Fusarium graminearum Genomics and Beyond

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4.1 Introduction

Diseases of cereal crops caused by pathogenic *Fusarium* species pose a great threat to global food production and safety. Head blight of wheat, barley, oats, and many other small grain cereal crops are caused by *Fusarium graminearum*. Ear rot and stalk rot of maize caused by *F. graminearum* and *Fusarium verticillioides* is a worldwide problem threatening both yield and quality of global corn production (White 1999). In addition to losses in yield and quality, infected grains also harbor fungal mycotoxins, many of which are potent carcinogens for humans and livestock and therefore cause for concern (Goswami and Kistler 2004). To understand the fundamental biology of pathogenic *Fusarium* species for the development of more effective disease management strategies, the genome of the major cereal killer, *F. graminearum* (Cuomo et al. 2007) and many other *Fusarium* species including *F. verticillioides, Fusarium oxysporum* (Ma et al. 2010), *Fusarium solani* (Coleman et al. 2009), *F. circinatum* (Wingfield et al. 2012), *F. fujikuroi* (Wiemann et al. 2013), and *F. pseudograminearum* (Gardiner et al. 2012), were sequenced and publicly released (reviewed in Ma et al. 2013). The open availability of these genomes has enabled further studies on fungal biology, genome evolution, pathogenicity, and the host–pathogen interactions using multiple omics approaches. In this chapter, we summarize recent research development and knowledge advancement regarding the *F. graminearum* genome since its public release in May 2003.

4.1.1 *Fusarium graminearum:* Reduces Crop Production and Contaminates Grains

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* Schwein (Petch)) is a filamentous fungus (Phylum Ascomycota, Subphylum Pezizomycotina, Class Sodariomycetidae, Order Hypocreales, Family Nectriaceae, and Genus *Gibberella*) capable of producing two types of asexual spores, macroconidia and chlamydospores. The ability of the fungus to produce microconidia has also been proposed (Xu, Purdue University, personal communication). The macroconidia are mononucleate and are typically slender, thick-walled, and banana-shaped with 5–6 septa. Similar to a number of other important fungal pathogens, *F. graminearum* is self-fertile and can also exhibit facultative outcrossing (Leslie and Summerell 2006). Sexual reproduction in *F. graminearum* gives rise to perithecia (Fig. 4.1), black and spherical fruiting bodies containing asci of typical eight ascospores. Both ascospores and macroconidia are

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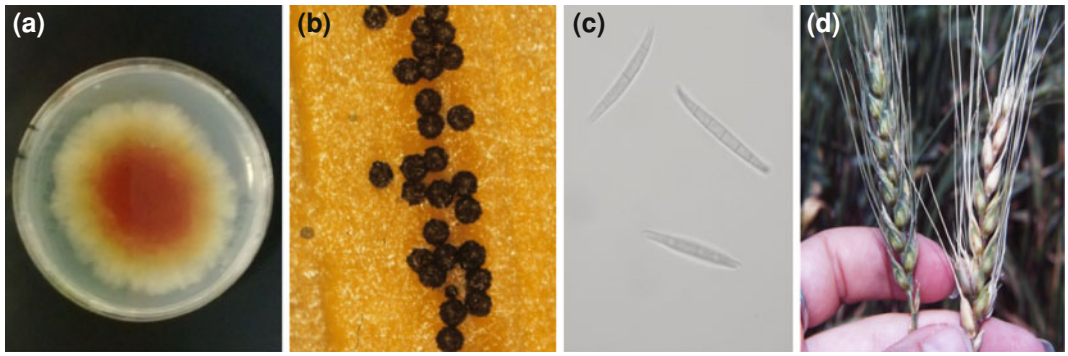

Fig. 4.1 **a** *F. graminearum* growing on potato dextrose agar. **b** peritheica, **c** macroconidia of *F. graminearum*. **d** Pictures of healthy and head blight of wheat infected by *F. graminearum*. (*Photos* **b** and **d** courtesy Dr. H. Corby Kistler at University of Minnesota)

infection propagules for the pathogen. However, ascospores forcibly discharged from perithecia are the primary inoculum for head blight infection of wheat and barley.

In addition to morphological characteristics, the species '*F. graminearum*' was defined primarily based on the association with cereal head blight. Members within this species complex were later divided into two groups (Leslie and Summerell 2006) based on their distinctive sexual reproduction strategies. The homothallic strains were grouped together and retained the species name *F. graminearum*, whereas *F. pseudograminearum* was used to delimit heterothallic strains requiring compatible mating types to cross. Further division was proposed based on molecular phylogenetics, resulting in 11 distinct lineages in the *F. graminearum species complex (FGSC)* (O'Donnell et al. 2000, 2004; Starkey et al. 2007). These species are *F. graminearum*, *F. gerlachii*, *F. asiaticum*, *F. vorosii, F. acacia-mearnsii*, *F. boothii*, *F. mesoamericanum*, *F. cortaderiae*, *F. brasilicum, F. austroamericanum, and F. meridionale*. The list of species within *Fg* complex has kept growing (Yli-Mattila et al. 2009; Sarver et al. 2011). However, the fact that these lineages are cross-fertile suggests that they probably still belong to a single biological species.

The most destructive disease caused by *F. graminearum* is Fusarium head blight (FHB) or scab on wheat, barley, oats, rye, rice, and many other small grain crops around the world (Goswami and Kistler 2004), resulting in annual crop losses in the billions of dollars. Although many Fusarium species were known to be associated with FHB, *F. graminearum* is indisputably the most common causal agent of FHB. In 1884, FHB was first reported at England and there have since been countless outbreaks in Europe, Asia, Canada, and the USA. In the USA alone, the direct and indirect economic losses resulting from the FHB epidemics in periods of 1991–1997 (Johnson et al. 1998) and 1998–2000 (Nganje et al. 2004) were estimated at 1.3 and 2.7 billion dollars, respectively. In China, FHB has devastated wheat production of over 7 million hectares and led to yield losses of 1 million tons during severe outbreaks (Bai and Shaner 2004).

