how the inhibition of HD activity promotes the infection of maize by C. carbonum race 1. Whereas other HSTs, such as T-toxin and victorin, inhibit central metabolic processes in sensitive tissues, leading to cell death, the process of histone acetylation/deacetylation is not well enough understood to be able to predict why inhibition of HD should cause disease susceptibility. Studies using yeast histone mutants and inhibitors of HD indicate that interference with histone acetylation is not lethal to cells but does perturb normal embryo development and normal gene regulation (Brosch et al., 1995). Most studies have concluded that actively transcribed chromatin is more highly acetylated than inactive chromatin, suggesting that inhibition of HD might lead to higher (but not necessarily normal) gene expression. One possible explanation for the action of
HC-toxin is that it permits infection to proceed by interfering with the induction of maize defense genes, such as those encoding pathogenesis-related proteins or cell wall-strengthening proteins (Figure 4; Brosch et al., 1995; Walton et al., 1996). From this perspective, HC-toxin could be called a suppressor rather than a toxin (Walton and Panaccione, 1993).

**Alternaria HSTs**

Based on ultrastructural and physiological studies, AF-, ACT-, and ACTG-toxin act at the plasma membrane, whereas ACR- and AT-toxin affect mitochondria (Otani et al., 1995). However, precise sites of action of these HSTs are not known. Tomato plants of genotype asc/asc are sensitive to AAL-toxin and also to the chemically related mycotoxin fumonisin, which is made by the unrelated pathogen *F. moniliforme*. Tomato plants of genotype Asc/Asc are less sensitive to both compounds. *F. moniliforme* is a pathogen of maize (against which fumonisin is nonselectively toxic) and not tomato. Fumonisin and AAL-toxin are also toxic to mammalian cells (Wang et al., 1996a, 1996b). Positional cloning of Asc is in progress (Vanderbiezen et al., 1996).

Fumonisin and AAL-toxin are analogs of a sphingosine

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**Figure 4.** Model for the Interaction between *C. carbonum* Race 1 (Tox2⁺) and Maize.

Elicitors indicate hypothetical, omnipresent, and nonselective fungal components that trigger plant defense responses. The defense genes, proteins, and chemicals that are actually important for the establishment of resistance in the *C. carbonum*–maize interaction are not known. HCTR, HC-toxin reductase.
precursor and inhibit ceramide synthase in several animal systems. The enzymes from tomato plants of genotype AsclAsc and ascasc are equally sensitive to AAL-toxin and fumonisin, and therefore, inhibition of ceramide synthase is not the basis of host selectivity (Gilchrist et al., 1995; D.G. Gilchrist, personal communication). The response of plant and animal cells to AAL-toxin, and also to KCN, arachidonic acid (a nonselective elicitor from Phytophthora infestans), and heat shock, resembles the process of apoptosis or programmed cell death (Wang et al., 1996a, 1996b; see Dangl et al., 1996, in this issue).

HC- and AAL-toxin have three factors in common. First, both HSTs are biologically active against nonhost organisms but not against other genotypes of the host plant; second, unrelated fungi make compounds that are chemically and pharmacologically similar to the HST; and third, these unrelated fungi either are not plant pathogens or are pathogens of species only distantly related to the host of the HST-producing fungus. Clearly, synthesis of a compound with host-selective toxic activity is not sufficient to make a nonpathogen into a pathogen; even HST-producing organisms need additional traits to be successful pathogens. The situation with AAL- and HC-toxin is in contrast to the situation with T- and PM-toxin, in which two unrelated fungi that produce chemically related compounds do have the same host range.

Other HSTs

A single semidominant gene in sorghum controls sensitivity to peritoxin and susceptibility to the producing fungus P. circinata. The toxic effects of peritoxin are prevented by pretreating cells or tissues with inhibitors of RNA and protein synthesis. This phenomenon suggests either that toxicity requires de novo protein synthesis or that it requires the presence of a protein with a high turnover rate, perhaps the protein to which peritoxin binds (Dunkle and Macko, 1995). A report of the identification and purification of a plasma membrane–localized HS-toxin (also known as helminthosporide) binding protein from sensitive sugarcane was not substantiated in key respects by later studies (Strobel, 1973; Lesney et al., 1982).

