Comparative Pathogenomics Reveals Horizontally Acquired Novel Virulence Genes in Fungi Infecting Cereal Hosts

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Abstract

Comparative analyses of pathogen genomes provide new insights into how pathogens have evolved common and divergent virulence strategies to invade related plant species. Fusarium crown and root rots are important diseases of wheat and barley world-wide. In Australia, these diseases are primarily caused by the fungal pathogen Fusarium pseudograminearum. Comparative genomic analyses showed that the F. pseudograminearum genome encodes proteins that are present in other fungal pathogens of cereals but absent in non-cereal pathogens. In some cases, these cereal pathogen specific genes were also found in bacteria associated with plants. Phylogenetetic analysis of selected F. pseudograminearum genes supported the hypothesis of horizontal gene transfer into diverse cereal pathogens. Two horizontally acquired genes with no previously known role in fungal pathogenesis were studied functionally via gene knockout methods and shown to significantly affect virulence of F. pseudograminearum on the cereal hosts wheat and barley. Our results indicate using comparative genomics to identify genes specific to pathogens of related hosts reveals novel virulence genes and illustrates the importance of horizontal gene transfer in the evolution of plant infecting fungal pathogens.

Introduction

Crop losses due to fungal pathogens represent one of the most serious threats to global food production. Staple cereal crops such as wheat, barley, rice and maize are subject to attack from a diverse array of fungal pathogens including biotrophs such as rust fungi that feed on living cells and necrotrophs such as Fusarium pathogens that kill host cells to obtain nutrients. Many Fusarium pathogens not only reduce crop yields but also produce mycotoxins that are harmful to humans and livestock when consumed in food and feed. A better understanding of the infection strategies used by these pathogens would help develop novel plant protection strategies. Comparative analysis of pathogen genomes offers a new and powerful approach to identify common and divergent virulence strategies as well as evolutionary history of pathogen lineages.

Shared virulence strategies may be used by different fungi to invade specific plant hosts. Presumably in many cases, the existence of common virulence strategies in different pathogen species may be explained by conservation of virulence gene function through vertical inheritance and/or exposure to common host defensive selection forces during pathogenesis on the same or related hosts. However, in some instances, horizontal gene transfer events have been identified in fungal pathogens and subsequently shown to have roles in pathogenicity [1–3]. A striking example of a locus-specific horizontal gene transfer event emerged from the sequencing of the wheat pathogen Phaeosphaeria nodorum (anamorph Stagonospora nodorum) genome, where a gene encoding a host-specific protein toxin (ToxA) was named by homology to a known toxin from another wheat pathogen Pyrenophora tritici-repentis. Functional analyses demonstrated that ToxA was necessary for virulence in both pathogens [1]. It was proposed that transfer of ToxA from P. nodorum to P. tritici-repentis resulted in the emergence of the tan spot disease of wheat caused by P. tritici-repentis in the 1930s [1,4]. In another example, genome analysis of the tomato vascular wilt pathogen F. oxysporum f. sp. lycopecrii revealed the presence of several supernumerary chromosomes. Non-sexual transfer of one of these chromosomes to a non-virulent and genetically diverged recipient strain was shown to be sufficient to confer virulence on tomato [2]. Recently, association genomics has been used to identify the fungal effector Avel (for Avirulence on Ve1 tomato) in Verticillium dahliae. Avel homologues were shown to
Cereals are our most important staple crops and are subject to attack from a diverse range of fungal pathogens. A major goal of molecular plant pathology research is to understand how pathogens infect plants to allow the development of durable plant protection measures. Comparing the genomes of different pathogens of cereals and contrasting them to non-cereal pathogen genomes allows for the identification of genes important for pathogenicity toward these important crops. In this study, we sequenced the genome of the wheat and barley pathogen *F. pseudograminearum* responsible for crown and root-rot diseases, and compared it to those from a broad range of previously sequenced fungal genomes from cereal and non-cereal pathogens. These analyses revealed that the *F. pseudograminearum* genome contains a number of genes only found in fungi pathogenic on cereals. Some of these genes appear to have been horizontally acquired from other fungi and, in some cases, from plant-associated bacteria. The functions of two of these genes were tested by creating strains that lacked the genes. Both genes had important roles in causing disease on cereals. This work has important implications for our understanding of pathogen specialization during the evolution of fungal pathogens infecting cereal crops.

Recently, a number of genes only found in fungi pathogenic on cereals have been identified through comparative genomics. These genes include those associated with a clade of sequences from unrelated species [6,7]. Such genes are best identified by gene phylogenies where a finding is consistent with multiple horizontal acquisition events [3].

Recent advances in genomics are revolutionizing the analysis of fungal species, which are particularly suited to analysis by the current generation of DNA sequencing technologies due to their relatively small genomes and in many cases minimal repetitive DNA contents. The *de novo* detection of shared virulence strategies without a priori information on the roles of shared genes in pathogen virulence offers an exciting possibility of uncovering new insights into the pathogenesis-related processes. The work of Klosterman et al [3] is one of the few examples where nothing was known about the role of the identified genes in pathogen virulence prior to being identified through comparative genomics. Therefore, it is reasonable to expect that further unbiased comparative genomic analyses will uncover examples of shared virulence genes or niche specialization genes in these pathogens, and ultimately provide insights into the co-evolution of virulence/niche specialization functions and the mechanisms of plant defense.

In this paper, we report the sequencing, assembly and annotation of the genome of *Fusarium pseudograminearum* (Aoki and O'Donnell) using Illumina sequencing and a comparative genomics analysis of its gene content. In many parts of the world, *Fusarium pseudograminearum* is the principal cause of Fusarium crown rot (FCR) of wheat and barley. FCR is significant in arid cereal-growing regions worldwide including South Africa [9], Northern Africa [10], the Middle East [11], Europe [12] and Australia [13] as well as the northwest of the United States of America [14]. In Australia, FCR is a chronic problem and among the most economically significant diseases of both wheat and barley [15,16].

The recent increase in incidence of FCR is ascribed to the increased use of conservation farming practices such as zero tillage and stubble retention. These practices permit the survival of fungal inoculum on residual plant matter across planting seasons. *Fusarium* root-rot is a related cereal disease caused by *F. pseudograminearum* as well as other fusaria [17,18]. *F. pseudograminearum* was initially distinguished from *F. graminearum* by host tissue preferences [19] and on the basis of molecular data was formally recognized as a separate species [20,21]. Multi-locus sequence analysis of diverse isolates has shown that *F. pseudograminearum* is a single phylogenetic species globally [22] in contrast to the *F. graminearum* species complex, which shows geographical structure [23,24]. In addition *F. pseudograminearum* is heterothallic whilst *F. graminearum* has a homothallic mating system. Whilsts *F. pseudograminearum*, *F. graminearum* and *F. culmorum* are present throughout the Australian wheat growing regions and can all cause FCR, *F. pseudograminearum* is the species most commonly recovered from plants showing FCR symptoms [25,26]. Field surveys in Australia have revealed that *F. graminearum* is the most frequently isolated species from wheat plants showing Fusarium head blight (FHB) symptoms [26], although *F. pseudograminearum* can also cause FHB disease. These observations suggest that *F. pseudograminearum*, while a broadly adapted cereal pathogen, may have evolved adaptations for niche specialization or infection processes that favor stem and/or crown infection.

We hypothesized that at least some genes in the *F. pseudograminearum* genome that were either uniquely or predominately present in other cereal fungal pathogens may have specialized functions related to cereal pathogenesis and niche specialization. Throughout the manuscript the term ‘niche specialization’ is used to encompass other potential aspects of the biology of these fungi for which are poorly understood such as saprophytic colonization of dead plant material, non-pathogenic interactions with other plants or potential endophytism of other hosts. A broad comparative genomics analysis indicated that the *F. pseudograminearum* genome contains genes that have strong homology to genes that are unevenly distributed across cereal pathogens while being apparently absent in other fungal genomes. Some of these genes shared by cereal pathogens also encode proteins with significant similarity to those from plant-associated bacteria. This finding is consistent with multiple horizontal acquisition events and indeed phylogenetic analysis of selected genes supported the hypothesis of horizontal gene transfer into diverse cereal pathogens. Functional analysis of two potentially horizontally acquired genes revealed important roles in the virulence of *F. pseudograminearum* on cereal hosts. Our results illustrate an important role for horizontal gene transfer in the evolution of cereal-associated fungi.
the genome of \textit{F. pseudograminearum} isolate CS3096 was sequenced using a combination of paired and single-end Illumina reads. De novo assembly of these reads resulted in a nuclear genome size of 36.8 Mbp assembled in 656 contigs with 50% of all nucleotides in contigs of 189 Kbp in length or greater (AFNW00000000). The average sequence coverage across these contigs was 179-fold. Compared to many other fungal genome assemblies using next generation sequencing technologies, the \textit{F. pseudograminearum} genome sequence assembly has a relatively high N50 (Table 1). Gene model predictions from three programs were combined to identify 12,448 protein coding gene (see materials and methods).