The *F. graminearum* disease cycle is well described in the review by Bushnell et al. (2003). Both ascospores and macroconidia can be disseminated by wind, rain, and insects to host plants and deposited on or inside of spike tissues. However, ascospores forcibly discharged from overwintering perithecia under favorable spring conditions are the sources of primary inoculum for the initiation of FHB on new wheat plants. A close association between FHB and wheat anthesis was observed, and extruded anthers are suggested as vulnerable sites for primary infection (Sutton 1982). Once having landed on a suitable host, fungal spores germinate and fungal hyphae enter the stomata. Cell wall degrading enzymes secreted by the

pathogen facilitate the entry of infection hyphae into epidermal cells. Furthermore, infection hyphae can penetrate into the xylem and phloem and move beyond the inoculation site through wheat vascular bundles in rachis and rachilla (Bushnell et al. 2003). As a result, infected florets become blighted and bleached, with pink macroconidia often visible on the outer surface of florets. Overwintering *F. graminearum* can persist on both living plants and dead plant debris (Bushnell et al. 2003). FHB-infected kernels are contaminated with mycotoxins, which include deoxynivalenol (DON), nivalenol (NIV), and acetyldeoxynivalenol (ADON) (more details in the Mycotoxin section). Animals intoxicated with trichothecence mycotoxins exhibit typical symptoms, such as vomiting, dizziness, abdominal pain, and diarrhea (Desjardins et al. 1993). Due to its toxicity, the Food and Drug Administration set strict guidelines on DON levels in grains intended for human (1 ppm) and animal consumption (1–10 ppm).

Head blight or "scab" of rice (HBR) is usually not a major problem in rice production, but could be severe under favorable conditions (Nyvall et al. 1999; Goswami and Kistler 2005). The disease has recently been reported in Asian countries (Carter et al. 2000; Desjardins et al. 2000). Since rice (*Oryza sativa*) is one of the most important cereal crops, there are growing concerns about this disease. Under the new classification, multiple *Fusarium* species are associated with HBR, including *F. graminearum* and *F. asiaticum* (Lee et al. 2009). Even though, no rice scab diseases have been reported in the USA, *F. graminearum* and a few other *Fusarium* spp. were reported to cause head blight on wild rice (*Zizania palustris*) in Minnesota (Nyvall et al. 1999). In addition, Goswami and Kistler (2005) have shown that several *F. graminearum* and *F. asiaticum* isolates pathogenic to wheat were also capable of causing rice scab. However, trichothecenes, an important virulent factor in wheat scab, were not detectable in the infected rice plants (Goswami and Kistler 2005).

Fusarium graminearum also frequently causes ear rot and stalk rot diseases on maize as known as Gibberella stalk rot and Gibberella ear rot, named after *G. zeae,* the teleomorph of *F. graminearum* (White 1999). Typically, ascospores and macroconidia can infect corn through silk channels. Different from Fusarium ear rot caused by *F. verticillioides* and *F. proliferatum,* where infection can spread throughout the ears, Gibberella ear rot develops from the tip of ears with pink or white mycelia colonizing the ears (White 1999). Cool and wet weather during and after the corn silking period favors disease development. The disease cycle is similar as FHB and contaminations of infected ears and stalks with DON and zearalenone (ZEA) are the major concerns with this disease.

Members within FGSC produce a variety of mycotoxins toxic to animals and humans, including two major groups of tricothecenes (Desjardins et al. 1993) and mycoestrogen zearalenone (Leslie and Summerell 2006), and many others (Leslie and Summerell 2006) such as aurofusarin and fusarin C. However, the ability to produce these secondary metabolites differs considerably among members. Given the lack of correlation between certain chemotypes and phylogenetic lineages (O'Donnell et al. 2000), horizontal gene transfer was proposed to be one of the explanations for the metabolic diversity in FGSC and many other fungal species alike (Bömke and Tudzynski 2009; Cambell et al. 2012; Ma et al. 2013)

Tricothecenes are sequisterpenoid fungal secondary metabolites. In addition to *Fusarium spp.,* they can be produced by more than 300 other fungal species, including *Trichoderma* (Nielsen et al. 2005), *Myrothecium* (Fernando and Bean 1986), and *Stachybotrys* (Andersen et al. 2002). Over 150 trichothecene mycotoxins have so far been identified and classified into four groups A, B, C, and D according to their chemical properties and fungal producers. Groups A and B are the most common and best studied trichothecene mycotoxins (Rocha et al. 2005). Type A tricothecenes include T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol (Kimura et al. 2007; Rocha et al. 2005). Type B tricothecenes include DON (also known as vomitoxin), NIV,

and their acetylated derivatives ADON (Kimura et al. 2007; Rocha et al. 2005). *F. graminearum* produces primarily type B trichothecenes during head blight infection of cereal crops. Because of its significance, the trichothecene profiles are used to classify *F. graminearum* strains into different chemotypes, such as DON, NIV, 3-ADON, and 15-ADON (Rocha et al. 2005). The toxicity of trichothecenes comes from the ability to bind to ribosomes and inhibit protein synthesis (Rocha et al. 2005). The toxin can cause severe mycotoxicoses to animals that are fed with contaminated grains even at low concentration (Beasley 1989). The toxic effects include growth retardation, immunocompromization, dizziness, diarrhea, vomiting, and feed refusal (Beasley 1989). Trichothecenes are also phytotoxic and cause necrosis, chlorosis, wilting, and other symptoms in many plants (Cutler Horace 1988). Trichothecenes are virulence factors for FHB disease. *F. graminearum* trichothecene biosynthesis mutant (*ΔTri5*) is unable to produce trichothecenes and has significanlty reduced virulence on wheat (Proctor et al. 1995a).

