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Technological Advancement

# Development of a *Fusarium graminearum* Affymetrix GeneChip for profiling fungal gene expression in vitro and in planta

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**Abstract**

Recently the genome sequences of several filamentous fungi have become available, providing the opportunity for large-scale functional analysis including genome-wide expression analysis. We report the design and validation of the first Affymetrix GeneChip microarray based on the entire genome of a filamentous fungus, the ascomycetous plant pathogen *Fusarium graminearum*. To maximize the likelihood of representing all putative genes (~14,000) on the array, two distinct sets of automatically predicted gene calls were used and integrated into the online *F. graminearum* Genome DataBase. From these gene sets, a subset of calls was manually annotated and a non-redundant extract of all calls together with additional EST sequences and controls were submitted for GeneChip design. Experiments were conducted to test the performance of the *F. graminearum* GeneChip. Hybridization experiments using genomic DNA demonstrated the usefulness of the array for experimentation with *F. graminearum* and at least four additional pathogenic *Fusarium* species. Differential transcript accumulation was detected using the *F. graminearum* GeneChip with treatments derived from the fungus grown in culture under three nutritional regimes and in comparison with fungal growth in infected barley. The ability to detect fungal genes in planta is surprisingly sensitive even without efforts to enrich for fungal transcripts. The Plant Expression Database (PLEXdb, http://www.plexdb.org) will be used as a public repository for raw and normalized expression data from the *F. graminearum* GeneChip. The *F. graminearum* GeneChip will help to accelerate exploration of the pathogen–host pathways that may involve interactions between pathogenicity genes in the fungus and disease response in the plant.


*Keywords:* Fusarium head blight; *Fusarium asiaticum*; *Fusarium boothii*; *Fusarium culmorum*; *Fusarium pseudograminearum*; *Fusarium verticillioides*; *Fusarium oxysporum*; RNA profiling

## 1. Introduction

DNA microarrays are one of the most useful technologies for functional genomic studies, allowing genome-wide analysis of transcript accumulation for individuals differing in genetic background and under a variety of environmental

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regimes (Stoughton, 2005). The first fungal microarrays were designed for genome-wide gene expression analysis of the ascomycetous yeast *Saccharomyces cerevisiae* and were followed by arrays designed for the fission yeast *Schizosaccharomyces pombe* (Lyne et al., 2003; Spellman et al., 1998). In *S. cerevisiae*, microarrays have been used to profile gene expression under different environmental, physiological, and developmental conditions, including temperature shifts, chemical treatments, pheromone responses, mutations in different genetic backgrounds, sporulation, and ploidy regulation (Chu et al., 1998; Galitski et al., 1999; Haugen et al., 2005; Roberts et al., 2000; Sudarsanam et al., 2000). The accumulation of a large amount of microarray data for *S. cerevisiae* has enabled the identification of transcription regulatory motifs and networks (Ihmels et al., 2004; Luscombe et al., 2004). The tiled microarray approach also has been used to study transcription regulatory elements, chromatin structure, and nucleosome positioning (Glynn et al., 2004; Yuan et al., 2005).

For pathogenic fungi, microarray analysis has great potential to systematically and efficiently identify genes required for different infection and developmental stages (Bryant et al., 2004; Lorenz, 2002). In the human pathogen *Candida albicans,* the whole-genome microarray has been applied to study drug resistance, cell wall synthesis, and pathogenic development (Andes et al., 2005; Hull et al., 2000; Kadosh and Johnson, 2005; Liu et al., 2005; Phan et al., 2000; Sohn et al., 2003). A partial genomic DNA array was developed for *Cryptococcus neoformans* and used for transcript profiling during temperature shifts and murine macrophage infection (Fan et al., 2005; Kraus et al., 2004). For plant pathogenic fungi, a *Magnaporthe grisea* array, now commercially available (http://www.agilent.com), has been used to study gene expression during in vitro growth and appressorium formation (Dean et al., 2005). However, there is no detailed published description on its design, representation of the *M. grisea* genome or sensitivity during plant infection. Also recently, long oligonucleotide microarrays have been designed for expression analysis of several other pathogenic fungi, including *Cryphonectria parasitica*, *Blumeria graminis*, *Histoplasma capsulatum*, *Metarhizium anisopliae*, and *Fusarium verticillioides* (Allen et al., 2003; Both et al., 2005; Hwang et al., 2003; Pirttila et al., 2004; Wang et al., 2005). However, in general, these arrays have represented only a fraction of the fungal genome as they are based on limited EST coverage.