The repeated sequence content of the \textit{F. pseudograminearum} genome, assessed using the RepeatMasker program, is only 1.6%, which is slightly higher than that of the \textit{F. graminearum} genome (0.7%) assessed using identical parameters, albeit that the sequencing and assembly methodology differed. RepeatMasker recognizes both simple sequence repeats and transposable elements present in RepBase [27]. Although approximately four times as many base pairs were flagged as being derived from Gypsy type Long Terminal Repeat (LTR) elements in the \textit{F. pseudograminearum} genome (26 Kbp) compared to \textit{F. graminearum} (6 Kbp), the difference in repetitive DNA content could mostly be attributed to a higher level of simple repeats and low complexity DNA (1.5% versus 0.41% of the genome, respectively). One high coverage contig in \textit{F. pseudograminearum} encodes an LTR-type retrotransposon with best match to \textit{F. oxysporum} transposon, probably present in 9–10 copies based on an average coverage of 1689 fold. This transposon matched to sequences in a number of different fungi and also to transposons in both monocot and dicot plants. The contig showed no polymorphism in the sequence read assembly across 5.5 Kbp, suggesting all copies are identical and thus could not be placed in other assembled contigs. Also not included in the overall repeat counts are the contigs that represent rRNA encoding genes. The Illumina sequencing approach used here was unable to resolve these repeats in \textit{F. pseudograminearum} and these currently appear in the assembly as high-coverage contigs.

The \textit{F. pseudograminearum} genome sequence assembly was also compared globally to that of \textit{F. graminearum} [28] by aligning the two genomes after masking simple repeat sequences and known fungal repetitive elements. In total, 89.8% of the \textit{F. pseudograminearum} genomic sequence could be aligned to the \textit{F. graminearum} genome at >70% nucleotide identity (Figure 1). An alignment with increased sensitivity was performed using a six frame translations of both genomes enabling an alignment of 94% of the \textit{F. pseudograminearum} genome to that of \textit{F. graminearum}. Thus, at least 6% of the low copy region of the genome (approximately 2.2 Mbp) appears to be completely unique to \textit{F. pseudograminearum}. Very few rearrangements between the \textit{F. pseudograminearum} and \textit{F. graminearum} genomes in the aligned regions were found (Figure 1A). The amount of aligned sequence between the two species decreases towards the ends of the \textit{F. graminearum} chromosomes (Figure 1B) and also in regions previously reported to be undergoing higher rates of genome innovation [20].

### Table 1. Summary of the \textit{F. pseudograminearum} genome sequence assembly in comparison to selected fungal genome sequences obtained by short-read sequencing and the reference genome for \textit{F. graminearum}.

| Species                  | Genome size (Mbp) | Technology | coverage | N50 (kb) | Reference   |
|-------------------------|-------------------|------------|----------|----------|-------------|
| \textit{F. pseudograminearum} | 37                | Illumina   | 179      | 180      | This work   |
| \textit{F. oxysporum} strain 5176 | 55                | 454        | 8 x      | 60       | [56]        |
| \textit{Pyrenophora teres} f. teres | 42                | Illumina   | *        | 26       | [77]        |
| \textit{Sordaria macrospora} | 39.9              | Illumina+454 | 95¹      | 117      | [78]        |
| \textit{Gremmeniella) clangera} | 30                | Illumina   | ~100     | 237      | [79]        |
| \textit{Puccinia striiformis} | 79                | Illumina   | 50       | 5        | [80]        |
| \textit{Fusarium graminearum} | 36.2              | Sanger (reference genome) | 10        | 5350     | [28]        |

¹not provided.

*85 x coverage with Illumina and 10 x with 454.

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and six of these 17 proteins were present only in a small number of other fungi (Table 2). The corresponding genes for these proteins are therefore good candidates for having been ancestrally acquired from bacteria. The GC content for each of these six genes was not obviously different to that of surrounding genes, possibly with the exception of FPSE_07765 (see below). Other candidates for cereal pathogen specificity, which to varying degrees showed distributions limited to cereal pathogens, were also identified through the BLAST matrix filtering. These are therefore also of interest with respect to putative functions in niche specialisation and virulence.

Figure 1. Alignment between *F. graminearum* and *F. pseudograminearum*. *F. graminearum* chromosomes are ordered in decreasing size. (A) Dot-plot representing a whole genome alignment between *F. graminearum* isolate Ph1 and *F. pseudograminearum* isolate CS3096. The alignment was generated with NUCmer, part of the MUMmer 3 comparative sequence analysis package. Sequences were pre-masked for known repetitive elements and simple repeats using RepeatMasker. The dot plot represents the best 1:1 alignment between the two genomes. Dots closest to the diagonal represent co-linearity between the two genomes. Red represents matches in the forward direction and blue is indicative of inversions in part of the mapped contig relative to the *F. graminearum* genome. (B) Distribution of gaps in the alignment between *F. pseudograminearum* and *F. graminearum*, relative to the *F. graminearum* genome. *F. graminearum* chromosomes were divided into 100 Kbp non-overlapping windows and the unaligned nucleotides in each window summed and expressed as a percentage.

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Table 2. *Fusarium pseudograminearum* genes that may be of bacterial origin and are candidates for roles in pathogenicity towards plants.

| Protein Name | Accession | BLASTp Score | e-value | Predicted Function | Best Bacterial Match Species | Number of Fungal Matches |
|--------------|-----------|--------------|---------|--------------------|-------------------------------|--------------------------|
| FPSE_00725   | 1099      | 63.2         | 0       | Amidohydrolase     | *Streptomyces violaceusniger* | 591                      |
| FpAH1        |           | 507          | 1       | Amidohydrolase     | *gamma proteobacterium*       | 416                      |
| FPSE_07765   | 1885      | 1673         | 1       | Aminotransferase   | *Micobacterium testaceum*     | 117                      |
|              |           | 1264         | 1       | Hydrolyase         | *Pathogen Segniliparus agsus* | 361                      |
| FPSE_07775   | 624       | 549          | 1       | NAD(P)H-dependent epimerase/hydrolase | *Oxalobacteraceae bacterium* | 403                      |
|              |           |              |         |                    |                               | 397                      |
| FPSE_11221   | 577       | 336          | 1       |                     |                               |                          |
|              |           |              |         |                    |                               |                          |
| FPSE_11233   | 1452      | 724          | 1       |                     |                               |                          |
|              |           |              |         |                    |                               |                          |

Proteins showing distribution limited to cereal pathogens in a BLAST matrix analysis were used as queries of the non-redundant protein database at NCBI and those for which specificity to pathogens was still evident and had strong matches in bacterial species, with a limited number of fungal matches were retained. Numbers indicate BLASTp bit scores from the reciprocal best blast hits. The score for the best bacterial blast hit is also shown.

* Cereal pathogen

*Number of fungal matches better than the first bacterial hit in GenBank's nr.

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Table 3. Examples of *Fusarium pseudograminearum* genes that may be of fungal origin and are candidates for roles in cereal pathogenicity.

| Fusarium pseudograminearum protein | Cereal pathogen | Putative function |
|-----------------------------------|----------------|------------------|
| FPSE_02381 299                    | Small secreted cysteine rich protein |
| FPSE_02497 1148 1044 677 632 654 | Choline dehydrogenase |
| FPSE_05718                        | Transcription factor |
| FPSE_05719 437                    | Cytochrome P450 monooxygenase |
| FPSE_05720                        | No reciprocal best blast hits in any fungi in the BLAST matrix. Encodes an amidase |
| FPSE_06956 365 254 113 133 98    | Small secreted cysteine rich protein |
| FpDLH1 541 502                    | Diene lactone hydrolase |
| FPSE_10646 274                    | Killer protein 4 homologues. Distributed in many ascomycetes. Calcium channel inhibitors |

Numbers indicate BLASTp bit scores from the reciprocal best blast hits.

*Cereals pathogen*

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towards plants. Examples of predicted genes from this latter category suggesting conserved roles in virulence towards cereals, and/or horizontal gene transfer are shown in Table 3. These candidate genes were subjected to more detailed analysis of sequence relationships as described below.