The best-characterized mycoestrogen toxin is zearalenone (ZEA) (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone), and is well reviewed by Zinedine et al. (2007). ZEA is produced by many *Fusarium* species including *F. culmorum, F. cerealis, F. equiseti, F. crookwellense*, *F. semitectum, F. sporotrichioides, and F. venenatum* (Leslie and Summerell 2006). ZEA contaminates primarily corn and several other small grain crops. Gibberella stalk rot and Gibberella ear rot result in significant levels of ZEA on infected corn ears and stalks. Structurally, mycoestrogen resembles the female sex hormone estrogen. Therefore, it can competitively bind to the mammalian estrogen receptor. Humans or animals that consume ZEA-contaminated products are at the risk of many reproductive disorders, such as infertility and abortion. Consumption of ZEA-contaminated food has been associated with human hyperoestrogenic syndromes. ZEA also has been associated with cancer in lab animals (Schoental 1974).

4.1.2 *F. graminearum* Genome: Smaller Genome Size and Ample Potential for Mycotoxin Production

As the first *Fusarium* genome sequenced (strain PH-1 NRRL 31084) (Cuomo et al. 2007), *F. graminearum* has been used as a model for understanding Fusarium mycotoxin production, pathogenicity, signaling transduction, transcription regulation, development, sexual and asexual reproduction, and many other biological processes.

A compartmentalized genome structure was one of the unique features revealed from the *F. graminearum* genome project (Cuomo et al. 2007; Kistler et al. 2013; Ma et al. 2013). As in all genomes, the subtelomeric regions of *F. graminearum* chromosomes are highly polymorphic between different isolates in the *F. graminearum* population. Interestingly, *F. graminearum* chromosomes also contain discrete segments in the middle of the chromosomes that have high single nucleotide polymorphism (SNP) rate and chromosomes that more frequently recombined within these regions. These regions are enriched for genes that are important in plant–fungus interactions, including secreted proteins and genes expressed specifically *in planta* (Cuomo et al. 2007). These segments were proposed to be indicators of where chromosomal fusion occurred, based on the facts that: (1) the discrete segments are subtelomeric like, (2) *F. graminearum* has the smaller number of chromosomes (Table 4.1), and (3) Chromosomal fragments outside these discrete segments are highly conserved with homologous chromosomes in other *Fusarium* species (Ma et al. 2010). These fusion events definitely contributed to the decrease of genome size, even though the significance of chromosomal fusion and genome size reduction is not fully known.

Lacking repetitive sequences is another noticeable feature of the *F. graminearum* genome (Table 4.1), which also contributes to the genome size reduction. The decrease of repeat content was partially attributed to the active

Table 4.1 Statistics of Fusarium genomes

Species	Genes	Strain	Size (Mb)	# og Chr.	Intergenic distance	% coding	% repeat	Ref.
F. verticillioides	14,179	7,600	41.7	11	1,379	42.8	1.76	Ma et al. 2010
F. oxysporum f. sp. *lycopersici*	17,735	4,287	59.9	15	1,977	35.4	27.44	Ma et al. 2010
F. graminearum	13,332	PH-1	36.2	4	1,182	49.0	0.67	Cuomo et al. 2007
F. pseudograminearum	12,488	CS3096	37	ND	−25	49.3	1.6	Gardiner et al. 2012
F. solani f. sp. *pisi*	15,707	77-13-4	54.4	17	−15	−15.0	15.14	Coleman et al. 2009

The 11 chromosomes of *F. verticillioides* reflects the fact that among 12 chromosomes detected in the *Fv* genetic maps, this genome assembly only mapped to 11 chromosomes
ND not detected

process of repeat induced point (RIP) mutation, in which duplicated sequences are subject to extensive mutation (Cuomo et al. 2007). Low repeat contents were also observed in other *Fusarium* genomes, such as *F. verticillioides* (Ma et al. 2010), *F. pseudograminearum* (Gardiner et al. 2012), and *F. fujikuroi* (Wiemann et al. 2013). In contrast, other *Fusarium* genomes, such as *F. oxysporum* (Ma et al. 2010) and *F. solani* (Coleman et al. 2009), have much higher repeat contents.

Genes controlling the biosynthesis of many fungal secondary metabolites, including trichothecenes and ZEA, are clustered together. The biosynthesis gene clusters typically include a terpene synthase (TS), or polyketide synthase (PKS), or nonribosomal peptide synthetase (NRPS) gene that are responsible for a fundamental step in biosynthesis of nonribosomal peptides, polyketides, or terpenes. Additionally, these clusters also include genes responsible for structural modifications of initial metabolites, for metabolite transport, and pathway-specific transcription factors that activate expression of genes in the clusters. Such distinctive features enable the identification of SM biosynthesis clusters and can be used to predict potential SMs synthesized in a genome. The *F. graminearum* genome analysis identified a total of 43 such clusters (Ma et al. 2010), including genes responsible for known secondary metabolites, such as trichothecenes, zearalenones, aurofusarin, fusarins (Desjardins et al. 2006). Importantly, this genome study revealed ample potential to produce many uncharacterized secondary metabolites.

The *F. graminearum* trichothecene biosynthesis core gene cluster (*Tri* cluster, Fig. 4.2) contains 12 genes, including two transcription factors, that are required for the DON production and full virulence of *F. graminearum* on host plants (Baldwin et al. 2010b). The two transcription factor genes, *Tri6* and *Tri10,* have been shown to regulate the expression of the cluster (Seong et al. 2009; Tag et al. 2001). All 12 genes share very similar expression profiles across all public available expression data available at PLEXdb (Guo unpublished data), supporting a tight regulation. Interestingly, these two transcription factors also regulate genes outside of *Tri* cluster, including many genes important for producing precursors essential for DON production (Seong et al. 2009). It is noted that gene cluster responsible for the synthesis of NIV has two extra genes *Tri7* (C-4 acetyltransferase) and *Tri13* (C-4 hydroxylase), which are nonfunctional in DON-producing strains (Lee et al. 2002).

The biosynthesis of ZEA is controlled by a gene cluster including two PKS genes (Gaffoor and Trail 2006; Lysoe et al. 2006), ZEB1 encoding an isoamyl alcohol oxidase (Kim et al. 2005) and ZEB2 encoding a bZIP transcription factor (Kim et al. 2005). Other secondary metabolites are also controlled by gene clusters.

