



A genomic fungal foray

Rapid progress in sequencing the entire genomes of fungal plant pathogens is promoting the development of new techniques for investigating these potentially devastating organisms. This approach is also revealing exciting new evolutionary glimpses.

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With a rapidly increasing global population it is essential to improve the characteristics of food crops. An increase in the yield, drought tolerance or hardiness of a crop, for example, could vastly increase food supplies. However, crop diseases constitute a serious constraint on yield. These diseases cause billions of dollars worth of crop losses worldwide every year and, in the worst cases, lead to devastating food shortages.

The problem of disease is not new. Most will be familiar with the Irish potato famine of the 1840s, caused by the potato late blight pathogen *Phytophthora infestans* (Agrios, 1997), but many other examples of dramatic crop losses are known. The black stem rust pathogen *Puccinia graminis tritici* caused the loss of 40% of the American wheat crop in 1954. Resistant and high yielding wheat varieties

were soon developed to alleviate the problem but the disease has since returned to prominence with the discovery of Ug99, a new and highly virulent strain, in Uganda in 1999. This strain is not controlled by the wheat varieties developed previously and so is currently spreading across Africa and could enter the Middle East (MacKenzie, 2009).

As well as mass food shortages, plant diseases can even lead to cultural alterations such as in the case of coffee rust (*Hemileia vastatrix*) in the 1870s. This rust disease decimated coffee production of Sri Lanka and led to the adoption of tea as the caffeinated drink of choice in Britain. Global banana production is currently under threat from black sigatoka (*Mycosphaerella fijiensis*), or black leaf streak, which can cause yield losses over 50% and result in prematurely ripening fruit which are dif-

Title image: A wheat crop showing symptoms of Fusarium Ear Blight (FEB). The infected ears have bleached spikelets (photograph courtesy of Rohan Lowe, Rothamsted Research).

difficult to export (Ploetz, 2001). Crop disease is now of increasing concern as world food supplies must continue to nourish a rapidly growing population.

Pathogens and plants

As the problem of disease grows, so new ways to protect crops are needed. Disease results from an interaction between a plant and a pathogen (the disease-causing organism) and therefore the fight against disease can be approached from two sides; that of the plant and that of the pathogen itself.

One new approach is to understand how pathogens cause disease. This information may then allow us to successfully modify the defences of the plant or the attack system of the pathogen in order to help reduce disease. One key aim of this approach is to understand the genetic basis of pathogenicity i.e. which of the genes in the genome of the pathogen allow successful colonisation of a plant and disease formation.

Sequencing the entire genomes of plant pathogens can help us identify those important genes. It is hoped that, in addition to providing a large amount of information about a particular species and opening the door for many experimental techniques, further insights into pathogenicity can be gained through the comparison of genomes of different species. This can lead to the discovery of chemical control targets to help combat the pathogen, so reducing disease incidence and severity, both in the crop and post-harvest.

Fungi constitute a large proportion of the plant pathogenic microorganisms present in the temperate and sub-tropical regions of the world. Plant pathogenic fungi exhibit a wide range of lifestyles and infection mechanisms and frequently produce harmful toxins. In 2002, the first fungal plant pathogen genome was sequenced (Dean *et al.*, 2005). *Magnaporthe grisea* was selected because this organism is responsible for the globally devastating rice blast disease (Agrios, 1997). Since then, the genome sequences of a number of pathogenic and related non-pathogenic fungal species have been made available, mainly through the efforts of the Broad Institute at MIT and Harvard and the DOE Joint Genome Institute (JGI), both located in the USA. The sequenced pathogenic species represent a wide range of infection biology types that can successfully cause disease on many different plant species. The non-pathogenic species provide a useful comparison and can be used to highlight factors required for disease.

Fusarium Ear Blight (FEB)

Our research focuses on Fusarium Ear Blight (FEB), a highly destructive and increasingly important disease of wheat and barley crops (Figure 1A-F). FEB, also referred to as head scab or head blight disease, is found in most wheat-growing areas of the world (in Leonard and Bushnell, 2003). The main causal agents of FEB are the fungi *Fusarium graminearum* and *F. culmorum* (Bai and Shaner, 2004; Snijders, 2004). As well as damaging grain, and so reducing product yield and quality (Snijders, 2004), many *Fusarium* species produce mycotoxins (such as deoxynivalenol (DON) (Figure 1 F), nivalenol (NIV) and zearalenone (ZEA)) which if eaten present a serious health hazard to humans and animals (Leonard and Bushnell, 2003).