The filamentous ascomycete *Fusarium graminearum*, the causal agent of *Fusarium* head blight (FHB) disease on wheat and barley, is one of the most destructive plant pathogens with great economic impact on agriculture throughout the world. With a remarkably complete draft whole genome sequence assembly available (*Fusarium graminearum* Sequencing Project), it is possible to design a microarray that can be used to explore the transcriptome at a genome-wide scale. Transcription profiling studies with whole genome microarrays may be the cornerstone for identifying essential elements of pathogen–host interactions and lead to the development of new targets for fungal control. The *F. graminearum* draft genome sequence assembly gives access to nearly all putative coding sequences, and is not limited to a subset of genes such as those shown to be expressed by EST analysis. Here we report the design and validation of the *F. graminearum* Affymetrix GeneChip and describe its first experimental applications.

## 2. Materials and methods

### 2.1. DNA chip concept and design

The genome sequence of *F. graminearum* was released in 2003 by the Broad Institute and subsequent automatic gene prediction was applied using the Calhoun annotation system (http://www.broad.mit.edu/annotation/fungi/fusarium/gene_finding.html). In addition to the automatically predicted set from the Broad Institute, a second automatic gene call set was developed at MIPS using FGENESH (www.softberry.com) with a matrix trained on fungal sequences of diverse origin (*Ustilago maydis*, *S. pombe*, and others). Surprisingly this matrix performed significantly better than FGENESH using a matrix trained on *Neurospora crassa* sequences even though *N. crassa* is more closely related to *F. graminearum* than *U. maydis* and *S. pombe*. In addition to the automated gene predictions, a subset of gene calls (7%) were manually improved focusing mainly on ORFs potentially related to pathogenicity (unpublished). Altogether 25,986 different calls are maintained in the *Fusarium graminearum* Genome DataBase (FGDB), covering most of the genes twice, as the number of valid ORFs is estimated to be around 14,100 (Güldener et al., 2006). To compile a set of sequences for the design of the GeneChip, the intrinsic redundancy needed to be reduced.

During the manual gene modeling and correction procedure, focusing on groups of genes potentially relevant in plant–pathogen interactions, it appeared that the MIPS draft gene call set performed better than the Broad set. When the exon structure of the 830 manually processed gene calls were compared with the initial calls, 55% of the Broad draft calls differed from the manual calls compared with only 23% of the MIPS draft calls. The MIPS draft gene calls appeared to have fewer erroneously fused gene calls and nearly no falsely added short 5′ and 3′ exons. A total of 706 Broad gene calls correspond to 2 or 3 MIPS draft or manually processed gene calls resulting in 1808 gene calls (e.g., see Fig. 1). Thus, together with 490 gene calls for which no corresponding Broad call exists, 2298 gene calls are present only in the MIPS sets. Therefore we produced a combined gene call set for the Affymetrix GeneChip design with the order of preference "manually processed new calls" (prefix "fg12") > "MIPS draft set" (prefix "fgd") > "Broad set" (prefix "fg"). To reduce the total number of gene calls, the ORF sequences without introns were truncated to 500 nucleotides towards the 3′ end and all the names of redundant calls were added to the preferred ones as an alias. This approach takes into account that probes