Gene_ID	Tri genes	Gene expression patterns	Annotation	Reference
FGSG_03532	Tri8		trichothecene 3-O-esterase	McCormick and Alexander, 2002
FGSG_03533	Tri7		hypothetical protein	Lee et al., 2002
FGSG_03534	Tri3		trichothecene 15-O-acetyltransferase	McCormick et al., 1996
FGSG_03535	Tri4		cytochrome P450	Hohn et al., 1995
FGSG_03536	Tri6		transcription factor	Hohn et al., 1999, Proctor et al., 1995b
FGSG_03537	Tri5		trichodiene synthase	Proctor et al., 1995a
FGSG_03538	Tri10		transcription factor	Tag et al., 2001
FGSG_03539	Tri9		hypothetical protein	Brown et al., 2001
FGSG_03540	Tri11		isotrichodermin C-15 hydroxylase	Alexander et al., 1998
FGSG_03541	Tri12		trichothecene efflux pump	Alexander et al., 1999
FGSG_03542	Tri13		hypothetical protein	Lee et al., 2002
FGSG_03543	Tri14		hypothetical protein	Brown et al., 2002; Dyer et al., 2005

Fig. 4.2 Schematic summary of trichothecene biosynthetic gene cluster in *Fusarium graminearum*. A diagram of the cluster shows all 12 trichothecene biosynthetic genes (*Tri* genes) modified according to (Rep and Kistler 2010). *Tri6* and *Tri10*, the two transcription factors regulating *Tri* genes are highlighted in *red*. Their functional annotation is summarized in the table together with the gene expression levels (*y-axis*) of all Tri genes in 198 samples representing 62 biological states (*x-axis*)

For instance, biosynthesis of aurofusarin ($C_{30}H_{18}O_{12}$), a secondary metabolite that contributes to the red/yellow pigment in fungal cultures, is controlled by a gene cluster harboring 10 genes, including two PKS genes and two transcription factor genes *aurR1* and *aurR2* (Malz et al. 2005).

4.2 Genetic Tractability

Fusarium graminearum can be easily cultured on a variety of media such as potato dextrose agar, carnation leaf agar, and carrot agar. Tractable genetic manipulation systems with remarkable phenotypic stability have been established for forward genetics screening for a genotype responsible for an observed phenotype, and for reverse genetics discovery of gene function by analyzing the phenotypic effects of manipulating a specific gene sequence.

Forward genetics depends on measurable phenotypic changes. Changing pathogenicity and reduced or lost toxin production are commonly used for screening genes of interest. Collectively, these studies identified and functionally characterized several genes in the trichothecene cluster (McCormick et al. 1996; Proctor et al. 1995a; Hohn et al. 1995, 1999; McCormick and Alexander 2002; Tag et al. 2001; Alexander et al. 1998, 1999; Brown et al. 2002). A gene cluster responsible for aurofusarin biosynthesis was also identified through random mutagenesis (Kim et al. 2005; Malz et al. 2005). The first large scale random mutagenesis study in *F. graminearum* was conducted using restriction enzyme-mediated integration (REMI) approach, which uncovered 11 genes associated with defective pathogenicity among 6,500 mutants (Seong et al. 2005). Subsequently, a high-throughput transposon-tagging mediated mutagenesis approach was developed in *F. graminearum* and identified 19 mutants (5.7 %) with altered phenotypes including novel pathogenicity genes (Dufresne et al. 2008).

Reverse genetics generates knockout mutants through homologous recombination. Applying reverse genetics to functionally characterize *F. graminearum* genes was greatly accelerated with the availability of the complete genome sequence. Many gene replacement vectors, containing some kind of selectable marker gene, the target gene sequence, or its flanking sequences, have been constructed for target gene disruption in protoplasts of filamentous fungi. After the transformation, homologous recombination events occur

between the wild-type target gene and the vector, which results in the replacement of the target gene with the marker gene. Initially, the insertion of the marker gene into the target gene depended on restriction enzyme digestion and subcloning DNA fragments onto plasmids. In recent years, subcloning-independent gene replacement approaches have emerged and triumphed. These methods typically use two or three rounds of PCR amplification, including overlapping PCR (Davidson et al. 2002), double-joint PCR (Yu et al. 2004), and split-marker (Catlett et al. 2003; Fairhead et al. 1996; Goswami 2011). In the split-marker deletion method, two constructs are required per transformation, each containing a flank of the target gene and roughly two-thirds of a selectable marker cassette. Homologous recombination between the overlapping regions of the selectable marker gene and between the flank regions and their genome counterparts results in a targeted gene deletion and replacement with an intact marker gene using only two rounds of PCR (Catlett et al. 2003; Goswami 2011). The ease of this procedure enables almost any molecular biology lab to generate constructs for targeted gene deletion within a few days. This approach is also ideal for high-throughput gene knockout studies and proves to be highly efficient for generating knockouts in a variety of filamentous fungi (de Hoogt et al. 2000; Fu et al. 2006; Li et al. 2011a; Wang et al. 2010; You et al. 2009) including *F. graminearum* (Son et al. 2011b; Wang et al. 2011a).

Fusarium graminearum transformation is usually mediated through either *Agrobacterium tumefaciens* (ATMT) (Malz et al. 2005) or polyethylene glycol (PEG) transformation (Proctor et al. 1995a). ATMT has been used to make knockout mutants in various fungal species with high efficiency. For ATMT, the marker gene and target gene or its flanking region are cloned into the T-DNA and transformed into *Agrobacterium tumefaciens*. Then, the fungal transformation is performed by co-incubation of the *A. tumefaciens* transformants with fungal hyphae or conidia. T-DNA carrying the gene disruption constructs is then introduced via *A. tumefaciens* infection, allowing the disruption of the target genes via homologous recombination. The transformed hyphae or conidia are subject to antibiotic selection and downstream PCR and Southern blot analysis. Alternatively, the gene replacement system can be introduced into *F. graminearum* through protoplasts mediated by polyethylene glycol (Goswami 2011). PEG-mediated transformation has been widely used in mutagenesis of *F. graminearum* with conidia and many other filamentous fungi with high efficiency. Efficiency of PEG transformation depends on the concentration and quality of protoplasts, DNA concentration, and linearity. Overall, comparing to ATMT transformation, procedures for PEG transformation tend to be simpler and more time-effective, while still able to generate large number of transformants.