### Putative Functions and Sequence Relationships of Selected Cereal Pathogen Genes

Amongst the *F. pseudograminearum* genes of potential bacterial origin (Table 2), the intronless gene FPSE\_07765 and its orthologues in *F. graminearum* and *F. verticilliodioides* encode proteins highly similar to a bacterial protein with both aminotransferase and homoserine kinase domains. FPSE\_07765 is 75% identical at the amino acid level across the entire length to a protein from *Microbacterium testaceum*, which is a bacterial endophyte of a variety of plants including the cereals sorghum and maize [29,30]. Other fungal hits to this sequence in GenBank only align across less than half of the protein and at much lower identities, with the best fungal match in *Trichophyton verrucosum* at only 31% amino acid identity and with only partial query coverage of 44%. Phylogenetic analysis of FPSE\_07765 identifies a *Fusarium* sequence clade embedded amongst a range of related bacterial sequences and strongly supports an origin of this gene via horizontal acquisition from bacteria, with retention in the cereal infecting fusaria (Figure S1). The GC content of FPSE\_07765 was 58% compared to the genome average of 51.8±3.9% (±SD) for coding sequences.

**FPSE\_11233**, another *F. pseudograminearum* gene with possible horizontal acquisition, encodes a putative secreted hydrolase that has reciprocal best BLAST hits only in cereal pathogens among the 27 selected genomes as well as hits in GenBank to putative pectin- and xylosyl-hydrolases in bacteria (best match is 38% identical with an e-value of 1e\^{-125} to a *Streptomyces hygroscopicus* protein). Further manual database queries showed FPSE\_11233-like sequences in the cereal endophyte *Chaetomium globosum* and the *Brassica* pathogen *Leptosphaeria maculans*. This gene sequence was analyzed further because, although *L. maculans* is not a cereal pathogen, it causes blackleg disease of canola which is a common rotation crop used in cereal farming systems [31], and *L. maculans* is related to other cereal pathogens in the *Plectosporales* family [32,33]. Again, phylogenetic analysis supported the relatedness of these fungal genes to bacterial sequences (Figure S2). However, any potential acquisition from bacteria is clearly extremely ancient as the sequence seems to have undergone considerable vertical diversification within the fungi following acquisition from bacteria. This hypothesis is not only supported by the phylogenetic analysis but also computational predictions, suggesting that the position and number of introns in FPSE\_11233-like sequences are not conserved between fungal species (data not shown). The placement of the *L. maculans* sequence outside of the Dothideomycete clade in the phylogenetic analysis (Figure S2) may also indicate a more complex mode of inheritance within the fungi.

A number of other enzyme-encoding genes of putative bacterial origin were also identified in the *F. pseudograminearum* genome, including genes encoding a hydrolase of unknown specificity (FPSE\_07775) and a NAD+ dependent dehydrogenase/eptimerase (FPSE\_11221). Two putative amidohydrolase encoding genes (FPSE\_00725 and FPSE\_05717) that have clear bacterial homologues were also identified in this analysis. One of the amidohydrolase genes, FPSE\_05717 hereafter termed *FphAH1*, also appeared to have a clear homologue in *P. nodorum* but not in any other fungus, suggesting a potential role for this gene in cereal pathogenesis and an unusual evolutionary history. The genomic organization, sequence diversity and virulence function of *FphAH1* will be described in more detail later in the manuscript.

Many *F. pseudograminearum* genes also had homologues in other fungal cereal pathogens but no clear bacterial matches, suggesting that these genes are either laterally inherited or rapidly diversifying and therefore have been selectively retained only in a limited number of pathogenic fungi. Examples of these (Table 3) include three genes (FPSE\_06956, FPSE\_10646 and FPSE\_02381) encoding small secreted proteins (candidate effector molecules) that were present in *F. pseudograminearum* and *F. graminearum* as well as other cereal pathogens. FPSE\_06956 had orthologous matches in the *Magnaporthe* and *Fusarium* lineages but no other matches in the BLASTmatrix or GenBank. FPSE\_10646 is a member of the killer protein 4 family (PFAM09044) of toxins that were shown to be extensively laterally shared between multiple fungal lineages including non-pathogens [34]. FPSE\_02381 is a member of a two-gene family encoding small secreted cysteine rich proteins in *F. pseudograminearum* and *F. graminearum* and has strong, albeit non-reciprocal BLASTp matches in *M. oryzae*. The other member of this family in *F. pseudograminearum*, FPSE\_05488, had reciprocal best BLASTp hits only in cereal pathogens, with the exception of a single match in the canola pathogen *Colletotrichum higginsianum* in the BLASTmatrix analysis (Dataset S1). Three other gene products shown in Table 3 (FPSE\_05718, FPSE\_05719 and FPSE\_05720) were encoded in a gene cluster in the *F. pseudograminearum* genome. Interestingly, *FphAH1*, which is predicted to be of bacterial origin (see above and Table 2), also seems to be part of this cluster. The function of *FphAH1* will be described in more detail later in the manuscript.

Also shown in Table 3 is a gene encoding a putative dienelactone hydrolase (FPSE\_08136), hereafter termed *FpDLH1*, with very strong orthologues in *F. verticilliodioides* and *C. graminicola* with 98% and 88% identities, respectively at the amino acid level. The next best BLASTp match (69% identical) of *FpDLH1* in GenBank, which is not a reciprocal best hit, is to *FGSG\_00089* from *F. graminearum*. The absence of strong reciprocal matches in all other fungi suggests that this gene may have been either shared between, or independently acquired from another donor species, by these *Fusarium* and *Colletotrichum* species through horizontal transfer mechanisms. The genomic organization and function of *FpDLH1* will be described in more detail later in the manuscript.

### FpAH1, a Horizontally Acquired Amidohydrolase Gene

*FphAH1* encodes a putative amidohydrolase (Pfam domain PF07969). The best BLASTp match (e-value 2e\^{-141}, 98% identical, 94% similar at the amino acid level) of *FphAH1* was to a predicted protein from the wheat glume blotch pathogen *P. nodorum* (encoded by *SNOG\_04819* hereafter termed *PnAH1*) [35]. The lack of *FphAH1* homologues in other fusaria and presence in *P. nodorum* was confirmed via hybridization of a *FphAH1* probe to genomic DNA of various *Fusarium* and a *P. nodorum* isolates (Figure S3). The overwhelming majority of subsequent BLASTp matches of *FphAH1* in GenBank were to predicted proteins from bacteria (Table 4). The next fungal match after *P. nodorum* was to the *F. graminearum* protein *FGSG\_10599* (25% identical 2e\^{-141} but this was much weaker than the bacterial matches (Table 4). *FGSG\_10599* also had another much stronger, indeed nearly identical (97%) protein (FPSE\_00725) encoded in the *F. pseudograminearum* genome. The *FPSE\_00725/FGSG\_10599* orthologs also had next best matches in bacteria. Phylogenetic analysis of these two *F. pseudograminearum* amidohydrolases strongly supports the hypothesis that they are of bacterial origin (Figure 2).

The *F. graminearum* genome contains six entries in this class of amidohydrolases (*PF07969*), all of which contain predicted orthologous matches with other genes in the *F. pseudograminearum* genome (Table S2). In other fusaria, clear orthologous
relationships exist between the remaining five amidohydrolases with Pfam domain PF07969 found in *F. pseudograminearum*, although both *F. oxysporum* and *F. verticillioides* contain additional members in this class of amidohydrolases (Table S2). In *P. nodorum*, only four proteins fall into this class of amidohydrolases. Thus *FPSE_00725* and *FpAH1* encode two amidohydrolases with extremely restricted distribution in fungi and are most likely of bacterial origin. BLASTp analysis of the other *F. pseudograminearum*

Figure 2. Phylogram of fungal amidohydrolases found in *F. pseudograminearum* that appear to be of bacterial origin and a number of bacterial amidohydrolases. Fungal sequences are highlighted in grey boxes. Numbers on branches indicate approximate likelihood ratio test branch support values.

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Table 4. Summary of BLASTp analysis of FpAH1 versus the non-redundant protein database (nr) at NCBI.

| Hit number¹ | Accession# | Species | % aa identity (similarity) | coverage of query (%) | e-value |
|-------------|------------|---------|----------------------------|-----------------------|---------|
| 1           | EAT87210 (SNOG_04819) | *P. nodorum* | 88 (94) | 100 | ~0 |
| 2           | EED35590 | Gamma proteobacterium NOR51-B | 38 (58) | 96 | 1e⁻¹³⁶ |
| 3           | EIIG62154 | *Bradyrhizobium* sp. WSM1253 | 37 (55) | 98 | 1e⁻¹³¹ |
| 4           | AD851002 | *Conexibacter woeae* DSM 14684 | 38 (57) | 96 | 9e⁻¹²⁶ |
| 5           | ABZ07000 | uncultured marine microorganism *HF4000_ANIW93N21* | 26 (46) | 98 | 9e⁻⁴⁷ |
| 161        | XP_390775 (FGSG_10599) | Second fungal hit (*F. graminearum*) | 25 (42) | 96 | 1e⁻²¹ |
| >250       | EED21309 | Third fungal hit (*Talaromyces stipitatus*) | 24 (40) | 91 | 2e⁻²⁰ |

¹BLASTp performed 18-May-2012.
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amidohydrolases identified a much wider distribution in fungi with FPSE_00474, FPSE_02365, FPSE_03227 and FPSE_11444 having more than fungal 30 hits of greater strength than the best bacterial hit (Table S2). FPSE_05738 was less widely distributed with seven hits of greater score than the best bacterial hit (Table S2) and may also be a candidate for acquisition from bacteria.

