

REVIEW

# Virulence Genes and the Evolution of Host Specificity in Plant-Pathogenic Fungi

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**In the fungal kingdom, the ability to cause disease in plants appears to have arisen multiple times during evolution. In many cases, the ability to infect particular plant species depends on specific genes that distinguish virulent fungi from their sometimes closely related nonvirulent relatives. These genes encode host-determining “virulence factors,” including small, secreted proteins and enzymes involved in the synthesis of toxins. These virulence factors typically are involved in evolutionary arms races between plants and pathogens. We briefly summarize current knowledge of these virulence factors from several fungal species in terms of function, phylogenetic distribution, sequence variation, and genomic location. Second, we address some issues that are relevant to the evolution of virulence in fungi toward plants; in particular, horizontal gene transfer and the genomic organization of virulence genes.**

Although the fungal kingdom has proven to be fertile ground for the emergence of plant pathogens, most fungi are not pathogenic toward plants. Also, plant-pathogenic fungal species are not evenly distributed phylogenetically across the fungal kingdom (Berbee 2001). This implies that the ability to colonize and adversely affect living plants arose multiple times during evolution. This ability tends to be host specific because most plant-pathogenic fungi have a limited host range. What works on one plant species apparently does not work on others.

To advance from these observations to a thorough understanding of the mechanisms and evolution of fungal plant pathogenicity, it is essential to identify the genes underlying that “special adaptive ability” that distinguishes virulent fungi from their sometimes closely related nonvirulent relatives. Many genes have now been identified in plant-pathogenic fungi that are required for pathogenicity (Baldwin et al. 2006). In this review, we will not address genes that have apparent orthologs in most or all fungal species, including nonpathogens, even when they are indispensable for the ability to infect. Instead, we will concentrate on genes that meet the following criteria: i) they were shown to be involved in virulence but not in any other process and ii) they have a limited or “patchy” phylogenetic distribution. This leaves a rather small set of genes that mainly encode small, secreted proteins and enzymes involved in synthesis of specific toxins. Henceforth, we will refer to these genes as “virulence genes.” We also include a few genes that only meet criterion 2 (i.e., without a demonstrated role in virulence) but

nevertheless are of value for the evolutionary processes under discussion.

## Scenarios of evolution.

One explanation for the limited phylogenetic distribution of virulence genes could be that they have an unusually high mutation rate, which would make it difficult to recognize orthologs. Such a high mutation rate can result from an arms race between a pathogen and its host plant involving virulence factors (from the pathogen) and their targets in the host (Maor and Shirasu 2005). Disease pressure will favor changes in host proteins that are a target for fungal attack, to neutralize the effect of virulence factors from the pathogen. The pathogen, on the other hand, will be pressured to keep updating its virulence factors, to continue to be able to attack the changing targets. The result is accelerated evolution of virulence genes in pathogens and virulence targets in plants. A second type of arms race occurs between plant resistance (*R*) genes and pathogen avirulence genes (Jones and Dangl 2006; Stahl and Bishop 2000). Some of the proteins that are produced by a pathogen during infection can be directly or indirectly recognized by the products of *R* genes, resulting in disease resistance. In such cases, the gene of the pathogen encoding the protein that is recognized is called an avirulence gene. Selection pressure on the pathogen population will favor escape of host recognition, whereas selection on the plant population will favor early recognition of the pathogen. In this way, mutation rates of avirulence genes and *R* genes accelerate relative to genes not involved in the arms race. For pathogens, consequences of these arms races can be i) loss of potential recognition targets, such as genes for secreted proteins or compounds without an obvious contribution to fitness, and ii) high mutation rates of secreted proteins involved in virulence, as a way to keep engaging their targets while escaping *R* genes in the plant host population.

Thus, both gene loss and high mutation rate may have resulted in the limited phylogenetic distribution of genes encoding secreted virulence factors. On the other hand, gene loss and mutation may be countered by gene gain through horizontal gene transfer (HGT), which would also contribute to a patchy distribution. HGT may occur either between species or among the different, vegetatively incompatible, clonal lines of an asexual species. More than one gene may be transferred in one HGT event, if these are present in clusters or on the same chromosome. Such clusters then would be analogous to pathogenicity islands of bacteria (Hacker and Kaper 2000). HGT could be very relevant for the evolution of fungal pathogenicity because receipt of a new virulence gene or virulence gene cluster could turn the recipient into a novel pathogen or allow

an existing pathogen to infect a new host. It also offers an alternative explanation (as opposed to gene loss) for the patchy distribution of virulence genes.

To assess to what extent each of the above mechanisms may have contributed to the evolution of fungal pathogenicity toward plants, current knowledge concerning virulence genes of several fungal species will be discussed in the following sections, with a focus on clues regarding their origin and evolution.