Due to the risk posed by such toxins, strict guidelines have been implemented so that grain with DON levels higher than around 1 part per million (ppm) is rejected. The new EU toxin limits, in effect from 1 July 2006, are shown in Table 1. Infected grain is therefore harder to market and is more difficult to process, further increasing the impact of this disease on the wheat and barley industry. In addition, fusarium fungal spores can remain in soil on debris from a previous season's cereal crop, aiding disease persistence and making disease control more difficult.

Since the early 1990s, the consequences of annual FEB infections have amounted to losses of millions of tonnes of grain, and hence billions of dollars in China and the USA. Over 7 million hectares of wheat in China have been affected by FEB (8% of the total crop in 1998 according to *www.desertcontrol.com*), with severe epidemics causing losses of over 1 million tonnes. Epidemics in wheat and barley in the USA and Canada from 1991 to 1997 resulted in around \$1.3 billion of direct losses and \$4.8 billion of accumulated economic impact (Bai and Shaner, 2004). This has led to FEB being considered by the US Department of Agriculture (USDA) as the worst plant disease in the USA since black stem rust devastated cereal crops in the 1950s (Agrios, 1997; Cuomo *et al.*, 2007).

In Europe, *Fusarium* species are found in all the cereal-growing areas, causing reductions in yield of 10-40% (Bottalico and Perrone, 2002). *F. culmorum* appears to dominate in northern Europe and *F. graminearum* in the south as this species prefers warmer conditions. However, the European Mycotoxin Awareness Network reports that in the last 10 years the latter

Figure 1A. A wheat ear exhibiting FEB infection. The production of fungal spores on the bleached spikelets can be seen as an orange colouration.

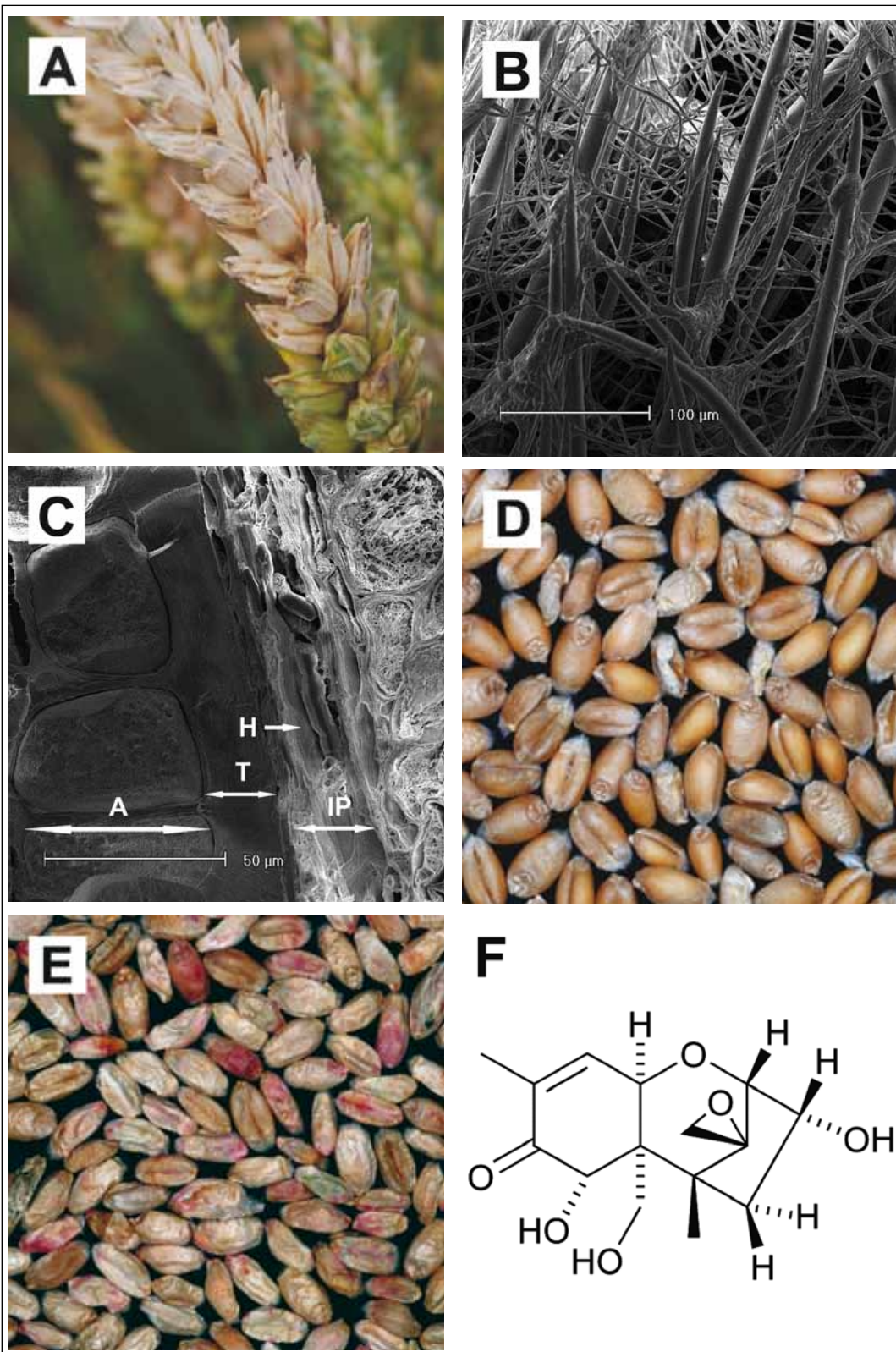
Figure 1B. A scanning electron microscope (SEM) image of FEB-infected wheat showing abundant *F. graminearum* hyphal filaments on the surface of the developing grain and amongst the grain hairs.