BIOCHEMISTRY, GENETICS, AND MOLECULAR BIOLOGY OF HST PRODUCTION

In the three species of Cochliobolus that have been genetically studied, production of their respective HSTs is under the control of a single locus in each species. For example, half of the progeny of a cross between C. heterostrophus race T (Toxl+; produces toxin) and C. heterostrophus race O (Toxl-; does not produce T-toxin) produce toxin and half do not (all known HST-producing fungi are haploid). The same is true for crosses between Tox2+ and Tox2- isolates of C. carbonum. Furthermore, in crosses between strains of C. victoriae that make victorin and strains of C. carbonum that make HC-toxin, one-quarter of the progeny produce HC-toxin (and infect maize only), one-quarter produce victorin (and infect oats only), one-quarter produce neither toxin (and infect neither plant), and one-quarter produce both toxins (and infect both plants). The toxin genes of C. heterostrophus, C. carbonum, and C. victoriae are called TOX1, TOX2, and TOX3, respectively (Walton and Panaccione, 1993). In recent years, major progress has been made toward understanding the nature of the TOX loci in Cochliobolus.

The study of HST biosynthesis can contribute to an understanding of the molecular basis of the evolution of new races, a phenomenon of major importance to plant breeders and growers. New races of pathogens are continually emerging, and the history of the appearance and spread of C. heterostrophus race T is particularly well documented. Before 1960, Tcms did not exist except as the occasional mutant plant; yet within 10 years of its widespread adoption in maize production, C. heterostrophus race T with strong specificity for Tcms had emerged as a major disease. An advantage of using the HST-producing species of Cochliobolus as model systems for the study of the evolution of new races of pathogens is that the critical evolutionary events are understood in molecular terms (Turgeon et al., 1995).

T-Toxin

The TOX1 locus of C. heterostrophus race T is well characterized genetically. Studies of TOX1 have been facilitated by the design of an assay for T-toxin using E. coli cells expressing T-urf13 from Tcms maize mitochondria (Dewey et al., 1988). Identification of TOX1 mutants has been further aided by expressing T-urf13 in C. heterostrophus race T (Toxl+) behind an inducible promoter. Upon induction of T-urf13 expression, only fungal isolates that cannot make T-toxin survive because the producers kill themselves (Yang et al., 1994).

TOX1 is tightly linked to a chromosomal translocation breakpoint (Tzeng et al., 1992; Chang and Bronson, 1996), but the significance of this fact to T-toxin synthesis and the evolution of race T is not yet known. In addition, Toxl+ isolates appear to have 1.2 Mb of DNA that is linked to TOX1 and absent in Toxl- isolates (Chang and Bronson, 1996). Using restriction enzyme–mediated integration mutagenesis, researchers cloned Tox1A, a gene that maps at the translocation and is required for T-toxin biosynthesis (Lu et al., 1994; Turgeon et al., 1995). The DNA of Tox1A is absent from Toxl- isolates of C. heterostrophus. The predicted product of the 78-kb open reading frame of Tox1A is a polyketide synthase whose most logical function is to biosynthesize T-toxin (Figure 2C).

Another gene required for T-toxin biosynthesis, Tox1B, has also been found. Surprisingly, it is not linked to Tox1A but is located on the other translocated chromosome of Toxl+ strains. Tox1B, which like Tox1A is absent in natural Toxl- isolates, encodes a putative decarboxylase. Condensation of acetate during the biosynthesis of polyketides creates an aliphatic acid with an even number of carbons. Because the T-toxin family consists of neutral compounds with an odd
number of carbons, a logical step in T-toxin synthesis is decarboxylation. Therefore, a plausible function for the product of this gene is to decarboxylate the product of the ToxA protein (Turgeon et al., 1995; O.C. Yoder, personal communication). The linkage of ToxA and ToxB (i.e., their appearance as a single locus, TOX1) in crosses between Tox1+ and Tox1− isolates is due to the translocation; in crosses between two isolates that are homozygous for the translocation, they segregate as two unlinked loci, both of which are necessary for T-toxin biosynthesis.

**HC-Toxin**

HC-toxin is the only HST whose biosynthetic enzymes have been studied. Two enzymes, which were later shown to be part of a single 570-kD polypeptide called HC-toxin synthetase (HTS), activate L-proline, L-alanine, and D-alanine by the ATP/PPi exchange reaction common to nonribosomal peptide synthetases (Walton and Holden, 1988). HTS also catalyzes the formation of amino acyl thioesters with these amino acids and epimerizes L-proline and L-alanine to the respective D-isomers. HTS activities are present only in HC-toxin—producing (Tox2+) isolates of *C. carbonum*, a finding explained by the subsequent discovery that Tox2− isolates lack the gene encoding HTS (see below). HTS has four amino acid–activating domains; therefore, by comparison with other peptide synthetases, it probably also activates Aeo or an Aeo derivative (Figure 2B) as well as polymerizing and cyclizing the tetrapeptide (Scott-Craig et al., 1992).