### PEP and PDA genes in *Nectria haematococca*.

In *Nectria haematococca* (anamorph *Fusarium solani*), the genes that are required for pathogenicity toward pea are located on a 1.6-Mb conditionally dispensable (CD) chromosome (Han et al. 2001). Similar to pathogenicity islands in bacteria, this CD chromosome contains genes contributing to virulence, a high density of transposable elements, and a different GC content compared with the rest of the genome, suggesting HGT from a species different from *Nectria* as a mode of dispersal for this chromosome (Liu et al. 2003; Temporini and VanEtten 2004). The virulence genes *Pea pathogenicity (PEP)1*, *PEP2*, and *PEP5* en *Pisatin demethylase (PDA) 1* are located within 25 kb of each other on the CD chromosome and all enhance virulence toward pea when transformed (individually) to a strain lacking the CD chromosome (Han et al. 2001). The function of the predicted Pep1 and Pep2 proteins is unknown; however, there is a homolog of *PEP1* in the plant-pathogenic fungus *F. graminearum*, whereas homologs of *PEP2* are present in many fungal species (H. VanEtten, *personal communication*). *PEP5* encodes a putative multidrug transporter, while *PDA1* encodes a pisatin demethylase that detoxifies the phytoalexin pisatin produced in the roots of pea. Several close homologs of *PDA1* have been found in *N. haematococca*. Most of these also are located on a 1.6-Mb CD chromosome (Funnell and VanEtten 2002), whereas two are located on a portion of a larger chromosome that also can be lost during meiosis (Funnell et al. 2002). Southern blot experiments have revealed that close homologs of the *PEP* and *PDA* genes are absent in several related species, except for *Neocosmospora boniensis* and several host-specific forms of the more distantly related species *F. oxysporum* (Temporini and VanEtten 2004). In *N. boniensis*, homologs of all genes are present whereas, in *F. oxysporum*, *PDA1* was present in only some isolates. Both *N. boniensis* and one of the isolates of *F. oxysporum* containing *PDA1* are able to infect pea (Temporini and VanEtten 2004). More genes involved in virulence toward pea may be present on the CD chromosome; therefore, the current ongoing analysis of this chromosome (H. VanEtten, *personal communication*) is highly interesting.

### SIX genes in *F. oxysporum*.

Several in planta-secreted proteins have been identified in *F. oxysporum* f. sp. *lycopersici*, the causal agent of tomato wilt disease. The first to be identified, secreted in xylem 1 (Six1), is a small cysteine-rich protein that contributes to virulence (Rep et al. 2004, 2005b). The *SIX1* gene is also the avirulence gene that matches the *I-3* resistance gene in tomato, and is located on one of the smallest chromosomes (approximately 2

Mb) (Rep et al. 2004). *SIX2*, of which the product is also secreted during infection, is located within 8 kb of *SIX1* (Houterman et al. 2007). In addition to Six1 and Six2, eight more fungal proteins were identified from xylem sap of infected plants, and the genes of at least six of these lie on the same chromosome as *SIX1* and *SIX2* (Houterman et al. 2007) (P. M. Houterman and M. Rep, *unpublished results*). This chromosome also harbors a homolog of *SIX1*, called *SIX1-H*, which is truncated by the insertion of a transposon (Rep et al. 2005a). Interestingly, even though *SIX1-H* is a pseudogene, there is evidence for diversifying selection having acted on *SIX1* and *SIX1-H* (presumably before inactivation of *SIX1-H* by insertion of the transposon): a higher number of single nucleotide polymorphisms in coding relative to noncoding sequences, preservation of the reading frames, and a high ratio of nonsynonymous over synonymous changes (Table 1).

No homologs of any *SIX* gene were found in databases, except for *SIX2*, of which a homolog is present in the closely related *F. verticillioides*. Although, as a species, *F. oxysporum* has a very broad host range, single isolates of this asexual fungus usually infect only one or a few plant species. Therefore, isolates have been grouped into formae speciales based on host specificity (Gordon and Martyn 1997). *SIX1*, *SIX2*, and an intervening gene, encoding a salicylate hydroxylase homolog (*SHH1*), were found to be present in all f. sp. *lycopersici* isolates but not in other formae speciales or nonpathogenic isolates. Interestingly, the f. sp. *lycopersici* isolates do not have a common ancestor within *F. oxysporum*, but appear mixed with isolates from other formae speciales in phylogenetic analyses (O'Donnell et al. 1998; H. C. van der Does and M. Rep, *unpublished observations*). One explanation for this is that the genomic region encompassing *SIX1* was present in ancestral *F. oxysporum* and subsequently lost in all clonal lines except a few that are now recognized as f. sp. *lycopersici*. However, the almost complete lack of polymorphisms in this region—in contrast to other sequences—favors the possibility of recent horizontal transfer of this region between clonal lines (H. C. van der Does and M. Rep, *unpublished observations*). Either way, an unresolved question is how the genomic region containing the *SIX* genes evolved in the first place and whether that was in *F. oxysporum* or in another species, subsequently arriving in *F. oxysporum* through HGT.