The predicted FpAH1 protein of 570 amino acid residues was encoded by an uninterrupted open reading frame of 1710 bp that was confirmed by RNAseq analysis of cDNA (data not shown). The P. nodorum genome annotation for PnAH1 contained a single intron, but it is likely that this prediction was not correct as the PnAH1 genomic region has a single uninterrupted open reading frame. In the coding region, FpAH1 was conserved between F. pseudograminearum and P. nodorum with 89% identity at the nucleotide level and 174 bp upstream of the predicted start codon and 85 bp downstream of the predicted stop codon could also be readily aligned.

The chromosomal complement of F. pseudograminearum has not been characterized and therefore the location of FpAH1 in the F. pseudograminearum genome is unknown. FpAH1 is present near the end of a ~90 kb sequence contig, the first 70 kb of which aligns with the end of F. graminearum chromosome 1, as shown in Figure 3. Interestingly, the genes surrounding FpAH1 did not have clear orthologues in the F. graminearum genome. On the contig containing FpAH1, 28 genes (FPSE_05686 through to FPSE_05714, excluding FPSE_05712) had clear orthologues in F. graminearum. However, FPSE_05715 to FPSE_05720 did not. Furthermore, three of the gene products (FPSE_05718, FPSE_05719 and FPSE_05720) were also identified in the BLASTmatrix analysis to be cereal pathogen-specific, suggesting that parallel selection or coordinated acquisition may have affected BLAST matrix analysis to be cereal pathogen-specific, suggesting FpAH1 and FPSE_05720) were also identified in the P. nodorum genome annotation for PnAH1 contained a single uninterrupted open reading frame of 1710 bp that was confirmed by RNAseq analysis of cDNA (data not shown). The P. nodorum genome annotation for PnAH1 contained a single intron, but it is likely that this prediction was not correct as the PnAH1 genomic region has a single uninterrupted open reading frame. In the coding region, FpAH1 was conserved between F. pseudograminearum and P. nodorum with 89% identity at the nucleotide level and 174 bp upstream of the predicted start codon and 85 bp downstream of the predicted stop codon could also be readily aligned.

The chromosomal complement of F. pseudograminearum has not been characterized and therefore the location of FpAH1 in the F. pseudograminearum genome is unknown. FpAH1 is present near the end of a ~90 kb sequence contig, the first 70 kb of which aligns with the end of F. graminearum chromosome 1, as shown in Figure 3. Interestingly, the genes surrounding FpAH1 did not have clear orthologues in the F. graminearum genome. On the contig containing FpAH1, 28 genes (FPSE_05686 through to FPSE_05714, excluding FPSE_05712) had clear orthologues in F. graminearum. However, FPSE_05715 to FPSE_05720 did not. Furthermore, three of the gene products (FPSE_05718, FPSE_05719 and FPSE_05720) were also identified in the BLASTmatrix analysis to be cereal pathogen-specific, suggesting that parallel selection or coordinated acquisition may have affected this region (Table 3). The putative function and position of the genes in this region in F. pseudograminearum are shown in Figure 3. Of the remaining 40 genes on the end of chromosome 1 in F. graminearum, only eight encoded proteins with reciprocal best BLAST hits in F. pseudograminearum, and these were distributed across six different contigs in the F. pseudograminearum assembly (data not shown). In contrast, PnAH1 is ~100 kb from the end of supercontig seven of the P. nodorum genome and appears to be surrounded by genes encoding proteins conserved in other fungi. In both P. nodorum and F. pseudograminearum, the GC content in the region of the PnAH1 and FpAH1 was similar to that of other gene rich regions of the respective genomes and some of the surrounding genes in the regions had introns. In summary, the genomic region containing FpAH1 has no equivalent sequence at the syntenic location in the F. graminearum genome nor the region containing PnAH1 in the P. nodorum genome.

**AH1 Sequence Phylogenies Indicate Ancient and Independent Horizontal Transfers into Fungal Pathogens**

The lack of close orthologues of FpAH1 and PnAH1 in other fungi and the presence of similar genes in bacteria (Table 4) based on BLASTp searches suggested an origin for these fungal genes via horizontal acquisition from bacterial species. Fusaria belong to the order/class Hypocreales/Sordariomycetes while Phaeosphaeria is in the distantly related Pleosporales/Dothidiomycetes. Acquisition of the gene may have occurred independently in both species or alternatively was horizontally transferred between the fungal species. To differentiate these possibilities, the sequence diversity of each gene was assessed in several globally sourced Fusarium and Phaeosphaeria isolates (Table S3). Isolates identified as F. pseudograminearum by multi-locus DNA sequencing [22] or by sequencing the elongation factor 1 alpha (EF1α) gene, were the only Fusarium sp. out of six tested that produced positive amplicons. PCR analyses of the distribution of FpAH1 in fusaria were confirmed for a limited number of isolates by hybridization analysis (Figure S5). PnAH1 sequences were PCR amplifiable from all P. nodorum isolates tested.
and also from two of four sister species, *P. avenaria f. sp. tritici* (Pat1) and Pat3, suggesting *PnAH1* was present in a common ancestor in this lineage.

In order to test the hypothesis of horizontal transfer between *F. pseudograminearum* and *P. nodorum*, a 500 bp region of the *AH1* gene was sequenced in isolate collections. A haplotype network showing the sequence relationships across both genera is shown in Figure 4. In *F. pseudograminearum*, seven haplotypes observed did not correspond to the geographic origin of isolates, consistent with global gene flow within the species as previously described [22]. Diversity in the *Phaeosphaeria* spp. sequences was more limited with only one haplotype observed in a global sample of *P. nodorum* and two haplotypes detected within each of the two *Phaeosphaeria* sister species containing *PcAH1* orthologues. There were no shared sequence haplotypes between *Phaeosphaeria* spp. and *F. pseudograminearum*. The presence of *AH1* orthologues in up to four related *Phaeosphaeria* spp. suggests that the acquisition of this gene occurred before the divergence of these species. Divergence of *PnAH1* between the *Phaeosphaeria* spp. was comparable to divergence observed at neutral sequence loci (M.C. McDonald, unpublished data). Furthermore, the haplotypes observed in the *Phaeosphaeria* species complex and *F. pseudograminearum* were distinct, suggesting independent acquisitions of *AH1*-like sequences by each lineage.

![Figure 4. Statistical parsimony haplotype network for *FpAH1*/*PnAH1* in *Fusarium pseudograminearum* and *Phaeosphaeria* spp. Haplotype bubbles are proportional to sample size shown in parentheses. Dots on line segments indicate single point mutations.](doi:10.1371/journal.ppat.1002952.g004)
FpAH1 Contributes to Virulence on Wheat and Barley

The likely independent acquisition and retention of AH1 orthologues by *F. pseudograminearum* and *P. nodorum* suggests that this gene may encode a protein that is necessary for virulence, at least in some hosts. To test this hypothesis, a functional analysis of the *FpAH1* and *PnAH1* genes was undertaken. *FpAH1* was expressed in both infected barley and wheat leaves and roots (Figure S4). The role of the *FpAH1* amidohydrolase in FCR of barley was also assessed by generating gene deletion mutants in *F. pseudograminearum* by replacing 635 nucleotides of the *FpAH1* locus with a geneticin resistance gene cassette (Figure S5A). There were no obvious defects in appearance or sporulation of the mutants. A culture time-course was used to compare the growth rates of one mutant to that of the parental strain and these were indistinguishable (Figure S5C). As shown in Figure 5, two independently-derived knock-out mutants consistently showed reduced virulence towards barley (cv. Golden Promise) across multiple independent experiments using a previously established FCR inoculation assay [36]. A second barley cultivar (cv Gairdner) also showed similar reduced virulence (data not shown). FCR disease severity caused by the mutant strains was significantly (*P*-value < 0.01) reduced compared to those caused by the wild type.