Figure 1C. SEM image of the inside of an FEB-infected wheat grain. *F. graminearum* hyphae are growing between the pericarp and testa layers. Double headed arrows indicate the different tissue layers. A = aleurone, T = testa, IP = inner pericarp, H = hyphae.

Figure 1D. Plump grain recovered at harvest from a healthy wheat ear.

Figure 1E. Wheat grain infected by FEB are small, shrivelled and show a pink colouration. Photographs D and E are courtesy of Rohan Lowe, Rothamsted Research.

Figure 1F. The structure of deoxynivalenol, one of the most potent and important toxins produced by *Fusarium* species. This toxin is harmful to both human and animal consumers of infected grain.



species has gradually been increasing in prevalence as maize production increases in northern Europe and climate change occurs. It is thought almost all wheat and barley grown in northern Europe is contaminated to some extent by fusarium mycotoxins (Bottalico and Perrone, 2002).

Overall, the situation in Europe appears to have worsened over the past 15 years, with increased levels of fusarium colonisation and infection (Magan *et al*,

2002). The recent adoption of higher yielding but more susceptible winter wheat varieties in western Europe has heightened the FEB problem (Snijders, 2004). Recent years have also seen a reported increase in FEB severity in spring barley, which appears to be related to a shift in environmental conditions, farming methods and crop varieties used (Bottalico and Perrone, 2002 and references therein).

In the UK, the proportion of disease due to

F. graminearum is increasing year on year and in 2002 exceeded that due to *F. culmorum* for the first time. *F. graminearum* now comprises a serious threat due to the high level of damage it can inflict (Jennings *et al.*, 2004). Surveys such as the Home Grown Cereals Association (HGCA) Crop Monitor 2005/2006 Winter Wheat Survey have highlighted the south west and East Anglia as the worst affected areas.

Life cycle and infection process

Fusarium spores are thought to be the main means of infecting wheat ears (Leonard and Bushnell, 2003). Spores infect the spikelets of the ear during flowering, the time when the host plant is most susceptible. The dispersal of spores from fungal colonies growing on crop debris can be aided by rain splash, wind and even insect vectors. Growth, reproduction and spore germination of fusarium are all affected by climatic conditions, with wet weather increasing the occurrence of the disease. The extent of disease development depends on plant age, spore levels, exposure duration and air temperature. However, there is an absolute requirement for a period of high moisture at crop flowering to permit the initial infection to occur.