### Genes for necrosis-inducing proteins

#### in *Rhynchosporium secalis* and *Pyrenophora tritici-repentis*.

From the barley pathogen *Rhynchosporium secalis*, three secreted proteins (NIP1, NIP2, and NIP3) have been isolated that are capable of causing necrosis in several plant species (Wevelsiep et al. 1991). NIP1 is also an avirulence factor, recognized by the *Rrs1* resistance gene in barley (Rohe et al. 1995). Different populations of *R. secalis* have been analyzed

**Table 1.** Evidence for diversifying selection acting on *SIX1* and *SIX1-H*

Region	Length <sup>b</sup>	Identity	Indels <sup>c</sup>	SNP <sup>a</sup>		
				Total	Syn.	Nonsyn.
Coding	560	91%	0	49 (8.8%)	5 (0/0/5) <sup>d</sup>	44 (23/15/6)
3' Non-coding	836 <sup>e</sup>	93%	8	51 (6.1%)	...	...

<sup>a</sup>Single nucleotide polymorphisms. Syn = synonymous, Nonsyn = nonsynonymous.

<sup>b</sup>Length in nucleotides; includes only the part of *SIX1* that could be aligned with *SIX1-H*.

<sup>c</sup>Insertions or deletions.

<sup>d</sup>Positions in codon in parenthesis.

<sup>e</sup>Excluding insertions; *SIX1* 3' noncoding is 853 bp and *SIX1-H* 3' noncoding is 841 bp.



for the occurrence of *NIP1*, *NIP2*, and *NIP3* as well as for mutations in the *NIP1* gene (Schurch et al. 2004). Gene deletion was independent among the three genes and, in many populations, isolates were present either containing or lacking *NIP1* or *NIP2*. This co-existence implies that loss of these genes does not cause a major virulence penalty. In contrast, *NIP3* was present in all but two isolates and its deletion reduced virulence (Schurch et al. 2004). For *NIP1*, strong indications for diversifying selection were found, despite its hardly detectable contribution to virulence and the fact that only few of the point mutations in *NIP1* are associated with loss of avirulence (Schurch et al. 2004). Thus, a satisfactory explanation for the apparent diversifying selection on *NIP1* is still missing.

*Pyrenophora tritici-repentis* causes tan spot disease on wheat and also produces necrosis-inducing proteins. These are host specific and, therefore, were called host-selective toxins (HSTs). ToxA was the first HST from this species to be identified. *TOXA* alone is sufficient to make a nonpathogenic isolate of *P. tritici-repentis* pathogenic on wheat (Ciuffetti et al. 1997). A related wheat-infecting fungus, *Stagonospora nodorum*, recently was found to harbor a close homolog of the *TOXA* gene (Friesen et al. 2006). Strikingly, other species, more closely related to *P. tritici-repentis* but with a different host range, do not have *TOXA* sequences, and the two *TOXA* sequences of *P. tritici-repentis* and *S. nodorum* are more similar than can be expected based on the rest of their respective genomes (Friesen et al. 2006). The shared region encompasses 11 kb, includes *TOXA* and a transposase gene, and is bordered by repetitive sequences in *S. nodorum*. Together, these observations strongly suggest HGT. The direction of this HGT is likely from *S. nodorum* to *P. tritici-repentis* because, in the former, 11 polymorphisms in *TOXA* were found and, in the latter, only 1. This is also in agreement with the relatively recent emergence of tan spot caused by *P. tritici-repentis* (Friesen et al. 2006). In the proposed recipient of *TOXA*, *P. tritici-repentis*, the gene does not seem to be present on a CD chromosome (Lichter et al. 2002).

Another HST gene of *P. tritici-repentis*, *TOXB*, is present in multiple identical copies in the genome (Martinez et al. 2004; Strelkov et al. 2002). The level of virulence of the fungus was suggested to correlate with the number of copies of *TOXB* (Strelkov et al. 2002). Each copy is close to a retrotransposon-like sequence and all are located on a 2.7-Mb chromosome and a 3.5-Mb chromosome (Martinez et al. 2004). Occasionally, in nonpathogenic isolates, one homologous locus, *toxh*, is present, of which the predicted mature gene product is 81% identical to *TOXB* (Martinez et al. 2004). In contrast to *TOXA*, sequences homologous to *TOXB* are present in a number of related fungi, from highly similar sequences in the close relative *P. bromi* to a distant homolog in *Magnaporthe oryzae* (L. Ciuffetti, *personal communication*), suggesting vertical transmission from an ancient common ancestor instead of HGT.