**Figure 5. Virulence assay of the *Fusarium pseudograminearum* amidohydrolase 1 mutants (∆FpAH1) compared to the parental strain towards barley cultivar Golden Promise at 21 days post inoculation (dpi) (A–B).** (A) Survival of plants in the assay 21 dpi. N = 3 with each replicate consisting of 13–15 plants divided between two paper towel rolls and maintained in separate vessels. The difference between wild type and each mutant was highly statistically significant (*P*-value < 0.01). (B) Representative rolls of plants from the assay. (C–D). Complementation of ∆FpAH1 (strains 96T681 and 96T682) restores virulence towards barley cultivar Golden Promise. (C) Proportion of plants surviving at 13 dpi. The total number of plants in each case was 30. (D) Photograph of assays at 13 dpi. doi:10.1371/journal.ppat.1002952.g005
strain. Genetic complementation of an FpAH1 mutant with a cassette containing FpAH1 under the control of the Aspergillus nidulans Tp6C promoter restored virulence towards barley (Figure 5C and 5D), providing further evidence that FpAH1 is required for virulence against barley.

*F. pseudograminearum* has a wide host range within cereals with no evidence for race specialization [37,38] for FCR disease on wheat. Unlike the experiments conducted on barley, highly replicated evidence for race specialization for FCR disease on wheat.

nidulans TrpC promoter restored virulence towards barley FpAH1 (Figure 5C and 5D), providing further evidence that FpAH1 is not pathogenic on barley and therefore was not tested on this host but mutant strains of this pathogen with deletions of the FpAH1 gene were also generated (Figure S5D) and tested on wheat plants in replicated leaf infection assays. No significant differences in virulence were observed between mutant and wild type strains on wheat (Figure S7). These experiments indicate that FpAH1 is required for full virulence on both wheat and barley but the importance of FpAH1 in pathogenesis remains unknown.

**Novel Genomic Context and Virulence Function on Cereals for the FpDLH1 Gene**

The *F. pseudograminearum* locus FpDLH1 encoding a dienelactone hydrolase was identified in the BLASTmatrix analysis as a cereal-pathogen associated gene with homologues detected only in *F. verticillioides* and *C. graminicola*. Strikingly, the gene (FPSE_08131 hereafter termed FpAMD1) immediately adjacent to FpDLH1 in the *F. pseudograminearum* genome encodes an amidase that is also present in the same divergently transcribed arrangement in the genomes of *F. verticillioides* and *C. graminicola* (Figure 7), indicating this is likely to be a two-gene cluster. The amidase family is much larger in ascomycetes than the dienelactone hydrolase family, making one-to-one orthologous relationships between species difficult to detect. However, for both FpAMD1 and FpDLH1, strong homology is detected between all three species at the nucleotide level (>80%) across the coding sequences of these genes. Furthermore phylogenetic analysis also supports the grouping of both of these genes in the three species in clades incongruent with expected evolutionary relationships (Figure S8). Thus, their genomic arrangement, discontinuous distribution in the fungi and strong homology to each other all suggest these genes represent a two-gene cluster that may have a common origin.

The FpDLH1 gene has the same intron-exon structure as another dienelactone hydrolase that is present in both *F. pseudograminearum* and *F. graminearum* (FPSE_08131 and FGSQ_000089, Figure 7). This intron arrangement is not a generic feature of dienelactone hydrolase encoding genes in *F. pseudograminearum*, indicating a possible gene duplication event in the Fusarium lineage. Furthermore, FPSE_08131 had reciprocal best BLAST hits in all Fusaria included in the analysis, with the exception of *F. solani*, as well as in two Magnaporthe spp., and the intron-exon structure was maintained in all species. The synteny in this region is also well conserved in the *F. pseudograminearum*-F. graminearum comparison (albeit split into two regions that align near the ends of different chromosomes in *F. graminearum*) but outside of this comparison the synteny weakens with the orthologues of the genes flanking this two-gene cluster in *F. pseudograminearum* appearing on multiple different contigs in *F. verticillioides* (Figure 7). In the *F. verticillioides*-F. pseudograminearum comparison, the conservation of FpDLH1/FvDLH1 (98% identical at the amino acid level) is much greater than the more widespread dienelactone hydrolase (FPSE_08131 and its *F. verticillioides* orthologue FVEG_12623) at 75% identity, suggestive of either an intra-fusarium transfer or strong conservation and selection. In *C. graminicola* the DLH1-AMD1 gene cluster is located on chromosome 2 in a region where only two of 11 genes flanking the cluster have clear orthologous relationships between *C. graminicola* and the closely related *C. higginsiana* (data not shown). However, three of the nine genes on one flank of the *C. graminicola* DLH1-AMD1 gene cluster have orthologues present in the flank adjacent to FpDLH1-FpAMD1 (Figure 7). These genomic associations suggest an ancient relationship in these regions.

Both FpDLH1 and FpAMD1 are expressed during infection of root by *F. pseudograminearum* and leaf tissue with higher expression in wheat than in barley (Figure S4). The role of FpDLH1 in fungal pathogenesis was assessed by creating knockout strains of two different *F. pseudograminearum* strains (CS3096 and CS3427), where the FpDLH1 gene was replaced by the geneticin resistance cassette (Figure S9). No obvious differences in sporulation were observed in the mutants nor were there differences in growth rate compared to their respective parents (Figure S9C). However, FpDLH1 mutants in both strain backgrounds showed significantly reduced virulence towards both wheat and barley in both root-rot and FCR assays (Figure 8 and Figure S10), suggesting that FpDLH1 contributes to fungal virulence against cereal plants.

**Discussion**

The new DNA sequencing technologies are well suited to characterizing low copy, gene-rich regions of fungal genomes that are relatively small in size. Using Illumina technology it was possible to assemble de novo an almost complete sequence of the *F. pseudograminearum* genome. A large proportion (~94%) of the *F. pseudograminearum* genome showed high similarity to *F. graminearum* and a great deal of co-linearity was observed. Alignment of the *F. pseudograminearum* genome sequence to the chromosomes of *F. graminearum* revealed that regions of poor sequence match were concentrated in specific regions, such as the ends of chromosomes and what are thought to be regions of ancient chromosome fusion in this Fusarium lineage and probable regions of genome innovation [2,28]. Although more strains will need to be sequenced to confirm the species-specificity of these regions of the *F. pseudograminearum* genome, it is possible that the genes contained in these regions may be responsible for various phenotypes that distinguish *F. pseudograminearum* from *F. graminearum*, such as its propensity to cause FCR rather than FHB, its broad geographical adaptation in arid cereal production areas [39].

We hypothesized that genes in the *F. pseudograminearum* genome that were either uniquely or predominantly present in other cereal fungal pathogens may have a specialized function related to cereal pathogenesis and niche specialization. To identify these genes, we undertook a BLASTmatrix analysis and found that many genes present in the *F. pseudograminearum* genome were also present exclusively in cereal pathogens. 214 of these genes appeared to be conserved in the three cereal-infesting Fusaria but had no equivalent matches in the genomes of three Fusarium that infect dicotyledonous plants. These genes may have undergone specialized selection in these cereal pathogen *Fusarium* lineages but been diversified or lost in other fusaria. Several *F. pseudograminearum* genes also appeared to have equivalents in cereal pathogens outside the fusaria, and these were present mostly in other necrotrophic or hemibiotrophic Ascomycete fungi. Two of these genes, encoding an amidohydrolase (FpAH1) and a dienelactone hydrolase (FpDLH1) were selected for functional analysis in *F. pseudograminearum*. In both cases, we demonstrated roles in
Figure 6. Fusarium root-rot virulence assay of the *Fusarium pseudograminearum* amidohydrolase 1 mutant (ΔFpAH1) towards wheat cultivar Kennedy (A and B) and barley cultivar Golden Promise (C and D). N = 16 individual plants. CS3096 is the parental strain for the FpAH1 mutant (96T492), which was complemented with FpAH1 driven by the TrpC promoter (96T681). Error bars represent the standard error of the mean. Different letters on A and C indicate statistically significant differences (P<0.05) in pair-wise t-tests. B and D are the plants used to score the shoot length shown in A and C.