Infection is first seen as the formation of brown spots on spikelets. The spikelets then become bleached and this bleaching subsequently spreads up and down the ear (Figure 1). Soon after the fungus infects the spikelet, the production of mycotoxins in the hyphae commences. Hyphal filaments of the fungus grow over and into the developing grain leading to damage and contamination with toxins (Figure 1). As the fungus spreads, it destroys starch granules in cells, damages cell walls, and causes death of infected cells. After several days, the fungus produces new spores that can remain on plant debris and infect subsequent crops (Leonard and Bushnell, 2003).

Combating FEB

Many approaches have been employed in attempts to combat FEB and so reduce disease occurrence, mycotoxin accumulation and symptom severity. As yet, a complete cure or preventative measure for FEB has not been found. Resistance in wheat is at best incomplete and differs between varieties. Often the most resistant varieties possess the worst agronomic characteristics. Both transgenic and conventional breeding approaches to increase resistance are under investigation (Snijders, 2004).

Table 1. In July 2006, throughout the EU, limits were imposed on the permitted levels of the fusarium-produced mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) in the food and feed chain.

	Legal Limit (ppb)	
	Deoxynivalenol (DON)	Zearalenone (ZEA)
Unprocessed wheat	1250	100
Flour	750	75
Finished products	500	50
Infant food	200	20

(Food Standards Agency, www.food.gov.uk)

Studies of possible fungicide control measures for *F. graminearum* and *F. culmorum* have provided some promising yet mixed results. The success of fungicide application is complicated because the results are dependent on which other fungal species are present and the effect of the fungicide upon these species (Aldred and Magan, 2004). For example, fungicide applications may remove competing fungi from the ear, which then permits the 'clean' ear to be colonised more extensively by pathogenic *Fusarium* species. Another problem arises because some fungicides may actually stimulate mycotoxin production when applied under certain conditions (Aldred and Magan, 2004). Even ensuring a good application of fungicides to each ear in the dense crop still remains technically challenging.

New fungicide targets are being sought to help combat FEB. To achieve this, a number of *F. graminearum* genes have been experimentally disrupted and/or deleted in the search for factors required by the fungus to cause disease (Figure 2A). Such gene products, if accessible to chemical intervention, may subsequently become targets for fungicides (Figure 2B).

Sequencing the genome

The infection biology of *Fusarium* species on cereal floral tissue has been determined predominantly from studies involving *F. graminearum*. In 2003, the genome sequence for this species was made available at the Broad Institute of MIT and Harvard, and a report on the genome was recently published in *Science* (Cuomo *et al.*, 2007). This new resource has greatly enhanced the possibilities for investigation of this important pathogen.

The genome sequence of *F. graminearum* strain PH-1 (a North American isolate) revealed a number of important features: *F. graminearum* possesses very little repetitive DNA sequence, at least a factor of 15 less than that of other related fungi. Few recently duplicated genes were found and mobile genetic elements called trans-

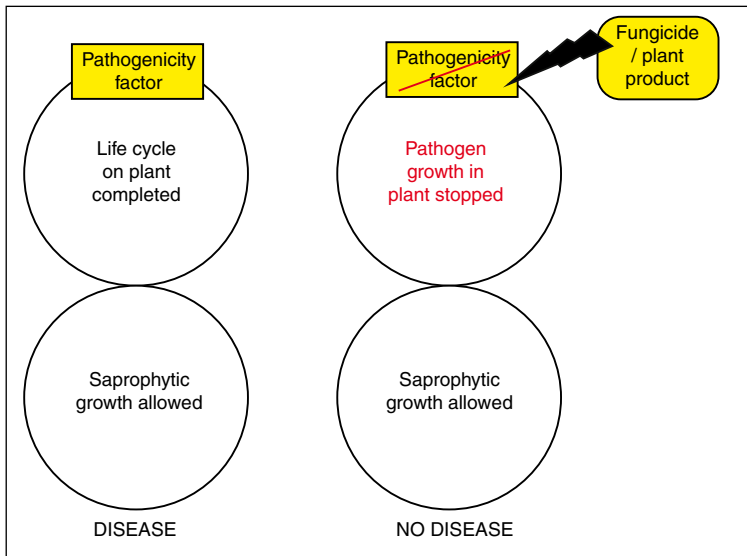
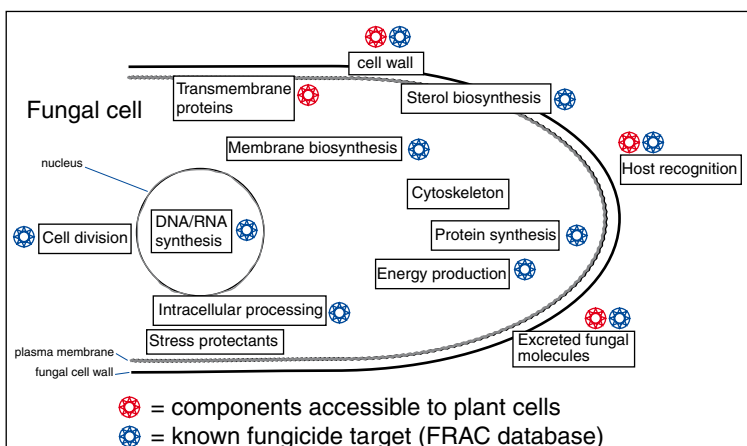