#### Small secreted proteins of *Cladosporium fulvum*.

The tomato leaf mold fungus *Cladosporium fulvum* (syn. *Passalora fulva*) secretes several small proteins into the apoplast of its host (Thomma et al. 2005). Some of these trigger *R*-gene-mediated resistance and are called avirulence (Avr) proteins, whereas others with initially no matching *R* gene in cultivated tomato were called extracellular proteins (Ecps). No close homologs have been found in any other organism for any of the four Avrs (*Avr2*, *Avr4*, *Avr4E*, and *Avr9*) and four Ecps (*Ecp1*, -2, -4, and -5), although distant relatives of *Ecp2* are present in the genomes of several ascomycetes (M. Rep, *unpublished observations*). *Ecp1* and *Ecp2* are required for full virulence (Laugé et al., 1997), and some of the Avrs also contribute to virulence, as demonstrated recently with RNAi-

silencing experiments (B. Thomma, *personal communication*). Although thought to reproduce mainly asexually, recent population genetic analysis suggests that recombination does occur in *C. fulvum* (Stergiopoulos et al. 2006). The *Ecp* genes are present in all isolates of a worldwide collection and show very little variation on the protein level (Thomma et al. 2005; P. de Wit, *personal communication*). In contrast, much more variation exists in the four identified *Avr* genes. In isolates that are virulent on plants carrying the resistance gene *Hcr9-4E*, the matching *Avr4E* gene was either absent or contained the same two point mutations, both leading to amino acid changes. All avirulent strains investigated had identical *Avr4E* sequences (Westerink et al. 2004). For *Avr9*, only deletion of the entire gene has been found in isolates that are virulent on plants carrying the matching resistance gene *Cf-9* (van Kan et al. 1991). In contrast to *Avr4E* and *Avr9*, functions have been assigned to the gene products of *Avr2* and *Avr4*. *Avr2* is an inhibitor of the plant apoplastic protease Rcr3 (Rooney et al. 2005). Again, no variation was found in the *Avr2* gene in avirulent isolates whereas, in 28 virulent isolates, five different insertions or deletions (polymorphism in the number of basepairs), one non-sense mutation, and one retrotransposon insertion were found (Luderer et al. 2002). *Avr4* is a chitin-binding protein that protects fungi against lytic activity of plant chitinases (van den Burg et al. 2006). In isolates virulent on *Cf-4* plants, various single point mutations were found in the *Avr4* gene, but no large rearrangements or deletions (Joosten et al. 1997).

Taken together, sequence variation thus far has been observed only in *Avr* genes of isolates that are virulent on tomato lines carrying the matching *R* gene, with all mutations leading to amino acid changes. Therefore, it is very likely that these variations arose quite recently and are under positive selection in agricultural settings (P. de Wit, *personal communication*). The genomic context (clustering of virulence genes, enrichment in transposons, and so on) of the *Ecp* and *Avr* genes of *Cladosporium* spp. has not yet been elucidated.

#### Gene clusters for secreted proteins in *Ustilago maydis*.

In the corn smut fungus *Ustilago maydis*, a basidiomycete, much progress has been made recently in the identification of gene clusters for secreted proteins whose genes are upregulated during infection. The genes in the *Maize induced gene (Mig) 2* cluster are related to each other and encode secreted proteins of unknown function. An additional homolog, *Mig2-6*, resides at another locus. Deletion of all six *Mig* genes does not result in an alteration of virulence, as measured by the ability to form tumors (Basse et al. 2002; Farfings et al. 2005). The five *Mig2* genes in the cluster are flanked by two direct repeats of 138 and 139 bp. The cluster is rearranged in some strains



and is absent in the related *U. scitaminea*, which is pathogenic on sugar cane (Basse et al. 2002). More recently, analysis of the complete genome sequence of *U. maydis* has led to the discovery that 79 (18.6%) of the genes for secreted proteins are organized in 12 clusters of 3 to 26 genes, scattered throughout the genome (Kamper et al. 2006). Compared with flanking genes, a significantly high proportion of clustered genes (50 to 60% of the genes) is upregulated in tumor tissue (in planta). Four clusters are required for virulence whereas deletion of one cluster enhances virulence. Only 7 of the 79 clustered genes have a suggested function, of which one is a (disrupted) transposase. Of the proteins without suggested function, only two are conserved in other species, while the rest are specific for *U. maydis*. The *U. maydis* genome as a whole does not show signs of large-scale duplication events and has a low number of transposable elements (Kamper et al. 2006). This remarkable organization of in planta expressed (virulence) genes in dispersed clusters has not yet been found in other fungi.

