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Emergence of a new disease as a result of interspecific virulence gene transfer

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New diseases of humans, animals and plants emerge regularly. Enhanced virulence on a new host can be facilitated by the acquisition of novel virulence factors. Interspecific gene transfer is known to be a source of such virulence factors in bacterial pathogens (often manifested as pathogenicity islands in the recipient organism[1]) and it has been speculated that interspecific transfer of virulence factors may occur in fungal pathogens[2]. Until now, no direct support has been available for this hypothesis. Here we present evidence that a gene encoding a critical virulence factor was transferred from one species of fungal pathogen to another. This gene transfer probably occurred just before 1941, creating a pathogen population with significantly enhanced virulence and leading to the emergence of a new damaging disease of wheat.

Host-specific toxins are important virulence factors and determinants of host range in plant pathogenic fungi. Classic examples include the HV toxin produced by *Cochliobolus victoriae*[3] that destroyed Victoria oats carrying the *Pc2* crown rust resistance gene and T-toxin that, in the southern corn leaf blight epidemic of the early 1970s, severely affected maize carrying the Texas male-sterile cytoplasm[4]. Interspecific transfer of toxin genes has been suggested in fungal pathogens[5], but direct evidence for this has never been reported.

The best-characterized proteinaceous host-specific toxin is ToxA produced by *Pyrenophora tritici-repentis*, causal agent of tan (or yellow) spot in wheat (*Triticum aestivum*). Virulence of the fungus on wheat genotypes carrying a dominant susceptibility gene *Tsn1* (ref. 6) is dependant on the production of the toxin, henceforward called *P. tritici-repentis* ToxA[7]. Sensitivity to the purified toxin colocates to the same genetic locus as susceptibility to the disease. Transfer of the *P. tritici-repentis ToxA* gene into avirulent isolates of the fungus results in virulence[8]. Fungi that lack *P. tritici-repentis ToxA* give an indistinct lesion on wheat genotypes containing *Tsn1*, and wheat genotypes that lack *Tsn1* are significantly less susceptible to the most common races of the pathogen.

Stagonospora (syn. *Septoria*, teleomorph *Phaeosphaeria*) *nodorum* is a major wheat pathogen in many parts of the world causing Stagonospora nodorum blotch of wheat[9]. Quantitative trait loci (QTLs) for resistance to *S. nodorum* have been found on many chromosomes in different wheat genotypes. Recent evidence has shown that proteinaceous toxins contribute to pathogenicity[10,11]. Semipurified preparations of culture filtrates induced necrosis in appropriate wheat cultivars, and genetic analysis of the reaction indicated colocation of toxin-insensitivity loci with QTL for disease resistance.

A homolog of *P. tritici-repentis ToxA* in the *S. nodorum* genome

A search of the recently acquired genomic sequence of *S. nodorum* identified a predicted gene (SNU16571.1) with 99.7% similarity to *P. tritici-repentis ToxA*. For comparison, the genes for glyceraldehyde 3-phosphate dehydrogenase and the ITS region from the two species are 80% and 83% similar, respectively, consistent with taxonomic placements in the same family but different genera[9]. No other known proteins show significant ($E < 0.005$) similarity to ToxA. RT-PCR analysis delineated the transcript and showed that the gene, like *P. tritici-repentis ToxA*, comprises three exons and two introns. The high degree of sequence and structural similarity between the gene

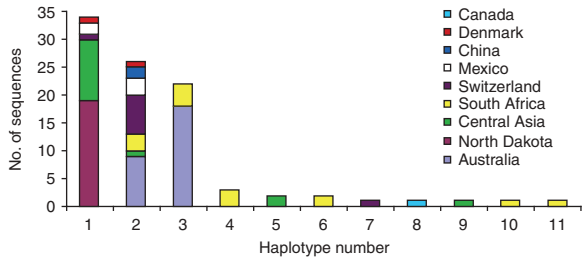

Figure 1 Geographical distribution and frequencies of observed *S. nodorum ToxA* haplotypes.

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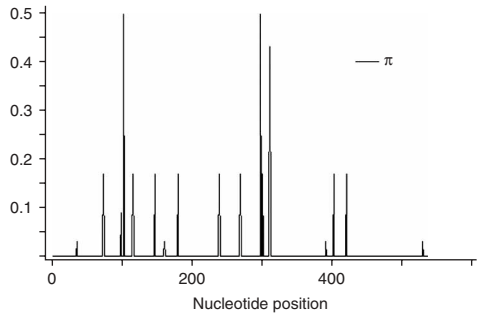

Figure 2 The *S. nodorum ToxA* locus in *S. nodorum*. Sliding window showing nucleotide diversity across the coding regions of the gene.

designated *S. nodorum ToxA* and *P. tritici-repentis ToxA* suggests a recent common ancestry.