Figure 2A. The definition of a pathogenicity factor is one whose deletion or mutation prevents completion of the life cycle of the pathogen on a host plant species while still allowing the pathogen to grow on dead or decaying matter. Such factors may be targets of host plant defence systems or provide important fungicide targets.

posons were minimal and non-functional. The lack of repetitive sequence is thought to be due to a combination of the low number of transposons, the self-fertility of *F. graminearum* (repetitive DNA is rarely gained from crosses) and the presence of a specific genetic mechanism, already known to be present in several other species, called repeat-induced point mutation (RIP) which efficiently eliminates repetitive DNA. RIP is thought to act as a defence mechanism for the genome in which duplicated sequences are selectively mutated before meiosis in the sexual cycle.

The *F. graminearum* genome was found to contain a larger number of genes predicted to encode proteins for transcription factors, hydrolytic enzymes and transmembrane transporters than the related Ascomycete species *Neurospora crassa*

Figure 2B. Possible fungicide and host defence targets. A number of external components of the fungal cell are accessible to plant defence mechanisms, while these and other internal components are currently being targeted by developing fungicide chemistries. These fungicides and their targets are listed in the Fungicide Resistance Action Committee (FRAC) database (see list of useful URLs).



(orange bread mould), *Magnaporthe grisea* (the causal agent of rice blast disease) and *Aspergillus nidulans* (a soil fungus).

Unlike other plant pathogenic fungi, *F. graminearum* contains only four large chromosomes. Once the genome sequence became available, it was possible to locate the previously identified genetic markers (small sequences in the genome whose position relative to other important features and each other can be calculated mathematically to help determine the location of these features) in the sequence because of the lack of repetitive DNA (Figure 3 row 1). This had not been possible for the previously published fungal plant pathogen genomes because of the lower quality of sequence information obtained as well as the abundance of repetitive sequences. These features also limited the ability of these other fungal genomes to be completely assembled from the overlapping fragments of sequence obtained in the sequencing project.

The sequence of strain PH-1 was also compared to that of a second strain of *F. graminearum*, GZ3639 (another North American isolate) and the location of any differences in sequence between the two was plotted (Cuomo *et al.*, 2007). This analysis revealed the presence of 10,495 single nucleotide polymorphisms (SNPs) where the two sequences show a difference in a single base pair (Figure 3 row 2). These SNPs tended to be clustered together, with many present near the ends of the four chromosomes at regions called telomeres. However, a few regions in the middle of three of the four chromosomes also showed high SNP density.

This led the authors to suggest that the large chromosomes are the result of fusion of previous, smaller chromosomes in the progenitor species. This may help to explain the low number of chromosomes in *F. graminearum*. The regions of highest SNP density also correlated with high **recombination frequency*** (Figure 3 row 3) and low **GC content**, further suggesting that these regions represent former telomeres.

Genes that were found to be specific to *F. graminearum* also tended to occur more frequently in the SNP-dense regions, as did genes specifically expressed during the first six days of barley ear colonisation (Figure 3 row 4). Such genes included those encoding predicted plant cell-wall degrading enzymes and several with similarity to known disease causing factors.