#### Avirulence genes in *M. oryzae*.

For several avirulence genes of the rice blast fungus *M. oryzae* (teleomorph *Pyricularia oryzae*), a contribution to virulence on some hosts or under some circumstances is suspected but not yet demonstrated. Nevertheless, their inverse role in virulence (namely avirulence), including the role of some of them in restricting virulence on nonhost plants, makes their study relevant for the understanding of the evolution of (host-specific) virulence of this fungus.

Although *M. oryzae* isolates from different host plants can be crossed in the laboratory, field isolates that are pathogenic to rice constitute a single lineage. This lineage probably originated during early rice domestication in Asia and subsequently diversified through predominantly clonal reproduction (Couch et al. 2005). The *AVR1-CO39* gene, which matches the *R* gene *Co39* (Farman et al. 2002), occurs only in *M. oryzae*, not in its sister species (Tosa et al. 2005). Remarkably, within *M. oryzae* isolates, *AVR1-CO39* is absent in most isolates that infect rice (Couch et al. 2005; Tosa et al. 2005) due to one of two types of genomic deletions and rearrangements that are associated with repetitive elements (Farman et al. 2002; Tosa et al. 2005). Evidently, *AVR1-CO39* is not required for virulence on rice, but its common presence in isolates that can infect other grasses, with conservation of avirulence activity when conferred to rice isolates, suggests a positive role in fitness for reproduction on some grass species (Peyyala and Farman 2006; Tosa et al. 2005). Its loss early in the lineage leading to current rice-blast isolates may have resulted from early exposure to presently rare *Co39*-containing rice (Tosa et al. 2005).

A similar situation has been observed for the *pathogenicity on weeping lovegrass (PWL)1-4* genes, two of which confer avirulence toward weeping lovegrass (Kang et al. 1995; Sweigard et al. 1995). *PWL* genes are predicted to encode small secreted proteins. In the laboratory, *PWL2* is easily lost due to spontaneous deletions in the order of 30 kb (Sweigard et al. 1995). All four *PWL* genes are close to repetitive elements, and *PWL1* is located close to a telomere (Kang et al. 1995). *PWL1* and *PWL2*, which are 75% identical at the amino acid level, confer avirulence toward weeping lovegrass, whereas *PWL4* (50% identical to *PWL2*) does so only when its expression is driven by the promoter of *PWL1* or *PWL2* (Kang et al. 1995). In contrast, *PWL3*, which is allelic to *PWL4*, is not functional. Interestingly, similarity between noncoding sequences upstream and downstream of *PWL3* and *PWL4* is higher (>98% identity) than between the open reading frames (85% identity). Moreover, between all four *PWL* genes, amino acid identity is lower than DNA sequence identity, indicative of diversifying selection (Kang et al. 1995). This, together with the near ubiquitous

presence and amplification of *PWL* genes in *M. oryzae*, strongly suggests some role in fitness, despite the absence of *PWL1* and *PWL2* in isolates that attack weeping lovegrass (Kang et al. 1995; Sweigard et al. 1995).

Another avirulence gene, *AVR-Pita*, encodes a putative metalloprotease that may act inside host cells and is recognized by the *Pita R*-gene product (Jia et al. 2000). No close homologs have been found in other fungi. The *M. oryzae* genome sequence (*Magnaporthe grisea* Sequencing Project, Broad Institute of Harvard and MIT) contains several distant relatives of *AVR-Pita*. Distant relatives also are present in the genome sequences of other fungi, including *N. crassa*, but these are more likely to be paralogs due to their low level of similarity. Like *PWL2*, *AVR-Pita* is easily lost under laboratory conditions, mainly due to deletions of 100 bp to >100 kb (Orbach et al. 2000). This high frequency of deletion may be related to its position, only 48 bp from the telomere repeat sequences. Also, insertion of a transposon in the *AVR-Pita* promoter has been reported to result in loss of avirulence (Kang et al. 2001). A similar event occurred in another avirulence gene of *M. oryzae*, *ACE1* (avirulence conferring enzyme 1), in which a composite retrotransposon had inserted in a virulent isolate (Fudal et al. 2005).

#### HST genes of *Alternaria alternata*.

Pathogenic isolates of the asexual fungus *Alternaria alternata* can be grouped in different pathotypes, depending on the host they infect (Thomma 2003). This host specificity is determined by the HSTs they produce. For example, the pear pathotype produces AK-toxin, while the strawberry pathotype and the tangerine pathotype produce AF-toxin and ACT-toxin, respectively. These three toxins are related in structure in that they all have an epoxy-decatrienoic acid backbone (EDT). In contrast, AM-toxin produced by the apple pathotype does not use this precursor but is the product of a nonribosomal peptide synthase.