4.3 Pathogenomics and Omics Approaches in the Postgenomic Era

Fusarium head blight diseases have been intensively investigated for nearly a century. Following the release of whole genome sequence of *F. graminearum*, different omics approaches have been applied to study both pathogen and its host and revealed new insights into infection biology and host resistance. This section will emphasize progress toward the understanding of *F. graminearum* pathogenomics. Research on genomics and genetics of host resistance to FHB has been reviewed previously (Bai and Shaner 2004; Bischof et al. 2011; Buerstmayr et al. 2009) and will not be discussed here.

4.3.1 Transcriptomics

The transcriptome is a collection of all transcripts in a cell or tissue at a given time. As a functional genomics approach, it provides a global view of transcript abundance in given cells or tissues under various environmental stimuli and developmental stages.

Conventionally, transcriptomics was investigated via microarray technology, where mRNAs isolated from biological samples are hybridized to DNA microarrays. A number of arrays have been reported. The first generation Affymetrix Genechip for *F. graminearum* was developed (Guldener et al. 2006) after the release of complete genome sequence of *F. graminearum*. The chip integrated around 14,000 genes of *F. graminearum* based on the genome annotation from the Broad Institute and Munich Information Center for Protein Sequences (MIPS). It was used to profile the fungal transcriptome *in vitro* and *in planta* with high sensitivity (Guldener et al. 2006) and provided a global view of transcriptional regulation during infection of wheat (Bernardo et al. 2007; Golkari et al. 2007; Jia et al. 2009; Lysoe et al. 2011b; Zhang et al. 2012) and barley (Boddu et al. 2006, 2007; Guldener et al. 2006). In the same year, multiple arrays with specific interest were developed to study the gene expression alteration in *mat1-2* mutant strain (Lee et al. 2006), and during perithecium development (Qi et al. 2006). Several other microarray chips based on ESTs or whole genome have been developed for transcriptome analysis of *F. graminearum* during different developmental and plant infection periods (Carapito et al. 2008; Golkari et al. 2007). Most microarray chips are made by Affymetrix using short oligonucleotide probes (25 mers), whereas chips designed by Agilent employing longer probes (60 mers) are also available (Becher et al. 2011). Many of these microarray-based transcriptomic data are available at PLEXdb (www.plexdb.org), a public database currently containing over 200 samples covering 19 different *F. graminearum* microarray experiments of more than 60 data points. With the advancement of array technology, a new Affymetrix exon array that contains nine plant pathogenic fungal genomes and tiling probes for the *F. graminearum* genome was produced, enabling the comparative study across multiple species (www.plexdb.org). The nine selected genomes are *F. graminearum, F. oxysporum* f.sp. *lycopersici, F. verticillioides, F. solani, Ustilago maydis, Puccinia graminis, Magnaporthe oryzae, Pyrenophora tritici-repentis,* and *Verticillium dahliea.*

With rapid technical advancement and drastic cost reduction of various sequencing technologies, sequencing entire transcripts (RNA-seq) has become a fast and cost-effective replacement of microarray technology. Compared to microarrays, RNA-seq enables digitalized quantification of transcripts in almost any given biological sample. It is more sensitive and has no dependency on a reference genome. At the time of this review, two *F. graminearum* transcriptome analysis using RNA-seq have been reported for studying alternate splicing (Zhao et al. 2013) and for comparative studies of perithecium formation in *F. graminearum* and *F. verticillioides* (Sikhakolli et al. 2012). Taking advantage of this new development, many laboratories have generated or are generating RNA-seq data. As will be described in later sections, these transcriptomic analyses have enriched our understanding of *F. graminearum* biology and pathogenesis, providing theoretical guidance for managing disease and controlling mycotoxin production.

4.3.2 Proteomics

Proteomics seeks to capture information on the entire compliment of proteins in an organism. Affected by posttranscriptional regulation or modification, the abundances of a transcript and its protein product are not always well correlated. Complementary with transcriptomic analyses, proteomics provides a more accurate measure of active cellular processes. The method starts with isolation and purification of total proteins, which are then typically separated by liquid chromatography or by 2D electrophoresis gels (e.g., PAGE gel). Protein fractions are then digested and characterized using mass spectrometry (MS) methods, such as Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) and electrospray ionization mass spectrometry (ESI-MS), etc. Peptide mass to charge ratios determined from MS spectra are then searched against predicted proteins from the genome analysis. Several

F. graminearum proteome studies have been reported (Kwon et al. 2009; Lee et al. 2008b; Paper et al. 2007; Phalip et al. 2005; Rampitsch et al. 2010, 2012; Taylor et al. 2008). Most studies focus on research topics, including secretomes/exoproteomes and phosphoproteomes. Secretome studies have identified secreted proteins species, such as cell wall degrading enzymes (CWDEs) and effectors that are likely to play critical roles in fungal virulence (Brown et al. 2012; Paper et al. 2007; Phalip et al. 2005; Yang et al. 2012). Phosphoproteomics of *F. graminearum* revealed important posttranslational modification processes, such as phosphorylation and ubiquitination (Rampitsch et al. 2010, 2012).

4.3.3 Sexual Reproduction

Fusarium graminearum is the only homothallic species in the genus *Fusarium.* Its sexual reproduction plays a pivotal role in disease manifestation of *F. graminearum*. Understanding molecular mechanisms underpinning sexual reproduction, especially perithecia and ascospore development, is an important area in *F. graminearum* research. The *F. graminearum* genome encodes both the *MAT1-1* and the *MAT1-2* loci, whereas all other fertile *Fusarium* species are heterothallic harboring either *MAT1-1* or *MAT1-2* and strains with differing *MAT* genes that are sexually compatible. The homothallic lifestyle likely evolved in the *F. graminearum* lineage from a self-sterile ancestor, potentially providing advantage in establishing infection without the dependency of searching for a mating partner (Ikeda 2010). Interestingly, population surveys have revealed low levels of gametic disequilibrium (Gale et al. 2002), suggesting that outcrossing is a mechanism to maintain population diversity.