* Note: terms in **bold** are explained in the Glossary of Specialist Genetic Terms

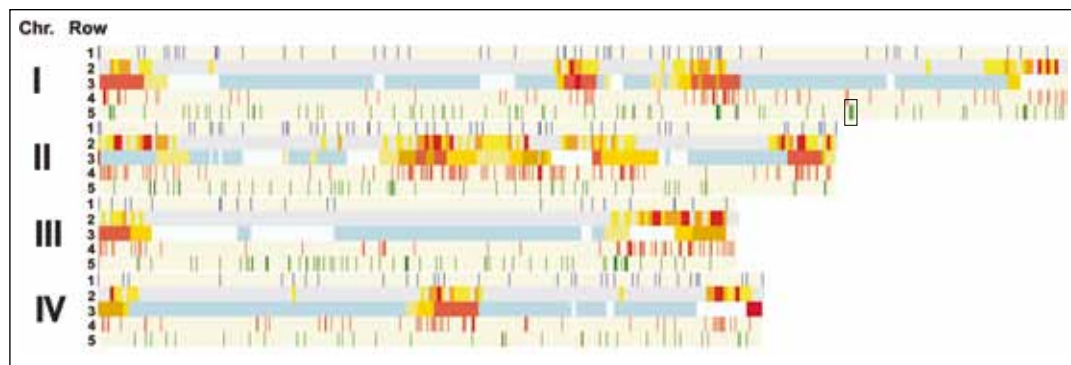


Figure 3. A display of the four *Fusarium graminearum* chromosomes and different gene and genetic information using the OmniMapFree software. Chromosomes I-IV are 11.9, 9.0, 7.7 and 8.1 Mb (megabases) long, respectively. The specific map developed for this species is called FgraMap. Each *F. graminearum* chromosome has been drawn as five consecutive horizontal rows. Row 1: The position of each genetic marker (see text) in the genome of the sequenced strain PH-1 is shown as a thin vertical blue line. Row 2: The density of single nucleotide polymorphisms (SNPs) between the fully and partially sequenced strains (PH-1 versus GZ3639) is revealed as a colour code where, per 50,000 base pair segment, the highest density of the SNPs is coloured red, the next highest density as orange, the next yellow and the lowest density of the SNPs as grey. The absolute SNP density range was from 152 to zero SNPs per 50,000 base pairs (bp). Row 3: The frequency of recombination in a cross between the sequenced strain PH-1 and a second USA strain called MN00-676 is shown as a colour code where recombination frequency increases through the colours azure, light blue, khaki, gold, golden rod, tomato and crimson. The absolute recombination frequency ranges from zero to >8 cM (centimorgan) between consecutive genetic markers (see row 1). Row 4: The location of each of the *F. graminearum* genes expressed only *in planta* is shown as a thin red vertical line on the chromosomes. Row 5: The distribution of *F. graminearum* genes with similarity to experimentally verified pathogenicity genes in a range of plant and animal pathogenic species (www.phi-base.org, version 3.1). The location of each gene is shown as a thin green vertical line on the chromosomes. The black boxed region indicates an example of a micro-region rich in this gene type.

The conserved core genes, such as those involved in basal transcription and protein translation, tended to occur away from these regions.

Overall, these genomic features make the landscape of the *F. graminearum* chromosomes very different from that of previously-sequenced fungi.

New insights

The availability of a sequenced genome for *F. graminearum* has allowed a rapid acceleration in the acquisition of knowledge regarding the disease causing ability of this species.

Using the genome sequence, a whole range of experimental possibilities is now available. For example, to assist with plant breeding or genetic engineering, *F. graminearum* targets for intervention by plant defences can be searched for in the genome and investigated. Crop species can then be bred or adapted accordingly so that host resistance to the fungus may be increased. A reporter gene sequence (one that encodes a protein whose production can be measured easily) can be fused to different *F. graminearum* promoter sequences (regions of DNA that control the level of activity of the gene) to monitor specific changes in gene activity when the fungus is growing under different conditions or during plant infection. In addition, an Affymetrix GeneChip has been

developed for *F. graminearum* so that the expression of thousands of genes can be analysed simultaneously (Güldener *et al.*, 2006) and those which may be co-ordinately regulated can thereby be identified.