Several of the genes required for the production of these toxins have been cloned. All of them appear in clusters, and some were shown to reside on CD chromosomes. In the pear pathotype, four genes, *AKT1*, *AKT2*, *AKTR-2*, and *AKT3-2*, were cloned and shown to be required for both AF-toxin production and virulence on pear (Tanaka and Tsuge 2000; Tanaka et al. 1999). All these sequences are located on a 4.1-Mb chromosome, along with multiple nonfunctional *AKT* homologs (Tanaka and Tsuge 2000). Homologs of several of these *AKT* genes were detected in tangerine and strawberry pathotypes, which produce the related ACT-toxin and AF-toxin, but not in other pathotypes or nonpathogenic isolates (Tanaka and Tsuge 2000).

The strawberry pathotype produces AF-toxin I, effective against both pear and strawberry, as well as AF-toxin II, effective only against pear. Consequently, this pathotype can infect both plant species. The genes for AF-toxin production are located on a 1.05-Mb CD chromosome (Hatta et al. 2002). Isolates in which this CD chromosome is lost show diminished synthesis of AF-toxin I and II and the EDT precursor and also are no longer pathogenic on strawberry or pear. The *AFT1-1*, *AFTR-1*, and *AFT3-1* genes are clustered and were cloned based on their homology to genes required for toxin production in the pear pathotype (Hatta et al. 2002). This cluster also contains *AFTS-1*, which is unique to the strawberry pathotype and is required for pathogenicity toward strawberry only and not to pear, because deletion of *AFTS-1* results in specific loss of AF-toxin I production and not AF-toxin II (Ito et al. 2004a). Further analysis of the cluster revealed the presence of additional potential *AFT* genes as well as five disrupted transposable elements, all unique to the CD chromosome (Hatta et al. 2006). At least one of these transposable elements is represented

only in the pear, strawberry, and tangerine pathotypes. Together with the homology between the *AFT* and *AKT* genes, this shared transposon suggests a common origin of the toxin biosynthesis gene clusters of these pathotypes (Hatta et al. 2006).

The genes required for the production AM-toxin in the apple pathotype also are located on a relatively small dispensable chromosome that can vary in size from 1.1 to 1.8 Mb (Johnson et al. 2000; Johnson et al. 2001). Loss of this chromosome results in loss of pathogenicity toward apple and loss of AM-toxin production (Johnson et al. 2001). AM-toxin is a cyclic peptide and requires for its synthesis a nonribosomal peptide synthase encoded by the *AMT* gene. At least one additional copy of *AMT* is present on the same chromosome, but this copy apparently is not functional (Johnson et al. 2000).

*A. alternata* is not known to have a sexual cycle; therefore, different pathotypes are not assumed to recombine meiotically. Moreover, single pathotypes do not form monophyletic groups. This indicates that virulence toward a specific host may have been acquired multiple times (Kusaba and Tsuge 1994, 1995). This, combined with the presence of toxin genes on dispensable chromosomes, suggests that pathogenic clonal lines of *Alternaria* can arise through HGT of CD chromosomes.

### HST genes in *Cochliobolus* spp.

The situation is different for the genes that are required for the production of the host-selective T-toxin in *Cochliobolus heterostrophus*, which is associated with high virulence on certain genotypes of maize (Baker et al. 2006; Rose et al. 2002; Yang et al. 1996). Three genes have been shown to be required for T-toxin production: *PKS1* (Yang et al. 1996), *DECI* (Rose et al. 2002), and *PKS2* (Baker et al. 2006). These genes, together with a fourth gene, *RED1*, that is not required for T-toxin production (Rose et al. 2002), reside in approximately 1.2 Mb of highly repeated, AT-rich DNA which is unique to T-toxin-producing strains (race T) and absent in nonproducing, race O strains. This “extra” DNA in T-toxin-producing isolates resides at the breakpoints of two chromosomes that are reciprocally translocated relative to race O (Baker et al. 2006). Remarkably, apart from *PKS1* and *PKS2*, all 23 other *PKS* genes of *C. heterostrophus* are shared between race T and race O (Baker et al. 2006). Homologs of at least *PKS1*, *DECI*, and *RED1* are absent in other fungal pathogens and these genes have a different codon bias than the rest of the *C. heterostrophus* genome, suggesting that the extra DNA in race T has arrived through HGT, an event that may have initiated the reciprocal translocation of the two chromosomes (Rose et al. 2002; Yang et al. 1996). Interestingly, *PKS1* is orthologous to *MzmPKS1* of *Mycosphaerella zae-maydis*, also a maize pathogen (Baker et al. 2006).