We PCR-screened 755 isolates of *S. nodorum* originating from nine geographical populations for the presence of *S. nodorum ToxA* and obtained 183 positive amplicons (24%). The frequency of *S. nodorum ToxA* ranged from 100% in Australia to 5% in China. Previous studies have shown that *P. tritici-repentis ToxA* is present in the large majority of *P. tritici-repentis* isolates collected from wheat worldwide[12,13].

We tested for the presence of *ToxA* in worldwide collections of five closely related species that attack oat and barley, *S. avenaria* (36 isolates), *P. gramininea* (2), *P. avenae* (1), *P. hordei* (1) and *P. teres* (18) (**Supplementary Table 1** online), using the same PCR conditions and primers. We did not obtain any positive *ToxA* amplifications. We used DNA blotting on a sample of these isolates, and all hybridizations were negative. These results suggest that the *ToxA* genes are specific to the two wheat-adapted pathogen species.

ToxA diversity in *P. tritici-repentis* and *S. nodorum*

To analyze *ToxA* sequence diversity, we sequenced 95 *S. nodorum ToxA* and 54 *P. tritici-repentis ToxA* amplicons from geographically diverse pathogen populations. Patterns of sequence variation in the 534-bp coding region of *ToxA* differed considerably between the two species. We found only one haplotype in *P. tritici-repentis*. In contrast, we found 11 *S. nodorum* haplotypes (**Fig. 1**), differing at 11 polymorphic sites. The average gene diversity at the *S. nodorum ToxA* locus was 0.77 (ref. 14). A sliding window plot of nucleotide diversity, π (ref. 14), across the 95 *S. nodorum ToxA* sequences showed that nucleotide diversity in the coding region was highest ($\pi = 0.5$) at two different regions (**Fig. 2**). Among the 11 mutations found in the 11 haplotypes, five mutations were synonymous and six were nonsynonymous. The high nucleotide diversity in *S. nodorum ToxA* indicates that the gene has been in the *S. nodorum* genome for a long time, whereas the lack of variation in *P. tritici-repentis ToxA* suggests a very recent introduction into this genome. *S. nodorum ToxA* and *P. tritici-repentis ToxA* sequences differed at only four fixed nucleotide sites, resulting in two predicted amino acid changes. This contrasting pattern of nucleotide diversity in the two species is consistent with interspecific transfer of the gene from *S. nodorum* into *P. tritici-repentis*. We hypothesize that the high frequency of *P. tritici-repentis ToxA* in current *P. tritici-repentis* populations is due to selection on wheat populations containing *Tsn1*.

An 11 kb region is present in both species

We amplified the region flanking *P. tritici-repentis ToxA* by conventional and thermal asymmetric interlaced (TAIL) PCR, sequenced it and compared it with the *S. nodorum* sequence. A region of 11 kb was essentially collinear with contig 416 of *S. nodorum*. Sequence similarity ranged from 80%–90% at the peripheries to 98%–100% in the central region (**Fig. 3**). A transposase sequence of the hAT family[15] was highly conserved, although other known features of transposons were not found. A region of 197 bp was absent in the *S. nodorum* sequence. The upstream and downstream regions in *S. nodorum* contained repetitive AT-rich sequences. The corresponding flanking sequences in *P. tritici-repentis* had no similarity to these repeats but instead were ~87% similar to single-copy DNA on unlinked contigs in the *S. nodorum* genome (**Fig. 3**). The ToxA genes are present on 3.0-Mb[16] and 2.35-Mb (data not shown) chromosomal bands in *P. tritici-repentis* and *S. nodorum*, respectively.

S. nodorum ToxA interacts with the wheat gene *Tsn1*

We generated *S. nodorum ToxA*-disrupted mutants in isolates SN15 and Sn2000. Protein blot analysis (**Supplementary Fig. 1** online) and infiltration bioassays of culture filtrates on the *Tsn1*-containing line BG261 showed that the mutants did not produce detectable *S. nodorum* ToxA, whereas *S. nodorum* ToxA was produced in SN15, Sn2000 and the ectopic transformants (**Fig. 4**). Toxicity of culture filtrates on a *Tsn1* (ToxA-sensitive) wheat line was completely eliminated in all *S. nodorum ToxA*-disrupted mutants. Infection assays were conducted on a population of wheat lines segregating for *Tsn1* showing that *Tsn1* was highly associated with the disease phenotype in wild types and ectopic strains but was completely eliminated in all the *S. nodorum ToxA-disrupted* mutants (**Fig. 4**).