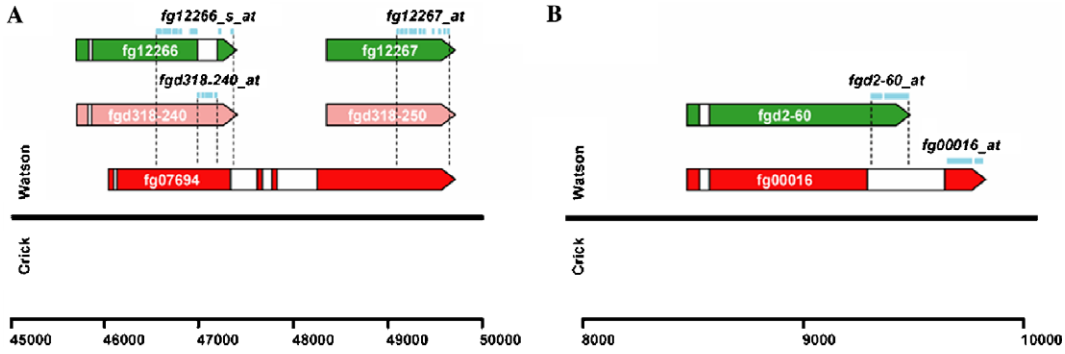


Fig. 1. Design of microarray probe sets for disparate gene models in the *F. graminearum* genome. (A) A Broad Institute call (fg07694—red arrow), two MIPS draft calls (fgd318-240 and fgd318-250—pink arrows) and two MIPS manually corrected calls (fg12266 and fg12267—green arrows) correspond to the region of the Broad Institute assembly, contig 318, Watson strand, between bp 45,000 and 50,000 (scale shown below). The terminal 600 bp of fg07694, fgd318-250 and fg12267 are identical and represented by the probe set indicated by blue boxes above fg12267_at. The terminal 600 bp of fg12266 and fgd318-240 differ by the prediction of an intron for fg12266. A probe set designed corresponding to the intron region of fg12266 (fgd318-240_at – blue boxes) is specific for the last 600 bp of fgd318-240. The probe set flanking the predicted intron (fg12266_s_at) corresponds to the 3′ region of both fg12266 and fgd318-240. (B) A Broad Institute call (fg00016—red arrow) and a MIPS draft call (fgd2-60—green arrow) correspond to the region of the Broad Institute assembly, contig 2, Watson strand, between bp 8000 and 10,000. The 600 bp at the end of fgd2-60 and fg00016 differ by the prediction of an intron for fg00016. A probe set designed corresponding to the intron region in fg00016 (fgd2-60_at—blue boxes) is specific for the last 600 bp of fgd2-60 whereas probe sets flanking the predicted intron (fg00016_at) would correspond only to the 3′ region of fg00016. Using RNA from *F. graminearum*-infected barley, 144h after inoculation (data not shown), probe set fgd2-60_at but not fg00016_at was detectable, implying that fgd2-60 is the correct gene call.

are designed mostly from the last 600 nucleotides of each sequence and through this process, focuses on differences in this region between analogous gene calls (Fig. 1; www.affymetrix.com/support/technical/other/custom_design_manual.pdf). This resulted in a set of 16,926 calls. This set of full-length ORF sequences along with an additional 611 ESTs, one 18S rRNA (GenBank AY188924) and 18 5S rRNA sequences (Rooney and Ward, 2005), as well as control sequences of wheat, barley, maize and rice were submitted to Affymetrix for initial computation of probe sets (see Table 1). Candidate probe sets were "hard pruned" against sequences used to develop the barley, wheat and rice Affymetrix GeneChips to reduce the likelihood of cross-hybridization between fungal probe sets and host RNAs. After three rounds of chip design proposals, probe sets of 18,069 entries were approved for mask design. Custom Affymetrix GeneChip microarrays with high density, 11 μm features in a 64 format were manufactured from this design.

Table 1
Number of probe sets and corresponding sequences tiled on the *Fusarium* GeneChip according to categories

| Sequence category | No. of probe sets | No. of sequences |
|---|---|---|
| (1) MIPS manually corrected calls (fg12 prefix) | 413 | 388 |
| (2) MIPS 'draft' calls (fgd prefix) | 13647 | 13280 |
| (3) Broad Institute calls (fg prefix) | 3165 | 2772 |
| (4) Broad Institute short gene calls (fgs prefix) | 371 | 339 |
| (5) ESTs | 213 | 204 |
| (6) rRNA (5S and 18S) | 19 | 19 |
| (7) *Tri7* and *Tri13*[a] | 2 | 2 |
| (8) Contig/Affx/1415[b] | 213 | 213 |
| (9) Barley/wheat/maize[c] | 3/14/9 | 3/11/9 |
| Total (categories 1–9) | 18069 | 17240 |
| Total *Fusarium* (categories 1–7) | 17830 | 17004 |

[a] *Tri7* and *Tri13* are *F. graminearum* genes involved in trichothecene biosynthesis not found in the sequenced strain PH-1. Probe sets for these genes were based on gene sequences from GenBank Accession No. AF336365.