Reciprocal translocation also has been suggested to explain the pattern of distribution of the genes in *C. carbonum* that are required for the synthesis of the cyclic peptide HC-toxin, also an HST for maize (Walton 2006). These genes are located at one locus, called *TOX2*. Seven different genes at this locus are currently known (*TOXA* to *-G*) and all are present multiple times in the genome (Ahn et al. 2002). In some strains, they are located within 600 kb on a 3.5-Mb chromosome, except for one copy of *TOXE* that resides on a 0.7-Mb chromosome (Ahn and Walton 1996; Ahn et al. 2002). Up to approximately 2 Mb of this chromosome is dispensable and meiotically unstable; however, the remaining approximately 1.4 Mb is never lost (Pitkin et al. 2000). This remaining part always contains at least one non-*TOX2* gene, *EXG2*, coding for a glucanase. In other *Tox2<sup>+</sup>* isolates, all *TOX2* genes are located on a 2.2-Mb chromosome, while *EXG2* is located on a 2.0-Mb chromosome (Ahn et al. 2002). All natural *Tox2<sup>-</sup>* isolates, as well as other *Cochliobolus* spp., completely lack the genes for HC-toxin

biosynthesis (Panaccione et al. 1992). Taken together, the chain of events leading to the present location of *TOX2* genes in *Tox2<sup>+</sup>* *C. carbonum* was proposed to be HGT followed by a reciprocal translocation event (Ahn et al. 2002). Either the entire 2.2-Mb chromosome with duplicated *TOX2* genes arrived through HGT or, as proposed by Ahn and associates (2002), a smaller part arrived and duplication occurred subsequently in *C. carbonum*.

### Questions of inheritance.

In the introduction, two evolutionary processes were proposed to account for the patchy distribution of virulence genes: i) vertical inheritance combined with an arms race resulting in frequent gene loss and accelerated evolution and ii) horizontal transfer of individual genes, gene clusters, or chromosomes across species or vegetative compatibility groups (VCGs). From this short survey of current literature, it becomes clear that both evolutionary processes operate in nature. Accelerated evolution and gene loss, supporting the arms race model, was suggested for *NIP1* of *R. secalis* and avirulence genes of *Cladosporium fulvum* and *Magnaporthe oryzae*. Diversifying selection also has been noted for *SIX1* and *SIX1-H* in *F. oxysporum* (Table 1) and for the *AvrL567* genes of the basidiomycete *Melampsora lini* (flax rust) (Dodds et al. 2004). It should be noted that, in the case of toxin-producing fungi, diversifying selection is expected to operate on the toxin and not directly on the amino acid sequence of the toxin-producing enzymes. This might be reflected in different compositions of the gene clusters; for example, in the presence or absence of genes responsible for modification of the toxin. A related scenario may be envisaged for the Ace1 avirulence protein of *Magnaporthe oryzae*, which is predicted to produce a secondary metabolite that interacts with the host plant. The fact that Ace1 is a rare fusion of a polyketide synthase (PKS) and a nonribosomal peptide synthase (NRPS) might be interpreted to reflect “diversifying selection” on overall gene architecture rather than sequence (Bohnert et al. 2004).

Next to adapting targets or discarding arms that are self-defeating, new arms likely have been obtained through HGT. Arguments for HGT are i) the existence of CD chromosomes or CD chromosomal regions, ii) higher sequence identity of virulence genes between different species or VCGs relative to the rest of the genome and, iii) differences in GC content or codon usage. To varying degrees, these phenomena have been observed in *N. haematococca*, *F. oxysporum*, *A. alternata*, *Cochliobolus heterostrophus*, and *C. carbonum*. HGT between *F. oxysporum* and other *Fusaria* was suggested before to explain the distribution of the *Fot1* transposon (Daboussi et al. 2002). An exceptional case of HGT is the sharing of *ToxA* genes between *Pyrenophora tritici-repentis* and *S. nodorum*, because of the small segment that is shared (11 kb) and its correspondence to emergence of a new disease.

However, whatever the mode of inheritance of virulence genes, the question remains: what is the origin of virulence genes? Are they derived from nonvirulence genes with another function? Did they arise de novo in the fungal kingdom? Were they obtained through HGT from outside the fungal kingdom? If so, did they serve as virulence factors before HGT? One example of possible transfer of a virulence gene between fungi and bacteria is the gene for tomatinase, an enzyme that detoxifies the antimicrobial compound tomatine in tomato and related species. This gene shows unexpected high similarity between fungal and bacterial pathogens on solanaceae (Kaup et al. 2005; Kers et al. 2005). Moreover, it is present in a pathogenicity island in the bacterium *Streptomyces* (Kers et al. 2005) and has a patchy distribution among isolates of *F. oxysporum* (Ito et al. 2004b). Fungal genes for enzymes involved



in toxin biosynthesis obviously can share a common ancestor with related genes in fungi that are involved in synthesis of secondary metabolites that serve other functions. However, the origin of genes for small secreted proteins is much harder to trace.