The production of mutant fungi in which a particular gene has undergone deletion to test for a role in disease has now become far simpler thanks to the available genome sequence. The location of the genes of interest can be predicted and the available DNA sequence used to design DNA segments that are then targeted to delete each gene and replace the gene sequence with one allowing the mutants to be selected. For example, a gene for resistance to the compound hygromycin, which normally kills fungi, can be introduced in place of the deleted gene (Figure 4). In this way, new fungal strains in which single genes have been deleted can be selected for by growing on a medium containing hygromycin.

The predicted genes in the genome can also be compared to genes whose function is already known in other species to try and determine their role in the life of the fungus. If a chosen gene is shown to be important for the disease-causing ability of the pathogen, then it can be investigated as a potential target for chemical control, such as fungicides.

A predicted function, or similarity to other genes of known function, in another

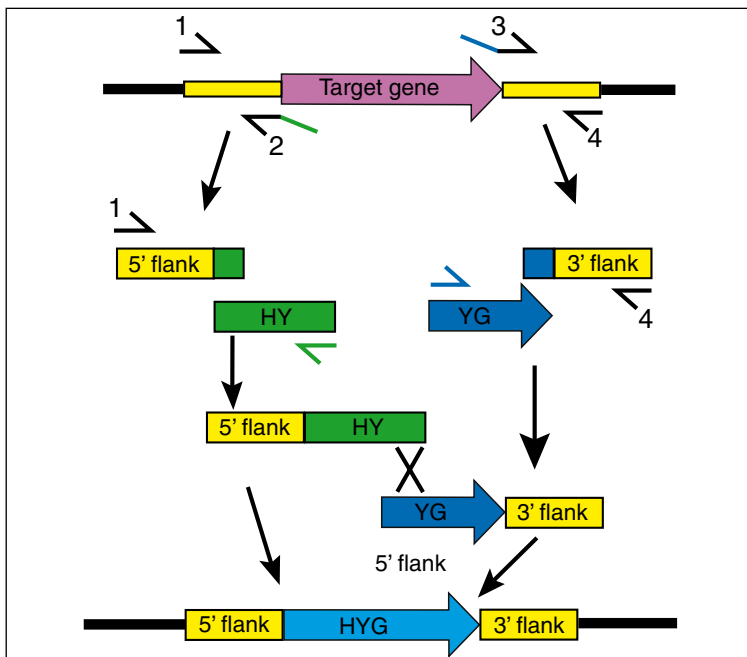


Figure 4. Targeted deletion of a chosen gene in *F. graminearum*. Amplification of regions either side of the target gene using the polymerase chain reaction (PCR) primer sequences 1-4 (small DNA sections used to start an amplification reaction) allows their attachment to an antibiotic resistance gene (in this case for the antibiotic hygromycin, HYG). This can be achieved by cutting and joining using restriction enzymes and DNA ligase or, as shown in the figure, using extra sequences on the primers to join the flanks and HYG gene. The method illustrated is known as the split marker technique (Catlett *et al.* 2003) as the antibiotic resistance gene is present as two different overlapping sections. One section is joined to each of the two flanking regions. The two DNA fusion products made are then inserted into experimentally prepared fungal fragments known as protoplasts (fungal cells with a plasma membrane but lacking a cell wall), resulting in deletion of the target gene and replacement with the antibiotic resistance gene via DNA recombination. The antibiotic can then be used to select for successful deletion events.

species is usually required for a gene to be chosen for targeted deletion. Genes are often chosen for deletion because they encode proteins belonging to a particular class. For example, a protein kinase which may control cellular signalling events or a secreted protein which may come in contact with plant cells during the infection process, or a protein that forms part of a pathway, such as the toxin biosynthetic pathways or cellular signalling pathways. So far, however, more than 75% of the computer-predicted genes in the *F. graminearum* genome do not yet have a predicted function. In addition, around 30% of the genes predicted in *F. graminearum* and the rice pathogen *Magnaporthe grisea* appear to be unique to these two species (Xu *et al.* 2006).

By combining the information base for *F. graminearum* with that of other fungal plant pathogens, together with data from published genomes, it is now possible to use a suite of novel routes to try and locate factors which are involved in the disease-causing ability of a pathogen (Figure 2A). For example, the Pathogen-Host Interactions database (PHI-base, Winnenburg *et al.* 2008) already contains

information on 687 genes (in Version 3.1, April 2008) proven to affect the outcome of a particular pathogen-host interaction (i.e. pathogenicity genes).