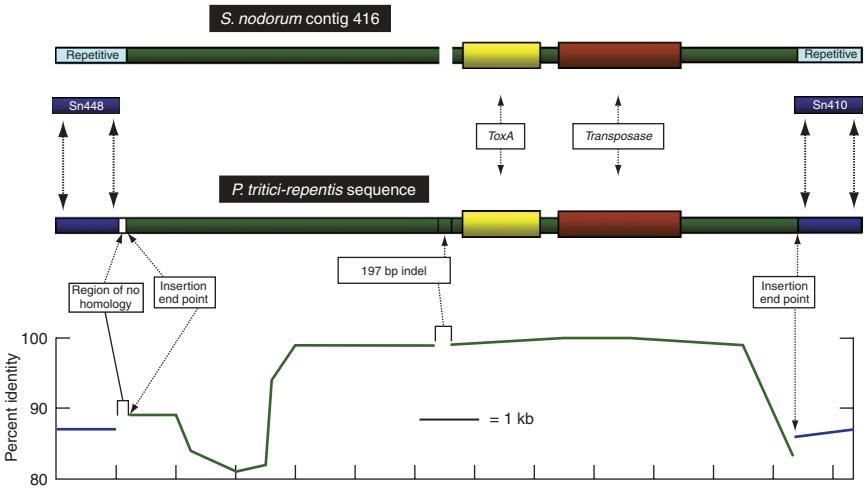

Figure 3 The *ToxA* loci in *S. nodorum* and *P. tritici-repentis*. The sequence of *S. nodorum* contig 416 compared with the *P. tritici-repentis* sequence obtained by conventional and TAIL PCR (green bar). The positions of the ToxA and transposase genes are indicated. The *S. nodorum* sequence is flanked by AT-rich sequence that is multiply repeated in the *S. nodorum* genome (light blue bar). The flanking *P. tritici-repentis* sequences are similar to unlinked *S. nodorum* contigs 410 and 448 (dashed lines). The percentage similarity between the *S. nodorum* and *P. tritici-repentis* sequences are shown below.

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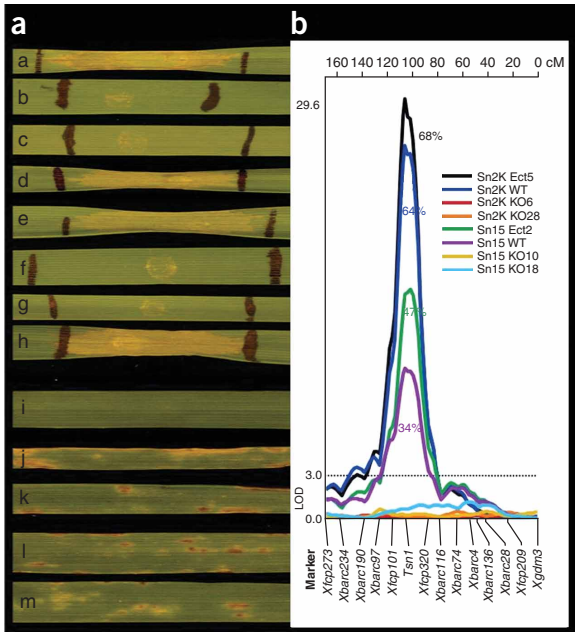

Figure 4 Functional and genetic analysis of interaction between *S. nodorum* ToxA and the wheat gene *Tsn1* (**a**) Bioassay of culture filtrates from *ToxA* dysfunctional mutants and controls on wheat line BG261 (ToxA-sensitive). a, SN15 wild type; b, SN15KO10; c, SN15KO18; d, SN15ECT2; e, Sn2000 wild-type; f, Sn2000KO6; g, Sn2000KO28; h, Sn2000ECT5. Fungal inoculation reactions with avirulent isolates Sn791087 (i) and Sn791087 transformed with *P. tritici-repentis ToxA* (j and k) and *S. nodorum ToxA* (l and m). (**b**) Interval regression map of chromosome 5BL indicating the association of *Tsn1* with reaction to Sn2K and SN15 toxA mutants, including wild-type and ectopic transformants 7 d after inoculation. The dotted line represents the lod significance threshold of 3.0, and the R^2 value for each significant QTL peak is given for each line. A centimorgan (cM) scale is shown at the top.