### Clustering and HGT.

An intriguing phenomenon is that, in several pathogenic fungi (putative), virulence and avirulence genes are present in clusters. It should be noted, however, that there are large differences between these clusters. On one side, in the basidiomycete *U. maydis*, genes encoding small secreted proteins—at least some of which have a virulence function—are present in clusters that are distributed randomly across the genome. In contrast, in the ascomycetes *N. haematococca*, *F. oxysporum*, *A. alternata*, and *Cochliobolus* spp., genes for (putative) virulence factors reside in relative close proximity on the same chromosome, interspersed with transposable elements and (occasionally) other genes. An extreme form of genomic organization was found recently in *Leptosphaeria maculans*, in which a genetically defined cluster of avirulence genes turned out to be present in large regions consisting almost exclusively of retrotransposons and their remnants, interspersed with blocks of “housekeeping” genes (Fudal et al. 2007; Gout et al. 2006).

Regardless of these differences, the question is how clustering arose during evolution. We see, broadly speaking, two possible scenarios, which are not mutually exclusive. In the first scenario, virulence genes arise or arrive in random positions in the genome, and clustering results from a combination of random gene shuffling and selection for clustering of virulence genes. In this case, there must be an adaptive advantage of clustering over dispersed location of virulence genes. The alternative is that particular genomic regions are somehow “receptive” for either de novo emergence or insertion of virulence genes. It may be that the high density of transposable and repetitive elements frequently observed in the vicinity of (putative) virulence genes somehow facilitates accumulation of virulence genes. Transposons are known to be able to mediate chromosomal rearrangements (Wöstemeyer and Kreibich 2002). They might facilitate the insertion of new sequences or recombination events that lead to new (chimeric) genes. A recent indication for this is provided by the analysis of chromosome 7 of *M. oryzae*, in which transposable elements were found concentrated in subchromosomal regions that display high recombination rates, more gene duplications, and a high rate of gene sequence evolution (Thon et al. 2006).

Especially if clustering of virulence genes has resulted from random shuffling, there must be a strong selective advantage of clustering. One theory is that clustering is correlated with concerted regulation of gene expression; for instance, through a different chromatin structure of the cluster compared with the rest of the genome (Bok et al. 2006). One may imagine, for instance, “unfolding” of a complete pathogenicity island once contact with a living plant has been sensed. A different chromatin state of clusters or CD chromosomes has not yet been demonstrated indisputably. At least the dispensable chromosome of *N. haematococca* appears to have the same chromatin structure as the other chromosomes (Taga et al. 1999). One problem with gene expression serving as a selective principle is that there are no strong arguments to assume that clustering is really necessary for co-regulation of genes, because numerous examples of co-regulated genes exist that are not clustered (Walton 2000).

Another possible selective advantage of clustering is propensity for HGT. If virulence toward a certain plant species requires several virulence genes, it is a selective advantage for

these genes (i.e., not necessarily for the fungus) to be close together, or at least on the same chromosome, so that HGT actually leads to gain of virulence of the receiving fungal VCG or species. Here the term “selfish cluster” (Lawrence and Roth 1996; Walton 2000), or even “selfish chromosome” is appropriate. Examples of such selfish chromosomes might be the CD chromosomes of *Nectria* and *Alternaria* spp. Particular sequence characteristics or chromatin structures, perhaps related to the presence of transposons, may even promote HGT. The mechanism of HGT between fungi is still poorly understood but probably involves hyphal fusion between normally incompatible VCGs or fungal species. Normally, incompatibility reactions cause destruction of DNA in the fused hyphae (Glass et al. 2000); however, regions with particular structures perhaps may survive these events and become incorporated in the genome of the fusion partner. This might explain why one of the chromosomes of *Colletotrichum gloeosporioides* can be horizontally transferred between two vegetatively incompatible isolates, whereas the other chromosomes cannot (He et al. 1998). To take this speculation even further: selfish clusters or chromosomes may even encode factors that promote “promiscuous” hyphal fusion or survival of (particular) chromosomes or chromosomal regions after fusion. If there is some truth in this, the emergence of a new disease may be looked upon as a virulence gene, a group of virulence genes, or an entire CD chromosome having found a suitable new vehicle for increasing its offspring. The vehicle, a previously anonymous member of the microflora, now emerges as a new pathogen threatening crops or forests.

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## AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute of Harvard and MIT *Fusarium verticillioides* Sequencing Project: [www.broad.mit.edu](http://www.broad.mit.edu)