[b] Control hybridization and labeling probe sets with "Affy," "contig," or "1415" prefixes.

[c] Probe sets designed from *Fusarium* community-provided sequences for genes from major host plants of *F. graminearum*.

GeneChip microarrays differ from long oligonucleotide arrays generally by having a larger number of shorter oligonucleotide probes representing single genes. Most of the 18,069 probe sets on the *F. graminearum* GeneChip are composed of 15 or 14 pairs of 25-mer oligonucleotides derived principally from the last 600 nucleotides of each sequence. Each probe pair contains perfect matching oligonucleotides and mismatching oligonucleotides with a single substitution at the 13th nucleotide for statistical validation of specific transcript detection by each probe set. The number of probe pairs is reduced to a minimum of 8, only in case of short sequences or highly homologous gene families. Except for EST sequences, rRNAs and controls, mainly predicted ORF sequences were submitted for design and so the probes correspond to potential coding regions and not to 3′ untranslated sequences. If the submitted sequences were too short to fit 8 probes they were omitted from the final probe designs. If they had only minor sequence differences to an analogous ORF, probes were only designed for one of those ORFs. In total, 529 sequences were not present in the final GeneChip design including 453 predicted ORF sequences, 13 ESTs and 63 of the 339 "short genes" from the Broad Institute set (labeled with a "fgs" prefix). The placement of probes in the ORFs, their nomenclature and GeneChip probe coordinates can be viewed from any single gene entry of the FGDB in the 'GeneChip Info' section http://mips.gsf.de/genre/proj/fusarium/GeneChip/GeneChip.html.

### 2.2. Experimental validation of the chip

#### 2.2.1. Strains and culture conditions

*Fusarium* strains used in this study are *F. graminearum* strains NRRL 31084 (PH-1) and NRRL38661 (Butte 86), *F. asiaticum* NRRL 26156, *F. boothii* NRRL 29020, *F. culmorum* NRRL 25475, *F. oxysporum* f.sp. *lycopersici* NRRL 34936, *F. pseudograminearum* NRRL 28338, and *F. verticillioides* NRRL 20956. Cultures were grown at 25 °C in liquid complete medium (CM) (Harris et al., 1994) for 3 days prior to extraction of genomic DNA. For infection of barley, macroconidium preparation and inoculation of *F. graminearum* strain NRRL38661 were accomplished as described previously (Boddu et al., 2006; Seong et al., 2005).

#### 2.2.2. Fungal growth in culture

One ml suspensions of $10^8$ macroconidia of *F. graminearum* strain NRRL 31084 were inoculated into three 250 ml flasks containing 100 ml CM and cultivated at 25 °C for 24 h with shaking at 150 rpm. Mycelia of each flask were harvested and washed with sterile distilled water. The harvested mycelia were re-inoculated into either 100 ml CM (complete medium control), 100 ml minimal medium (Trail et al., 2003) prepared without a nitrogen source (MMN), or 100 ml minimal medium without a carbon source (MMC). After 12 h of cultivation at 25 °C with shaking at 150 rpm, mycelia were harvested and used for RNA extraction.

#### 2.2.3. Barley infection time course

The experiment described here also was used to examine barley transcript accumulation during *F. graminearum* infection (Boddu et al., 2006). Seeds from the barley cultivar Morex were planted in Scotts MetroMix 200 in 6-in. pots (four seeds/pot) and plants were grown in a growth chamber with 16 h day at 20 °C, and 8 h night at 18 °C. At pot level, the light intensity was $170 \pm 20\,\mu E\,m^{-2}\,s^{-1}$. Five milliliters of Osmocote 14/14/14 (Marysville, OH) with micronutrients was applied after one week. For the first 2 weeks, the plants were watered every 2 or 3 days and then every day.