Like T-toxin, production of HC-toxin by *C. carbonum* appears to be under the control of a single genetic locus, TOX2. The molecular genetic structure of TOX2 was approached by cloning the gene, called HTS1, that encodes HTS. HTS1 is part of a 22-kb region of contiguous DNA that is present only in Tox2+ isolates and that segregates genetically with HC-toxin production (Panaccione et al., 1992). The 22-kb region encodes two genes, HTS1 and TOXA. HTS1 has no introns in its 15.7-kb open reading frame (Scott-Craig et al., 1992); TOXA, which is just upstream of HTS1, has three introns and encodes a 2.1-kb mRNA. TOXA and HTS1 are transcribed in opposite directions, and their transcriptional start sites are 366 bp apart (Pitkin et al., 1996). The predicted product of TOX2 is a hydrophobic protein with strong similarity to transport proteins of the major facilitator superfamily, which includes antibiotic, sugar, and organic acid efflux pumps (Margar and Saier, 1993). A plausible function for the TOX2 product is an HC-toxin efflux pump that serves to protect *C. carbonum* against its own toxin (Pitkin et al., 1996; Walton et al., 1996).

HTS1 and TOXA are present in two functional copies in most Tox2+ strains (Panaccione et al., 1992; Pitkin et al., 1996). Disruption of both copies of HTS1 is necessary to cause loss of HC-toxin production, HTS activity, and specific pathogenicity (Panaccione et al., 1992). Just as ToxA1 and ToxB1 are absent in Tox1− isolates of *C. heterostrophus*, HTS1 and TOXA are absent in Tox2− isolates of *C. carbonum*. Another gene required for HC-toxin synthesis, TOXC, was found by analyzing novel regions of DNA that are present only in Tox2+ isolates. TOXC has a 6.5-kb open reading frame, is present in most Tox2+ isolates in three copies, and encodes a protein highly similar to the β subunit of fatty acid synthases from yeast and other filamentous fungi (J.-H. Ahn and J.D. Walton, manuscript submitted). The most plausible role for the product of TOXC is that it contributes to the synthesis of the decanoic acid backbone of the epoxy-containing amino acid Aeo of HC-toxin (Figure 2B). It had earlier been shown that Aeo is synthesized from acetate (Wessel et al., 1988).

Because the genes for HTS1, TOXA, and TOXC are restricted to Tox2+ isolates, it seemed possible that *Tox2+* isolates had evolved from a *Tox2−* isolate through the acquisition of the HC-toxin biosynthetic genes by horizontal gene transfer. PCR primers based on HTS1 were used to amplify cyclic peptide synthetase domains from the other fungi that make Aeo-containing cyclic peptides (Walton et al., 1996), but none of the domains found was closely related to that of HTS1. Therefore, if Tox2+ races of *C. carbonum* did evolve by horizontal gene transfer of the HC-toxin biosynthetic genes, it did not happen in the recent evolutionary past (Nikolskaya et al., 1995). Furthermore, the complex arrangement of the TOX2 genes does not support the hypothesis of recent horizontal gene transfer (see below; Ahn and Walton, 1996).

If HC-toxin biosynthesis requires three genes, each of which is duplicated, why does this trait appear to be controlled by a single gene, TOX2? Mapping by pulsed field gel electrophoresis indicates that all copies of TOXA, HTS1, and TOXC are on the same chromosome. In the laboratory strain SB111, this chromosome, at 35 Mb, is the largest of the ~13 chromosomes. The chromosomal locations of the two copies of HTS1 and the three copies of TOXC are distributed over 540 kb (Figure 5). Therefore, despite the apparent simplicity of its pattern of inheritance, TOX2 is a large and complex entity. It contains, at a minimum, multiple copies of three genes, some of which encode large, multifunctional enzymes, some of which are clustered, and all of which are physically linked. Furthermore, crossing over between the two copies of HTS1 is suppressed (Pitkin et al., 1996). The pattern of the organization of the genes of TOX2 does not suggest a simple mechanism by which it might have evolved (Ahn and Walton, 1996).