ToxA confers virulence to an avirulent *S. nodorum* isolate

P. tritici-repentis ToxA and *S. nodorum ToxA* were expressed in the avirulent (*ToxA*-lacking) *S. nodorum* isolate Sn79-1087. *ToxA*+ transformants were verified by DNA and protein blot analysis and toxin production. Sensitivity to culture filtrates and susceptibility to all four transformants cosegregated with *Tsn1* in the segregating wheat population (**Fig. 4**). The wild type isolate Sn79-1087 produced no visible lesions on any lines (data not shown).

Thus, both gene disruption and overexpression experiments clearly demonstrate that *S. nodorum ToxA* is necessary for complete virulence and is sufficient for induction of disease on wheat lines containing *Tsn1* and that the *S. nodorum* ToxA protein interacts (directly or indirectly) with the *Tsn1* gene product. This also shows that *S. nodorum ToxA* and *P. tritici-repentis ToxA* are functionally identical in their interaction with *Tsn1* despite the two predicted amino acid differences.

Transfer of *ToxA* from *S. nodorum* to *P. tritici-repentis*

In comparison to Stagonospora nodorum blotch caused by *S. nodorum*, tan-spot of wheat caused by *P. tritici-repentis* is a recently recognized disease. *P. tritici-repentis* (formerly known as *Helminthosporium tritici-vulgaris*) was first identified in 1902 from grass species and in 1928 from wheat, but was described as a saprophyte or occasional pathogen[17]. It was not until 1941 that typical necrotic tan spot symptoms were first described[18,19]. Following these US reports, these symptoms were next noted in Australia and Africa in the early 1950s[20,21]. It is significant that tan spot was initially called yellow spot in both the United States and Australia, a presumably accurate description of mild chlorotic disease symptoms. The 1942 reports describe leaf spots with light brown centers and yellow borders, a symptom perfectly in line with a current infection involving ToxA-induced necrosis. These early reports may have captured the first emergence of a new form of *P. tritici-repentis*, containing interspecifically transferred *ToxA*. In contrast, *S. nodorum* has been recognized as an abundant, regular and serious wheat pathogen in many parts of the world since at least 1889 (ref. 22).

ToxA is found in only 24% of *S. nodorum* isolates and 80% of *P. tritici-repentis* isolates (this study and refs. 12,13). The high frequency of deletion variants in both pathogens may reflect a balanced polymorphism that results from selection on wheat populations possessing or lacking *Tsn1*. This is analogous to the situation described earlier for the NIP1 avirulence-and-toxin protein in the barley scald pathogen *Rhynchosporium secalis*[23]. Balancing selection would also apply if there is a significant metabolic cost to produce ToxA, with selection operating against strains that make the toxin if hosts containing *Tsn1* are not present. Recent studies and unpublished work has shown that *S. nodorum* and *P. tritici-repentis* isolates can express several different host genotype–specific toxins[10,24,25]. We postulate that isolates lacking ToxA can use any one of these other toxins to infect different wheat cultivars.

Currently, epidemics of both pathogens occur annually, and mixed infection on the same leaf is common. It is therefore likely that the two fungi regularly come into close contact. It is possible that conidial anastomosis tubes (CATs) form between these species, facilitating genetic exchange[26]. We have observed CAT formation in *S. nodorum*, and CATs have been observed in *P. tritici-repentis* (Lynda Ciuffetti, personal communication).

The presence of a large region of almost identical DNA in both species, the presence on that region of a critical host range–enhancing toxin gene and its absence in related species is consistent with interspecific gene transfer. The pattern of high sequence diversity in *S. nodorum* and monomorphism in *P. tritici-repentis* and the recent emergence of the tan spot symptom lead us to suggest that the interspecific transfer of *ToxA* was from the former to the latter and occurred some time shortly before 1941. It remains possible that *ToxA* was transferred to *P. tritici-repentis* from a third, as yet undiscovered fungal (or non-fungal) species, but we have been unable to identify other possible donors. It is likely that *ToxA*-expressing *P. tritici-repentis* was globally distributed in shipments of infected grain, leading to widespread dissemination and setting the stage for the emergence of new highly pathogenic, tan spot–causing populations of *P. tritici-repentis* in wheat fields worldwide.