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Our analysis identified genes in the genome of *F. pseudograminearum* with matches in bacterial genomes, and a number of genes were restricted to other fungal pathogens of cereals. These findings are consistent with possible acquisition of these genes by horizontal transfer. These observations also suggest considerable genome plasticity in *F. pseudograminearum* and provide a number of candidates for further study of potential horizontal acquisition.
Compelling evidence for acquisition of a gene of bacterial origin and retention in cereal-infecting fusaria is illustrated by FPSE_07765 encoding a putative aminotransferase. Most matches to this aminotransferase were from bacteria, with the closest match showing a remarkable 75% amino acid identity to a predicted protein in the genome of Microbacterium testaceum, a common bacterial endophyte of cereals. Horizontal transfer from co-inhabiting endophytic bacteria into the Fusarium lineage with selective retention in cereal pathogens is a simple explanation for these strong, but restricted gene homologies and organismal relationships. Another significant bacterial match was the putative cell wall hydrolase encoded by FPSE_11233, with equivalent proteins present in many cereal pathogens. In this case, phylogenetic analysis indicated that all identified proteins from

Figure 8. Virulence assay of the *Fusarium pseudograminearum* dienelactone hydrolase 1 mutant (ΔFpDLH1) mutant towards wheat cultivar Kennedy in crown rot (A and B) and root rot (C and D) assays. For A and B N = 3 with each biological replicate consisting of 4 paper towel rolls consisting of 7–8 plants per roll. For C and D N = 15–16 plants. Error bars represent the standard error of the mean. Letters indicate statistically significant differences at P<0.05. CS3096 and CS3427 are the parental strains for the FpDLH1 mutants, 96T926 and 27T892 respectively. Mock treatments are inoculations performed with agar plugs that have not been colonized by *Fusarium*.
doi:10.1371/journal.ppat.1002952.g008
diverse fungal species clustered into a single clade. The biased occurrence of this gene in plant pathogens is likely due to selective retention after an ancient acquisition event during fungal evolution.

*FpAH1* represents a striking example of likely horizontal movement of a gene from a bacterium into the *F. pseudograminearum* genome. The only closely related gene to *FpAH1* in the fungi examined was *PsAH1* found in the genome sequence of *F. nodorum*, a pathogen of wheat. Sequencing of several globally distributed isolates of *F. pseudograminearum* revealed several distinct haplotypes for this gene. This suggests that acquisition of *FpAH1* was not recent or that there has been significant selection driving the creation of new alleles. There was no evidence of horizontal transfer directly between *F. pseudograminearum* and a *Phaeosphaeria* spp. The limited divergence of *PsAH1* between the *Phaeosphaeria* spp. orthologues, suggests that a common ancestor of these *Phaeosphaeria* species acquired the gene. The presence of the gene in at least two lineages of cereal pathogens suggests that the gene may play an important role in wheat pathogenesis.

Both *FpAH1* and *PsAH1* were clearly dispensable for growth and their retention in two otherwise unrelated fungal pathogens suggests a specialized function for these genes in fungal virulence. Indeed, a virulence function for *FpAH1* against two different cereal hosts was confirmed. However, *PsAH1* knockout strains did not show altered virulence against wheat. Pathogenesis in any one species is the sum of many different components and the relative contribution of these genes to pathogenesis in the different species may be substantially different. The genomic context of the genes in these two fungal pathogens was also very different. *PsAH1* is located in a region of the *F. nodorum* genome adjacent to several conserved genes. In contrast, *FpAH1* occurs at the end of a long contig in a cluster of genes found in other cereal pathogens, but not in *F. graminearum*, and it could be that *FpAH1* is functioning in *F. pseudograminearum* as part of this group of genes. It appears that most of the genes on the end of chromosome 1 in *F. graminearum* are absent from *F. pseudograminearum*. This observation provides further support to the notion that chromosome ends or ancient chromosome fusion sites are regions of genome innovation in the *Fusarium* lineage [28] and regions like this may have played a role in niche separation between *F. pseudograminearum* and *F. graminearum*. The role and reason for retention of *PsAH1* in *F. nodorum* however, remains unknown.

The two-gene cluster represented by *FpDLH1*-*FpAMD1* is likely to be of fungal origin in the genomes of *F. pseudograminearum*, *F. verticillioides* and *C. graminicola*. A comparison of the genomic context of the *DLH1*-*AMD1* genes in *F. pseudograminearum* and *C. graminicola* also supports a common origin with some related genes being present in flanking regions of this cluster in both pathogens. The conservation (~80%) of the nucleotide sequence across the coding regions of both genes between *C. graminicola* and the two *Fusarium* spp. suggests that any possible genetic exchange event between these lineages is ancient and has thereby allowed accumulation of considerable sequence divergence. The close physical proximity in the *F. pseudograminearum* genome of *FpDLH1* to another dienelactone hydrolase encoding gene, *EPSE_08131*, with identical intron-exon structure and with orthologues in other fusaria, suggests *FpDLH1* may have arisen vertically by gene duplication and subsequent diversification within the *Fusarium* lineage. The *FpDLH1*/*FvDLH1* and *FpAMD1*/*FvAMD1* orthologues of *F. pseudograminearum* and *F. verticillioides* show remarkable identity (98%) at the amino acid level but very different genomic context. However, currently, it is not possible to resolve whether an inter-species transfer had occurred between the *Fusarium* lineages, or alternatively whether strong *DLH1* and *AMD1* gene conservation with regional genomic rearrangements had occurred in these two cereal infecting fusaria. Further diversity surveys will be required to resolve the origin of *FpDLH1* and *FpAMD1* related genes in these cereal pathogens.

Both *FpAH1* and *FpDLH1* are thought to be catabolic enzymes based on conserved domain matches. Amidohydrolases are a diverse superfamily of enzymes that catalyze the hydrolysis of C-N bonds in small molecules. This family includes enzymes functioning in central metabolism (*eg* urease), enzymes that degrade xenobiotics (*eg* atrazine) as well as those that are known to catalyze reactions other than C-N cleavage, including P-O cleavage and also isomerisation [40]. Likewise, the dienelactone hydrolase family of enzymes appears to be large, with members involved in the degradation of chloroaromatic compounds [41,42] by bacteria and activation of produgs containing lactone-like side chains in humans [43]. The specific biochemical roles of *FpAH1* and *FpDLH1* during this particular host-microbe interaction remain elusive. However, the predicted catabolic activity of these enzymes suggests they could be either targeting specific plant defense compound(s) or involved in production of fungal toxin(s). Although the defensive compounds important in the response to *F. pseudograminearum* are currently unknown in barley and wheat, candidates may include hordatine, hordeine and gramine in barley and the benzoxazolinones in wheat [44–47]. All these compounds contain C-N bonds and are known to have antifungal properties [44–47]. However, fungal growth inhibition assays conducted in liquid culture medium with synthetic hordatine and gramine showed that while *F. pseudograminearum* is moderately sensitive to both compounds, *PsAH1* knockout strains were equally as sensitive as the wild type (data not shown). Likewise, the *FpDLH1* mutants were as sensitive as the wild-type to the benzoxazolinones, BOA (2-3H-Benzoxazolinone) and MBOA (6-methoxy-2(3)-benzoxazolinone). However, in *F. verticillioides* BOA detoxification is a two-step process with only one of the two responsible genes having been cloned [48]. A more detailed understanding of the barley and wheat metabolites involved in defense against *F. pseudograminearum* would be an important starting point to identify molecular mechanism(s) of *FpAH1* and *FpDLH1* mediated virulence.

Genomics is allowing increased discovery of examples of likely horizontal gene transfer events [6,7]. Our work provides additional evidence for this hypothesis although detection of horizontal gene transfers, particularly of ancient events, requires robust phylogenetic analyses [3,7,49]. The extent of the horizontal gene transfer phenomenon has not yet been fully ascertained amongst the sequenced fungal genomes but there will presumably be many more examples. A robust methodology needs to be developed to enable identification of horizontally acquired genes on a scale of kingdoms and beyond. The BLASTMatrix analysis presented here is one possible method for identifying these genes, although it is limited in its ability to cope with post-acquisition family expansion. It is also important to note that the analysis reported here was centered on *F. pseudograminearum* and limited by the species included in the comparative analyses. A broader systematic analysis for each fungal pathogen genome is warranted to obtain a more complete perspective of potential gene sharing and its relation to host range and virulence.

In summary, in this paper we report the first genome sequence for *F. pseudograminearum* and demonstrate that a broad comparative genomic analysis can identify genes that show a biased distribution in fungal pathogens of plants with putative roles in virulence processes or niche specialisation. Functional analysis in *F. pseudograminearum* of two such genes demonstrated novel virulence functions on cereals.
Materials and Methods

Fungal Isolates and Culture

The *F. pseudograminearum* isolate (CS3096) chosen for genome sequencing was originally isolated from a wheat crown collected in 2001 near Moree in Northern New South Wales, Australia [26]. Isolates for phylogenetic analysis of the *FbH1* gene were selected from a collection housed at CSIRO Plant Industry, Brisbane, Australia, consisting of isolates from Australia, New Zealand, Canada, United States of America and Turkey (Table S3). *Phaeosphaeria* isolates were selected from a collection housed at ETH Zurich and the Australian isolate SN15.