By mapping the contents of this database onto the *F. graminearum* genome using our chromosome visualisation software, OmniMapFree, the location of genes similar to those described in the database can be determined (Figure 3 row 5). This has allowed us to pinpoint close groups of potential pathogenicity genes in *F. graminearum* and so target these and other genes in these 'micro-regions' for further investigation. For example, in *F. graminearum* one such micro-region exists on Chromosome 1 (boxed region in Figure 3). In both plant and animal-attacking fungal species, genes required for disease causing ability are often shown to be clustered together, and so these micro-regions may provide a way to find novel pathogenicity genes.

F. graminearum is not the only *Fusarium* species for which a genome sequence is now available. *F. verticillioides*, a maize pathogen, and also *F. oxysporum* and *F. solani*, broad-spectrum pathogens which infect non-cereal species, have been sequenced. This has allowed a comparison to be made between these four related species at the Broad Institute website. This is useful in determining the degree of conservation of interesting features found in *F. graminearum* within the *Fusarium* species and for comparing properties such as repetitive DNA content, average inter-gene gap size, chromosome number and % GC content. Such features have been shown to influence gene activity in many species.

By broadening the comparison to other non-*Fusarium* species, for example the closely related *Epichloë festucae* (which grows in grasses but does not produce disease symptoms) and *Neurospora crassa* (which grows on tree bark after forest fires), clues about the evolutionary history of pathogenicity genes and gene clusters can be obtained. This may indicate features that are specific to *Fusarium* species and those broadly spread through many pathogenic species but absent from non-pathogenic species.

Similarly, such genes or gene clusters could also be found in non-pathogen species, suggesting differences in regulation or activation/inactivation events that are responsible for the different lifestyles of each species. This knowledge will provide insights into the pathogenic mechanisms of not only *F. graminearum* but also of the

other compared fungal species, many of which are also serious pathogens of globally important crop species.

Acknowledgements

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Useful URLs

- Broad Fusarium comparison site:
www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html
- Broad Institute: www.broad.mit.edu
- China's shrinking grain harvest:
www.desertcontrol.com/China's%20Shrinking%20Grain.htm
- European Mycotoxin Awareness Network:
www.mycotoxins.org
- Food Standards Agency (FSA):
www.food.gov.uk/foodindustry/farmingfood/fusariumadvice
- Fungicide Resistance Action Committee (FRAC) Database: www.frac.info/frac/index.htm
- Home Grown Cereals Association (HGCA) Crop Monitor: <http://cropmonitor.co.uk>
- Joint Genome Institute (JGI): www.jgi.doe.gov
- OmniMapFree: www.omnimapfree.org
- Pathogen-Host Interactions Database (PHI-base):
www.phi-base.org

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Glossary of specialist genetic terms

Recombination frequency

During meiosis (the division of cells to produce gametes, or reproductive cells), crossing over of DNA between pairs of homologous chromosomes alters the inheritance of specific traits. Here sections of DNA swap between the homologous chromosomes as the DNA breaks and rejoins in a recombination reaction. The further apart in the chromosome two features are, so the more likely a crossing over event can occur between them and the less likely they are to be inherited together. This forms the basis of mapping the positions of **molecular markers**. Some parts of a chromosome are inherently more likely to undergo recombination than others as recombination likelihood is affected by DNA sequence. This variation in recombination frequency is plotted for *F. graminearum* chromosomes in Figure 3 row 3. Recombination frequency is measured in **centimorgans** (cM), one centimorgan being equal to a 1% chance of two points in a genome being separated due to recombination by crossing over in a single generation.

GC content

DNA is comprised of strings of nucleotide molecules joined together. Each nucleotide can have one of four different base groups attached - adenine (A), cytosine (C), guanine (G) or thymine (T). In the DNA double helix, adenine binds to thymine and cytosine to guanine. The binding of C-G base pairs is stronger than that of A-T base pairs and so the distribution of A-T versus C-G base pairs alters the properties of the DNA. The GC content of a piece of DNA represents the proportion of base pairs that are C-G rather than A-T. Different GC contents can be indicative of different genome features, for example, pathogenicity gene clusters in bacteria often show a different GC content to the rest of the genome.