Sexual reproduction of *F. graminearum* requires the production of a pheromone and its recognition by a pheromone receptor, a process controlled by the mating-type (MAT) locus and the MAPK cascade, a major signaling pathway orchestrating the sexual reproduction in *F. graminearum* (Kim et al. 2008; Lee et al. 2008a). Upon pheromone binding to receptors, heterometric G proteins are activated, which then activates the MAPK pathway. The importance of the MAPK cascade has long been known in pheromone sensing and mating in yeast and filamentous fungi, such as *M. oryzae* and *N. crassa*.

Microarray analyses of *F. graminearum* during different sexual development stages showed that temporal gene expression changes occur during the development of perithecia (Qi et al. 2006; Hallen et al. 2007). Genes involved in metabolism and cell differentiation are highly expressed in developing immature perithecia, whereas genes responsible for cellular transport such as ion transport genes are highly expressed in mature perithecia (Qi et al. 2006; Hallen et al. 2007). The fact that genes involved in metabolism and cell differentiation are highly transcribed at early perithecial development, but downregulated in the mature perithecia stage indicates that the fungus undergoes substantial physiological and developmental changes and tight regulation of these processes during perithecium formation and ascospore development.

Table 4.2 includes genes known to be involved in *F. graminearum* sexual reproduction, including an F-Box protein *FBP1* (Han et al. 2007), a calcium ion channel protein *CCH1* (Hallen and Trail 2008), a siderophore synthetase gene *NPS2* (Oide et al. 2007), two chitin synthase genes *GzCHS5* and *GzCHS7* (Kim et al. 2009), a protein kinase gene *GzSNF1* (Lee et al. 2009), *ROA* (Min et al. 2010), a cyclin C-like gene *CID1* (Zhou et al. 2010), velvet proteins *FgVeA* (Jiang et al. 2011a) and *FgVelB* (Lee et al. 2012), a histone deacetylase gene *HDF1* (Li et al. 2011b), ATP citrate lyase *ACL* (Son et al. 2011a), transcription factors *MYT1* (Lin et al. 2011), *MYT2* (Lin et al. 2012), *FgStuA* (Lysoe et al. 2011a), and *ZIF1* (Wang et al. 2011b), and phosducin-like protein *BDM1* (Horevaj and Bluhm 2012).

Table 4.2 Summary of *Fusarium graminearum* genes functionally characterized recently

Genes	Annotation	Mutant phenotypes	Disease	Reference
FSR1	WD repeat protein	Female infertility, deterred perithecia formation	◎	(Shim et al. 2006)
RAS2	Ras GTPase	Slow growth, delayed conidia germination	◎	(Bluhm et al. 2007)
SID1	Siderophore synthase	Poor growth at low-iron medium	◎	(Greenshields et al. 2007)
FBP1	F-box protein	Perithecia absent	◎	(Han et al. 2007)
MES1	Hypothetical protein	Reduced conidiation, cell wall deposition	◎	(Rittenour and Harris, 2008)
GzGPA2	G protein α subunit	Increased chitin accumulation	◎	(Yu et al. 2008)
FTL1	Transducin-beta like protein	Reduced conidiation	○	(Ding et al. 2009)
FgTep1	Tensin-like phosphatase 1	Mycelia sensitive to lithium, reduced conidiation and germination	◎	(Zhang et al. 2010)
CID1	Cyclin C-like protein	Reduced growth and conidiation, increase pigmentation Reduced DON, female infertility	◎	(Zhou et al. 2010)
FgVeA	Velvet gene	Reduced aerial hyphae, hydrophobicity and DON production, increased conidiation and delayed germination	◎	(Jiang et al. 2011a)
FgRrg-1	Response regulator protein	Increased sensitivity to osmotic stress and fungicides	◎	(Jiang et al. 2011b)
FgPtc3	Type 2C protein phosphatase	Reduced aerial hyphae and DON, increased conidiation, increased resistance to osmotic stress	–	(Jiang et al. 2010)
HP1	Heterochromatin protein	Increased aurofusarin, decreased DON,	–	(Reyes-Dominguez et al. 2012)
HDF1	Histone deacetylase	Reduced DON, defective sexual reproduction and conidiation	◎	(Li et al. 2011b)
FgATG15	Autophagy-related lipase	Aberrant conidia shapes, reduced storage lipid degradation under N starvation	◎	(Nguyen et al. 2011)
EBR1	Zn_2Cys_6 transcription factor	Reduced radial growth, disrupted hyphal apical dominance	◎	(Zhao et al. 2011)
BDM1	Phosducin-like protein	Abnormal conidia germination and hyphal morphology. Reduced DON	◎	(Horevaj and Bluhm 2012)
FgVelB	Velvet gene	Reduced aerial hyphae and hyphal hydrophobicity, highly increased conidiation, increased resistance to osmotic stress, reduced DON production	◎	(Jiang et al. 2012)
FgERG4	Sterol C-24 reductase	Increased sensitivity to metal cations, increased resistance to cell wall degrading enzymes and sterol biosynthesis inhibitors, reduced DON	◎	(Liu et al. 2012)
FgOS-2	Protein kinase	Reduced *in planta* DON and ZEA production, higher *in vitro* DON production, defective sexual reproduction	◎	(Van Thuat et al. 2012)

(continued)

Table 4.2 (continued)