Sequencing and Assembly

Two lanes of single-end 75 bp and one lane of 100 bp paired-end sequencing was performed on an Illumina GAII, genome sequencer by the Australian Genome Research Facility, Melbourne Australia. Reads were imported into CLCBio Genomics Workbench 3 and quality trimmed (default parameters) prior to assembly. A total of 60 million paired reads and 33 million single end reads were used in *de novo* assembly plug in (version 3.03), again using default parameters with a minimum contig size of 500 bp. Low coverage (<40×) contigs (compared to the genome average of 179×) were excluded from further analyses. BLASTn comparison to the *F. graminearum* mitochondrial sequence and coverage information (3,500–6,000×) was used to separate nuclear and mitochondrial sequence. 29 contigs with total length 107 Kbp were identified in this process and excluded from the genome annotation but are included in the *F. pseudograminearum* genome submission to GenBank. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AFNW00000000. The version described in this paper is the first version, AFNW01000000.

Comparison to the *F. graminearum* Genome

Repeat masking was performed using RepeatMasker version open-3.2.8 [50], run in sensitive mode with cross_match version 0.990329 as the search engine and RepBase version 20090604 [27]. The species descriptor “fungi” was used.

Whole genome alignments were performed with the NUCmer and PROmer algorithm in the MUMmer package [51]. The minimum cluster length was set to 100 bp. The total length of the genome alignment between *F. graminearum* and *F. pseudograminearum* was calculated by summing the length of all non-redundant alignments extracted by using the show-coords program (part of the MUMmer package).

Gene Prediction

Protein coding genes were *ab initio* predicted in the *F. pseudograminearum* genome using FGENESH [52] based on the *F. graminearum* gene models as part of the MolQuest2 package from Softberry, AUGUSTUS [53] and GeneMark-ES version 2 [54]. AUGUSTUS was run with a training set of *F. graminearum* genes that had 100% nucleotide alignment and identity to the *F. pseudograminearum* genome. When the three programs disagreed in the splicing for the same gene, the following strategy was used to prioritize the predictions. When two of the three programs agreed, this prediction was taken. When all three programs disagreed for what appeared to be the same gene, priority was given in the order FGENESH, GeneMark and AUGUSTUS. Predicted transcripts encoding <20 amino acids were removed manually from the main prediction set. In total 17,503 unique transcripts were predicted, which after removing 4833 alternative predictions for the same gene left a putative set of 12448 unigenes.

BLASTMatrix Pipelines to Identify Genes Specific to *F. pseudograminearum* or Discontinuously Distributed in Fungi

The proteins coded by 27 different fungal genomes were downloaded from the Broad Institute (www.broadinstitute.org), Joint Genome Institute [55] or NCBI for the *F. oxysporum* strain 5176 genome [56]. Reciprocal best BLASTp hits were used to determine the orthologous protein relationships between *F. pseudograminearum* and each of the 27 fungal protein sets. This was performed using a pair-wise all versus all BLASTp analysis was conducted on the National Computational Infrastructure Specialized Facility in Bioinformatics Cluster located at the University of Queensland, Australia. Perl scripts obtained from http://sysbio.harvard.edu/csb/resources/computational/scriptome/unix/Protocols/Sequences.html were used to extract best reciprocal hits and the associated blast score from each species comparison BLASTp outputs. The *F. pseudograminearum–F. graminearum* relationship was treated as a special case and used to curate the dataset. In total, 11481 genes had clear one-to-one relationships between the two species, but differences in gene prediction (eg splice sites or predictions split/fused with respect to each other in each of the genomes) resulted in false identification of genes without orthologous matches in this analysis. These were accounted for by performing BLASTn analysis using *F. pseudograminearum* gene sequences containing introns as the query against the *F. graminearum* genomic sequence. Transcripts showing strong matches to the *F. graminearum* genome (match length greater than 50%) were classified as having different annotation between *F. graminearum* and *F. pseudograminearum*. 456 genes were removed from the analysis using this process.

Phylogenetic Analyses

Phylogenetic analyses were implemented using phylogeny.fr [57]. Briefly, multiple alignments were generated using MUSCLE [58] with default parameters, and curated using Gblocks [59]. Phylogeny was performed using PhyML [60] with the WAG amino acid substitution matrix [61] using an approximate likelihood-ratio test for branch support [62]. Trees were drawn using TreeDyn [63]. Trees were exported to Adobe Illustrator to allow shading of fungal branches.

Hybridization Analysis

A slot blot membrane was prepared using a Hoefer PR 648 apparatus using Amersham Hybond-XL membrane (GE Healthcare) prewetted with 0.2 M NaOH. Approximately 500 ng of DNA was prepared in 0.2 M NaOH to a final volume of 60 μL and incubated at 37°C for 15 min prior to application to the membrane. Following application of the DNA the membrane was cross-linked using a GS GeneLinker UV cross-linker (BioRad) with...
program TCS v1.2 was used to visualize the most-parsimonious protein haplotypes was also performed using this software. The sequences for each isolate was performed in SeqScape software Analyzer (Applied Biosystems). Alignment of forward and reverse 6

products were cleaned with the illustra Sephadex G-50 fine DNA PLOS Pathogens | www.plospathogens.org 18 September 2012 | Volume 8 | Issue 9 | e1002952

for gene deletion using AHKOscr1, AHKOscr2 and gpdAr prepared using the REDextract and Amp kit as per the (Fermentas), 0.4

m

Taq

DNA polymerase (Fermentas). For

FpAH1

Construction of

FpAH1

mutant 96T492 with pAN7.1 [70] and a construct described to expresses

FpAH1

mutant was complemented by co-transformation of

F. graminearum

described [69].

Complementation of

FpAH1

Mutant

The

ΔFpAH1

mutant was complemented by co-transformation of

F. pseudograminearum

targeting construct consisted of 1858 bp of sequence

described [68] and screened by using primers designed outside the

flanking DNA (PnAH1KO3’F and PnAHKO3’R amplified a 1519 bp region

downstream. These flanks were fused using overlap PCR with a

gyromycin resistance cassette amplified from pAN7.1, resulting in a deletion construct of 5.9 kb. The deletion cassette was transformed into

P. nodorum

isolate SN15 protoplasts as previously described [68] and screened by using primers designed outside the

flanking DNA (PnAH1KOScr-F and PnAH1KOScr-R). Copy number of the transformed construct was determined as previously described [69].

Growth Rate Determination

A microtiter plate assay to monitor growth was performed based on the method described by Schmelz [74]. Briefly, each well contained 200 μL of basal media [72] with 5 mM glutamine as the nitrogen source with a final spore concentration of 1 x 10^4 spores mL^{-1}. Absorbance at 405 nm was measured using an iEMS microplate reader. For the

FpAH1

mutant test the plate remained in the instrument for the duration of the assay at ambient temperature. In the case of the

FpDLH1

mutant assay after an initial reading the plate was incubated at room temperature and readings commenced 22 hours post inoculation.

Fusarium Crown Rot Virulence Assays

Assessment of fungal virulence during FCR was carried out using the method described previously [36]. For virulence testing of

FpAH1

mutant and complemented strains, isolates were inoculated on to 14 cm Synthetischer Na¨hrstoffarmer Agar plates from potato dextrose agar plugs stored as water cultures and allowed to grow for 14 days under 12/12 hour day/night cycle under white and black fluorescent light at room temperature (~22°C). Spores were harvested by flooding the plates with water
and adjusted to the same concentration. To test the role of \textit{FpDLH1} in virulence, spores were produced in shaking 1 L flasks containing 100 mL of 25\% Campbell’s V8 juice inoculated with a single plug taken from a 20\% V8 juice plate. Flasks were incubated at room temperature for 8 days and spores harvested by filtration through miracloth followed by centrifugation (5,500 x g) to remove media. Spores were resuspended in sterile water, counted and adjusted to the same concentration. Seeds were plated out two days prior to inoculation on wet paper towel in a 14 cm Petri dish and allowed to germinate on the laboratory bench. Germinated seeds were transferred to a 50 mL falcon tube containing 2 mL of the spore suspension and rolled gently to coat the seeds. Six to ten seeds were placed in a single paper towel, rolled up and tapped closed and placed in a jar containing water. Paper towel rolls were kept moist throughout the experiment. For the analysis of \textit{FpAH1} mutants a concentration of 1 x 10^5 spores mL^{-1} was used and plants were maintained on a laboratory bench without supplementary lighting. To test complemented plants were maintained on a laboratory bench without supplementation. \textit{FpAH1} kept moist throughout the experiment. For the analysis of mutants a concentration of 1 x 10^5 spores mL^{-1} was used and plants were maintained in a closed bench top biohazard cabinet with a cool mentary lighting. To test complemented plants were maintained on a laboratory bench without supplementation.