METHODS

Fungal materials. *S. nodorum* isolates SN15 (ref. 27) and Sn2000 (ref. 10) were used for gene disruption, and avirulent isolate Sn79-1087 (from J. Krupinsky, USDA-ARS, Mandan, North Dakota), was used for gene expression. Isolates of *S. nodorum* collected from wheat fields in South Africa, Australia, China, Central Asia, Denmark, Switzerland, Mexico, North Dakota and Canada were used to assess *ToxA* diversity (**Supplementary Table 1** online). Diversity at *ToxA* in *P. tritici-repentis* was characterized using *P. tritici-repentis ToxA*-producing isolates from the US, Canada, Brazil, Uruguay, Argentina and the Czech Republic[24]. Fungal DNA extraction was carried out as previously described[24,27]. The *P. tritici-repentis* race 2 isolate 86-124 was used for comparison of the *ToxA* flanking region.

Analysis of *S. nodorum ToxA* by RT-PCR. cDNA was synthesized from total RNA extracted from wheat leaves 3 d post-infection as previously described[27] and was amplified using the primers toxAa to toxAg (see **Supplementary Table 2**

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online). All PCR reactions were performed using the same conditions and an annealing temperature of 57 °C.

Vector construction. A *cpc*-1::hygromycin-resistance gene from pLP605KO[28] was cloned into the *Sal*I site of pBluescript (Stratagene) as pDAN. For the disruption vectors, *ToxA* was amplified from DNA of *P. tritici-repentis* isolate 86-124 using primers ToxA3 and ToxA4 with an annealing temperature of 62 °C. The insert was cloned into *Pvu*II-digested pUC19. This plasmid was digested with *Hin*dIII and *Sal*I to remove a 260-bp fragment of *ToxA*, into which was cloned the 2.7-kb *Hin*dIII/*Xho*I fragment of pDAN. Amplification with primers ToxA5 and ToxA6 at an annealing temperature of 57 °C gave a product that was cloned into the *Xba*I site of pBluescript. For the expression vectors, *ToxA* genes were amplified from *S. nodorum* SN15 and *P. tritici-repentis* 86-124 using native *Pfu* DNA polymerase (Strategene) and were verified and transferred to pDAN. The recombinant plasmids were confirmed by sequencing, linearized by *Xba*I (disruption vector) or *Eco*RV (expression vector), and transformed into fungal protoplasts[27]. Transformants were verified by DNA and protein blotting using standard techniques.

Production of culture filtrates and toxin bioassay. Culture filtrate production and toxin bioassays were as described[10]. At least two leaves were infiltrated for each replicate, and all experiments were replicated at least twice. Three days later, leaves were scored as insensitive or sensitive. Sensitive reactions were characterized by necrosis, whereas insensitivity showed no reaction within the infiltrated area.

Disease analysis and marker regression analysis. Conidial inoculations and disease analysis using 118 lines of a BR34 X Grandin (BG) F7:F9 wheat mapping population[29] were as described[11] with slight modifications. Each inoculation consisted of three plants, per line, per replicate.

Flanking regions of ToxA containing fragment in *P. tritici-repentis*. The *S. nodorum* contig 416 sequence was used to design primers every 1.1 kb to amplify sequence flanking the *ToxA* gene in *P. tritici-repentis*. Thermal asymmetric interlaced (TAIL) PCR was performed as described[30] to obtain *ToxA* flanking sequences not present in *S. nodorum*.

DNA sequence comparisons. Amplification of the *ToxA* coding regions used primers ToxA1 and ToxA2. PCR conditions were as follows: 2 min at 96 °C followed by 35 cycles of 1 min at 96 °C, 1 min at 60 °C, and 1 min at 72 °C, followed by 5 min at 72 °C. Sequences were aligned and edited manually using the program Sequencher 4.1 (Gene Codes). Further processing of sequence alignments was performed using BioEdit.

Database accessions. *S. nodorum ToxA* (GenBank DQ423483), and *P. tritici-repentis ToxA* (GenBank AF004369).

URLs. The *S. nodorum* genome sequence can be found at http://www.broad.mit.edu. The BioEdit sequence alignment editor can be found at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

T.L.F. supervised the work on SN2K; carried out the phenotyping and contributed to writing the paper; Z.L. performed the TAIL PCR and generated the ToxA transformants; S.M. supervised protein blot analysis and purification of ToxA; H.L. performed purification of ToxA; J.D.F. performed all host genetic analysis, including QTL analysis; J.B.R. constructed the vectors for transformation; P.S.S. generated the SN15 knockout; B.A.M. and E.H.S. conducted the population genetic analyses of the ToxA locus in both fungi and contributed to writing the paper; and R.P.O. carried out the sequence comparisons of ToxA and *S. nodorum*, supervised the work on SN15 and contributed to writing the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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