There are probably additional enzymes, and hence genes, required for HC-toxin biosynthesis. The structure of Aeo and the presence of TOXC suggest that Tox2− isolates probably also have a gene encoding a homolog of the α subunit of fatty acid synthase. Furthermore, several lines of evidence indicate that there is probably a positive activator gene necessary for full expression of TOXA and HTS1 (Walton et al., 1996). Weiergang et al. (1996) showed that HC-toxin production is developmentally regulated during infection. Mapping studies have tentatively placed the putative regulatory gene within 100 kb of copy 1 of HTS1 (Figure 5; J.W. Pitkin, A.N. Nikolskaya, and J.D. Walton, unpublished results).

TOX1 of *C. heterostrophus* and TOX2 of *C. carbonum* have interesting parallels and differences. Both TOX1 and TOX2 en-
code multiple biosynthetic genes required for synthesis of their respective HSTs. In both, the genes are absent in natural Tox− isolates. TOX1 and TOX2 differ in that the genes of TOX2 are duplicated, whereas those in TOX1 are not. Although the known genes of TOX2 are all on the same chromosome, the genes of TOX1 are actually on separate chromosomes and cosegregate because they are linked to a translocation.

Other HSTs

Mendelian and molecular genetic analyses of victorin production in C. victoriae are in progress (Turgeon et al., 1995). Presumably, a cyclic peptide synthetase similar to HTS is involved in the biosynthesis of victorin. However, because the normal 20 proteinogenic amino acids are not components of victorin, it is likely that several other enzymes are also required for its synthesis. A cyclic peptide synthetase gene that is unique to AM-toxin-producing strains of the A. alternata apple pathotype has been identified (K. Kohmoto, personal communication). HST production is not amenable to classic genetic analysis in any species of Alternaria, B. sacchari, or B. circinata because these fungi lack a known sexual stage. Nevertheless, the development of molecular genetic techniques for these fungi should facilitate future analysis of their HST genes (Tsuge et al., 1990). Production of Ptrl-toxin is presumably controlled by a single gene, because the toxin is a single polypeptide. Cloning of this gene is in progress (Tuori et al., 1995).

HSTs IN RELATION TO OTHER DETERMINANTS OF DISEASE SPECIFICITY

Although HSTs were for many years the only known agents of disease specificity, they are not central to most of the models of specificity that have been proposed over the years (Walton and Panaccione, 1993). There are several reasons for this. First, specificity in many pathogens is under monogenic control, and it has been generally assumed that secondary metabolites, such as most HSTs, would require multiple genes for their biosynthesis. However, multifunctional enzymes, gene clustering, and suppression of crossing over are characteristics of known HST biosynthetic pathways by which the synthesis of complex molecules can appear to segregate as single loci.

Second, HSTs are clearly positive-acting agents of virulence, whereas in many disease interactions, avirulence is genetically dominant to virulence. This has focused attention on a search for host-selective elicitors, defined as pathogen-derived, positive-acting chemical inducers of resistance, rather than as toxins. However, like most toxins, most elicitors kill plant cells and are nonselective. One of the few known host-selective elicitors is syringolide, a secondary metabolite whose synthesis is controlled by a single gene in Pseudomonas syringae (Keen et al., 1990). Furthermore, AAL-toxin has been proposed to induce apoptosis, a process under complex genetic and biochemical control that has also been proposed to underlie the resistance-associated and elicitor-induced hypersensitive response (see Dangl et al., 1996, in this issue).

Third, resistance and susceptibility in at least some diseases are cell autonomous, but HSTs, being typically small molecules, should diffuse and therefore affect nearby cells. However, there is now an example of a proteinaceous HST (Ptl-toxin; molecular mass of 13.2 kD), several examples of elicitors that are low molecular weight (e.g., the avr9 peptide of Cladosporium fulvum and syringolide of P. syringae), and several examples of elicitor-active proteins that can move readily in plants (e.g., a 22-kD fungal xylanase; Bailey et al., 1993).

For most of the toxic compounds produced by plant pathogenic organisms, there is no or only incomplete information about their involvement, if any, in promoting the development of a compatible or incompatible interaction. It is therefore arbitrary whether these compounds a priori should be called elicitors or toxins. Because cell death is a characteristic of both
compatibility and incompatibility, it is not by itself a very informative phenotype. A challenge to researchers is to establish why, if both classes of compound cause a similar kind of cell death, HSTs are agents of compatibility, whereas elicitors are agents of incompatibility (Walton and Panaccione, 1993; Wang et al., 1996a).

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