\textit{Fusarium} Root-Rot Virulence Assays

Fungal strains were plated from PDA plug water storage onto 20\% Campbell’s V8 juice 1.2\% agar plate and incubated for seven days at room temperature under white and black fluorescent light on a 12/12 hour day/night cycle. Seed for the assay were surface sterilized by soaking for 5 minutes in a 0.64\% sodium hypochlorite-10\% ethanol solution, followed by several rinses in sterile distilled water. Seed were plated onto three sheets of pre-wetted Whatmann no 3 12.5 cm filter disks in a 14 cm Petri dish. Seeds were incubated in the dark for 5 days at 4°C prior to germination at 20°C also in the dark. Germinated seedlings were distributed to Petri plates assembled with three filter papers wetted with 20 mL of sterile water prior to inoculation. Each plate contained 15–16 seedlings and was used for inoculation with one isolate. Inoculum consisted of agar plugs taken using either an inverted 1-mL pipette tip or a number 4 (6 mm) cork borer from the edge of the growing V8 agar plate. Plugs were placed on a single root per seedling about 1 cm below the seed with the mycelia in direct contact with the root. Plates were sealed with sealing film (PhytoTechnology Laboratories), incubated in a Thermoline illuminated incubator for 6 days at 20°C with 12 hours of light provided by fluorescent lamps. Assays were scored by measuring the length of the shoot.

\textit{Phaeosphaeria nodorum} Virulence Assays

Parental and mutant strains of \textit{P. nodorum} were tested on the susceptible wheat cultivar Amery as previously described [75]. Latent period sporulation was assessed using detached leaves as described previously [76].

\textit{In planta} Expression Assays

Gene expression during infection of wheat and barley by \textit{F. pseudograminearum} was performed using both a detached leaf infection assay and a root infection time course. For the latter, plants were inoculated as described for the root-rot virulence assay and root segments were harvested into liquid nitrogen at 24, 48 and 96 hours post inoculation. For expression in detached leaves, fourth leaf segments (7–8 cm long) of both hosts were taken from glasshouse-grown plants and each end of the cut leaf was sandwiched between water agar in a 14-cm Petri plate. Leaf segments were pierced at two points in a central region using a 200 \mu L pipette tip. A spore suspension of the wild type isolate (1 x 10^5 sp mL^{-1}) was placed on the wound sites and the plate was sealed with sealing film. Plates were maintained in a fluorescent bulb lit growth chamber (Thermoline) at 20°C with 12 hours of light. The whole leaf segment was harvested for RNA extraction. RNA extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Relative expression was compared to \textit{β-tubulin}. Primers used for gene expression analysis are shown in Table S4. qRT-PCR was performed as previously described [73].

Supporting Information

Dataset S1 BLASTmatrix analysis of \textit{F. pseudograminearum} proteins against 27 fungal species. The BLAST matrix contains the reciprocal best hit information in each of the 27 genomes for all 12448 \textit{F. pseudograminearum} proteins. The identifier, percentage identity, match length, e-value and bit score are given for each of the hits. The FpSpecific156 tab contains the locus identifiers for all 156 \textit{F. pseudograminearum} proteins that had no reciprocal best BLASTp hits in any of the 27 species. The FilteredBLASTmatrix239proteins tab contains the cereal pathogen specific set of 239 proteins and the bit score information for the matches across the 27 organisms. (XLSX)

Figure S1 Phylogenetic analysis of FPSE_07765 homologues in two other fusarias and bacteria. The next best match in any fungi in GenBank (\textit{Triticophyton verrucosum}) has been used as an out group. Fungal sequences are highlighted in grey boxes. Numbers on branches indicate approximate likelihood ratio test branch support values. (EPS)

Figure S2 Phylogram of FPSE_11233 orthologues in fungal and bacterial species. No additional fungal matches were identified in GenBank other than those shown here at the e-value cut off of e^-9. Fungal sequences are highlighted in a grey box. Numbers on branches indicate approximate likelihood ratio test branch support values. (EPS)

Figure S3 Slot blot hybridization of the \textit{FpAH1} gene sequence to six \textit{F. pseudograminearum} isolates (CS3096, CS3220, CS3270, CS3427, CS3487, and CS5834), five other fusaria (\textit{F. graminearum} Ph1 and CS3005, \textit{F. equiseti} CS3069, \textit{F. acuminatum} CS5907 and \textit{F. culmorum} CS7071), and \textit{P. nodorum} (isolate SN15). (TIF)

Figure S4 Expression of \textit{FpAH1}, \textit{FpDLH1} and \textit{FpAMD1} during infection of roots (A, C and E) and detached leaves (B, D and F) of wheat and barley. For root infection assays, N = 4 with each replicate consisting of 15 pooled roots and detached leaf assays N = 3 with each replicate consisting of a single leaf segment. (EPS)

Figure S5 \textit{AH1} gene knockouts in \textit{Fusarium pseudograminearum} and \textit{Phaeosphaeria nodorum}. (A) Targeting construct (top) and wild type genomic locus (bottom) for disruption of \textit{FpAH1}. 635 bp of the 3' end of \textit{FpAH1} was replaced by the neomycin phosphotransferase cassette driven by the \textit{Aspergillus nidulans} gpdA promoter. (B) PCR screen to detect successful homologous recombination and gene deletion. PCR was performed with three primers (AHKOscr1, AHKOscr2 and gpdAr) as
Figure S6 Virulence assay of the Fusarium pseudograminearum amidohydrolase 1 (FpDLH1) mutants compared to the parental strain (CS3096) towards wheat 25 days post inoculation. (A) Survival of plants in the assay 25 days post inoculation. N = 3 with each replicate consisting of three or four paper towel rolls each with eight plants maintained in separate vessels. (B) Representative rolls of plants from the assay FCR assay (cultivar 2–49).
(TIF)

Figure S7 Phaeosphaeria nodorum virulence assay with PnAH1 mutants. (A) Disease scores in leaf blight assays (B) Sporulation of mutants and wild type (SN15) after seven days of infection.
EPS

Figure S8 Phylogenetic analysis of the dienelactone hydrolase (A) and amidase (B) proteins. Sequences were selected based on reciprocal best blast hits in the BLASTmatrix analysis to (A) FPSE_08131 and FpDLH1 and (B) to FpAMD1. Numbers on branches indicate approximate likelihood ratio test branch support values.
EPS

Figure S9 Fusarium pseudograminearum dienelactone hydrolase 1 (FpDLH1) knockout strategy and locus. (A) Targeting construct (top) and wild type genomic locus (bottom) for disruption of FpDLH1. 891 bp of the FpDLH1 gene was replaced by the neomycin phosphotransferase cassette driven by the gpdA promoter. [B] PCR screen to detect successful homologous recombination and gene deletion. PCR was performed using three primers (DLHKOscr1, DLHKOscr2 and gpdAr2) as indicated in part A. The lower band indicates the presence of the wild type locus and the absence of this band and the presence indicated in part A. The upper band is indicative of successful targeting. (C) Growth of wild type and one indicated in part A. Absence of the smaller wild type band and indicated in part A. The presence of the upper band is indicative of successful targeting. (C) Growth of the mutant and parental strain are indistinguishable in culture. Error bars represent the standard error of the mean for three biological replicates.
EPS

Figure S10 Virulence assay of the Fusarium pseudograminearum dienelactone hydrolase 1 mutant (AfpDLH1) mutant towards barley cultivar Gairdner in crown rot (A and B) and root-rot (C and D) assays. For A and B, N = 3 with each biological replicate consisting of four paper towel rolls consisting of 5–6 plants per roll. For C and D N = 15–16 plants. Error bars represent the standard error of the mean. Letters indicate statistically significant differences at P<0.05.
(TIF)

Table S1 Numerical summary of BLASTMatrix comparative analysis of F. pseudograminearum proteins against those of 27 other fungi.

Table S2 Putative orthologous amidohydrolase encoded in three Fusarium genomes. Numbers in parentheses are percentage similarities at the amino acid level to the F. pseudograminearum sequence.

Table S3 Isolates used in this study and the database accession numbers of sequences from these isolates either generated here or accessed from previous publications.

Table S4 Primers used in this study. Lowercase letters indicate regions of homology for use in lambda phage mediated recombination or overlap PCR. Underlined sequences indicate restriction endonuclease target sites for use in cloning.

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Author Contributions
Conceived and designed the experiments: DMG JMM KK SC PSS. Performed the experiments: DMG AGR PSS LG CMCM. Analyzed the data: DMG MCM PSS JBM KK. Contributed reagents/materials/analysis tools: MM BAM. Wrote the paper: DMG KK JMM.
Computational analysis: DMG MM.

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