Genes	Annotation	Mutant phenotypes	Disease	Reference
AMT1	Arginine methyltransferase	Slightly reduced vegetative growth, increased resistance to oxidative stress, reduced DON	◎	(Wang et al. 2012)
FgStuA	Fungal transcription factor	Reduced spore production, perithecia absent, conidiophores and aberrant macroconidia, reduced DON	◎	(Lysoe et al. 2011a)
FGL1	Lipase	Decreased extracellular lipolytic activity	◎	(Voigt et al. 2005)
GPA1	G protein α subunit	Defective sexual reproduction, increased DON and ZEA	●	(Yu et al. 2008)
MAP1	MAP kinase	Defective sexual reproduction Reduced DON	○	(Urban et al. 2003)
GMPK1	MAP kinase	Reduced conidiation, defective sexual production	○	(Jenczmionka et al. 2003)
MGV1	MAP kinase	Defective sexual reproduction, self-incompatible, reduce DON production	○	(Hou et al. 2002)
Fgp1	Wor1 like protein	No DON production, abnormal asexual and sexual spore development	○	(Jonkers et al. 2012)
ZIF1	b-ZIP transcription factor	Reduced DON production, defective sexual reproduction	◎	(Wang et al. 2011b)
Tri12	Major facilitator super family protein	Reduced DON production, reduced radial growth on trichothecene induction medium	◎	(Menke et al. 2012)

○ Nonpathogenic; ◎ reduced virulence; ● full virulence;—not available

4.3.4 Host–Pathogen Interaction

To overcome plant defense, pathogens such as *F. graminearum* probably have evolved an effective and powerful arsenal to establish infection. Like many filamentous fungi, environmental cues are typically sensed by G protein coupled receptors (GPCRs) on the cell membrane, which subsequently activate downstream intracellular signaling pathways. A total of 84 *F. graminearum* GPCRs have been predicted (Ma et al. 2010) and several of them are upregulated in the early stages of wheat infection (Zhang et al. 2012). The signals are then quickly passed to G proteins and the MAPK pathway (Hou et al. 2002). Penetration into the plant tissue is a critical step during early infection. The *F. graminearum* genome is enriched for cutinase genes, which may enable the pathogen to degrade the cuticle and penetrate the plant epidermis. Indeed, these genes are upregulated during infection of barley (Cuomo et al. 2007). *F. graminearum* is also likely to produce several lipases that dismantle the long-chain fatty acids found in cuticle layers to assist cuticle degradation. In fact, Voigt et al. (2005) showed that a secreted lipase *FGL1* by *F. graminearum* is essential for virulence on both wheat and maize. This lipase *FGL1* along with MAPK*GPMK1* is also under transcriptional regulation by the *RAS2* protein in *F. graminearum* (Bluhm et al. 2007). The process is clearly orchestrated by the MAPK cascade and signal RAS proteins.

The mycotoxin DON, is a known virulence factor for *F. graminearum* infection of wheat and barley, as DON nonproducing mutants typically have attenuated disease symptoms (Proctor et al. 1995a). However, DON production is not required for successful penetration, since

Tri5 mutants can still form appressoria-like structures (Boenisch and Schafer 2011) like the wild-type strain. Using a Tri5-GFP-tagged *F. graminearum* strain, the expression level of *Tri5* was monitored to evaluate the induction of trichothecene pathway during *F. graminearum* colonization of wheat heads (Ilgen et al. 2009). It was found that no or very low level of expression of DON biosynthesis gene *Tri5* was observed in infected anthers where the initial colonization occurs. In contrast, *Tri5* was highly expressed in rachis node, where *F. graminearum* uses biosynthesized DON to destroy this major physical barrier, so that the fungal growth is spread throughout the whole spikes and heads (Ilgen et al. 2009). This suggests that DON is produced in a tissue-specific manner during *F. graminearum* infection of wheat heads.

Large-scale functional analyses have been conducted on the entire predicted set of transcription factors (Son et al. 2011b) and protein kinases (PK) (Wang et al. 2011a). The phenotypes of the 657 transcription factor mutants are documented at the *F. graminearum* transcription factor phenotype database (FgTFPD) (http://kropbase.snu.ac.kr/cgi-bin/Fusarium/Fusarium_main.cgi). Among the 657 transcription factors analyzed analyzed, 170 showed phenotypic changes in the deletion mutant, including 73 for mycelial growth, 41 for pigmentation, 105 for sexual reproduction, 69 for ZEA production, 55 for DON production, 41 for conidiation, 62 for virulence, and 49 for stress response. In total, 42 (of 116) PK genes (Wang et al. 2011) are associated with *F. graminearum* virulence and mutants of 22 PK genes, including MAPK genes Gpmk1, Mgv1, and CPKA gene are nonpathogenic or defective in colonizing the plants.

Table 4.2 lists other genes regulating *F. graminearum* pathogenesis reported so far. Most of these genes are essential for full virulence or pathogenicity of *F. graminearum*. Many genes regulate asexual and sexual reproduction besides virulence, indicating normal sporulation and germination required for fungal virulence on plants. Remarkably, there is a strong correlation between defects in DON production and virulence in many of these mutants, confirming the importance of DON in disease symptom development in head blight. In fact, according to the FgTFPD, 35 transcription factor mutants are defective in both virulence and DON production (Sun et al. 2011b). In addition, mutants that have increased sensitivity to environmental stresses, such as oxidation, heavy metals, and antifungal compounds tend to have attenuated virulence as these mutants are likely to be vulnerable to the plant defense actions (Table 4.2).

Fusarium graminearum gene expression is dynamically regulated along the infection course and genes differentially expressed *in planta* are significantly enriched in regions high in single nucleotide polymorphisms (SNPs) in the genome. Over 70 % of them are unique to *F. graminearum* and have unknown functions (Lysoe et al. 2011b). Zhang et al. (2012) investigated *F. graminearum* transcriptomics during infection of wheat coleoptiles at different time points combining laser capture microdissection and microarray analyses, which provided transcriptional regulation at single cell-type resolution (Zhang et al. 2012). The study identified 344 genes differentially expressed during fungal invasive growth *in planta*. Among those, 134 genes encode putative CWDE. These genes exhibited a unique expression profile, depicting increased expression levels at 16 and 64 h after inoculation (HAI), respectively. These two time points correlate to fungal intercellular growth at 16 HAI and at 64 HAI when the infection transits into a necrotrophic stage. Interestingly, the second increase at 64 HAI is much higher suggesting extensive cell wall degradation involved in fungal necrotrophic growth. No DON biosynthesis genes are induced during the coleoptile infection suggesting that DON might not be required for the coleoptile infection. However, an unknown secondary metabolite gene cluster, FG3_54 predicted through genomic study (Ma et al. 2010), was induced at 64 HAI, which may produce an unknown toxin that contributes to the late stage of fungal infection (Zhang et al. 2012).

Transcriptomic studies on knockout mutants provide information on regulatory network rewiring after removing targeted genes (Baldwin

et al. 2010a; Gardiner et al. 2009; Hallen and Trail 2008; Jonkers et al. 2012; Lee et al. 2011; Seong et al. 2009). Transcriptomic analyses of mutants of *tri6* and *tri10*, the two transcription factors encoded in the *Tri* gene cluster, revealed that in addition to genes in the trichothecene biosynthesis gene cluster, these two transcription factors also regulate many other genes, including isoprenoid biosynthesis, disease and virulence, ABC transporter expression, and secondary metabolism (Seong et al. 2009). Jonkers et al. (2012) applied microarray analysis on the deletion mutant of Fgp1 (a Wor1-like protein) and showed that Fgp1 positively regulates *Tri* cluster, butenolide cluster, and NPS8 cluster genes during plant infection (Jonkers et al. 2012).

4.3.5 Molecular Detection and Quantification

Importantly, wheat plants infected with *F. graminearum* may be asymptomatic but still contaminated by mycotoxins (Sinha and Savard 1997). Therefore, it is imperative to be able to detect *F. graminearum* in a timely manner and to quantify mycotoxins in harvested crops beyond morphological identification. For host–pathogen interaction and host resistance studies, it is also valuable to be able to monitor the colonization progression and to quantify fungal biomass. Many molecular detection approaches were developed to quickly and accurately detect *F. graminearum*, including PCR (Bluhm et al. 2002; Li et al. 2005; Niessen et al. 2004; Niessen and Vogel 1998; Wang et al. 2008), real-time PCR (Atoui et al. 2012; Bluhm et al. 2004; Brandfass and Karlovsky 2008; Halstensen et al. 2006; Horevaj et al. 2011; Moradi et al. 2010; Nielsen et al. 2012; Reischer et al. 2004), LAMP (loop-mediated isothermal amplification) (Abd-Elsalam et al. 2011; Niessen and Vogel 2010), and oligonucleotide microarrays (Kristensen et al. 2007b; Nicolaisen et al. 2005). With the available genome sequence, molecular detection can be applied to any given gene of interest. So far genes used for PCR detection include *gaoA* (galactose oxidase) (Wilbert and Kemmelmeier 2003) and trichothecene and zearalenone biosynthesis genes (Bluhm et al. 2002; Horevaj et al. 2011; Nielsen et al. 2012; Niessen and Vogel 1998).

Standard PCR methods are unable to quantify fungal DNA and biomass in plant materials. In the past decade, real-time PCR has been favorably used for both detection and quantification owing to its high accuracy, sensitivity, and speed and it was embraced to detect and quantify *F. graminearum* (Atoui et al. 2012; Bluhm et al. 2004; Burlakoti et al. 2007; Demeke et al. 2010; Dyer et al. 2006; Horevaj et al. 2011; Nielsen et al. 2012; Reischer et al. 2004). There are two major real-time PCR technologies: *TaqMan* and SYBR Green, reviewed previously (Heid et al. 1996; Rebrikov and Trofimov 2006; Wong and Medrano 2005). Similar to conventional PCR, real-time PCR uses gene-specific primers to amplify the target gene. Florescence tags are amplified through every cycle, offering a real-time quantification of the PCR product. Oligonucleotide array (Kristensen et al. 2007b; Nicolaisen et al. 2005) and SNP approaches (Kristensen et al. 2007a) were developed to differentiate *F. graminearum* from other closely related *Fusarium* species. The array hybridization results can be visualized using a colorimetric Silverquant for easy detection (Kristensen et al. 2007b).

4.4 Systems Biology and Future Perspectives

A *F. graminearum* protein–protein interaction (FPPI) database that interconnects 7406 proteins into 223,166 protein–protein interactions was created combining interaction-ortholog and domain–domain interaction (DDI) algorithms (Zhao et al. 2009). Based on this framework and utilizing differential gene expression before and after infection, pathogenicity-related subnetworks were constructed centered on several seed pathogenicity genes in the core FPPI and (Liu et al. 2010).

Fusarium graminearum candidate regulatory motifs were identified by systematically searching for evolutionarily conserved regulatory motifs (Kumar et al. 2010). These studies established a foundation to investigate global regulatory networks in the system.

The next wave of pathogenicity-related studies could focus on identifying and characterizing *F. graminearum* effectors. Comparative proteomic analyses identified secreted proteins during the *F. graminearum* infection process (Paper et al. 2007; Yang et al. 2012), including cell wall and starch degrading enzymes. Secretome studies identified more potential novel effectors (Brown et al. 2012), many of which are *F. graminearum* specific (Brown et al. 2012). Information on these predicted secreted proteins provides a foundation for future experimental studies to verify their function in pathogenesis.

Fusarium graminearum could also be developed into a model system to study genetic mechanisms that control genome stability. Repeat Induced Polymorphisms (RIP) can effectively mutate repeated DNA in the genome. However, RIP, as first discovered in the *N. crassa* genome, only introduces mutation in the repeats with sequence identity above 80 %. The *N. crassa* genome is littered with low sequence identity repeats below 80 %. The phenomenon of almost complete elimination of repeats in the *F. graminearium* genome suggests the existence of additional mechanism(s) for removing repeats. Understanding genetic mechanisms that control the invasion or expansion of repeats will enhance our understanding of genome stability.

In summary, *F. graminearum* genomics has provided detailed genetic information about this destructive fungus. Improved genomic and functional annotations, detailed phenomics and enriched transcriptomics and proteomics data will open up new possibilities for applied systems biology approaches. In the near future, such approaches will enhance investigations of the regulatory networks essential to comprehend cellular functions and pathogenesis.

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