The *F. graminearum* strain NRRL38661 (Evans et al., 2000) was used for all inoculations. Spray inoculations were conducted on spikes at 2–3 days after emerging from the boot (plant ontology: 7.03-anthesis completed GRO:0007104). An airbrush (Model VL; Paasche Airbrush, Harwood Heights, IL, USA) adjusted to a pressure of 82.8 kPa was used to conduct the spray inoculations. The two inoculation treatments were a freshly-prepared spore suspension of $2 \times 10^6$ macroconidia per ml in 0.04% (vol/vol) Tween 20 in water or water plus Tween 20 (mock). All inoculations were conducted at 3 p.m. To ensure proper disease severity, the spikes were bagged in clear plastic for 72 h. Three biological replicates were conducted at 24, 48, 72, 96, and 144 h after inoculation (hai). RNA from water treated (mock) spikes from the 144 h time point was used as a control. For each biological replication, eight spikes were randomly sampled at 24, 48, 72, 96, and 144 hai. At each of the time points, spikes were sampled at 3 p.m.

#### 2.2.4. Nucleic acid extraction, RNA labeling, and hybridization

Total RNA was isolated from fungal mycelia and from infected barley using TRIzol™ reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Total RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Genomic DNA was isolated from different *Fusarium* species for DNA labeling following a CTAB method (Saghai-Maroof et al., 1984).

Ten micrograms of total RNA were treated according to the conventional Affymetrix eukaryotic RNA labeling protocols (Affymetrix, Santa Clara, CA). Prior to labeling, the Agilent 2100 bioanalyzer™ (Agilent, Palo Alto, CA) was used to examine the RNA quality. One GeneChip each was used for the three biological replications in each experiment (CM, MMC and MMN) and at each replication for the time points of the barley infection experiment.

For genomic DNA labeling, 10 μg of genomic DNA was fragmented by digestion with 0.5 U of DNase I (Amersham Biosciences, P/N 27-0514-01) in One-Phor-All Buffer (Amersham Biosciences, P/N 27-0901-02) for 5 min at 37 °C into average size 20–30 bp. After heat inactivation of DNase activity (98 °C for 15 min) and prior to labeling, fragmentation of genomic DNA was checked by gel electrophoresis on a 4% Metaphor agarose gel (Cambrex Bio Science, Rockland, ME). Gel was stained with SYBR Gold (Molecular Probes, P/N S-11494) for 30 min. The fragmented DNA was labeled in 1× terminal transferase reaction buffer with 20 U of terminal deoxynucleotidyl transferase (Promega, P/N M1875) and 300 μM of Labeling Reagent (Affymetrix, P/N 900542) for 60 min at 37 °C. Two GeneChips (two replications) were used for each DNA.

The chip hybridizations, washes, and chip reading followed standard Affymetrix procedures (http://www.bipl.ahc.umn.edu/affymetrix.html) in use at the Biomedical Image Processing Facility at the University of Minnesota.

#### 2.2.5. Analysis of microarray hybridization data

The hybridization signals were scanned with a GeneChip GCS 3000 scanner (Affymetrix, Santa Clara, CA) and the cell intensity (CEL) files were obtained from software GCOS 1.2 (Affymetrix, Santa Clara, CA). CEL files were loaded into the Expressionist Pro software version 1.0 (Genedata, San Francisco, CA). When present, defective areas on the chip and outliers were masked while the files were processed for further analysis. For experiments involving fungal RNA or RNA from infected plants as a starting material, the Robust Multichip Analysis (RMA) algorithm (Irizarry et al., 2003) was used for condensing the data as implemented in the Refiner segment of Expressionist. Data were normalized using the RMA global normalization algorithm with the 100 control probe sets (having an "AFFX" prefix) and 13 plant probe sets (with a "Contig" prefix) as reference points. The above mentioned 113 probe sets and an additional 147 control probe sets which are not of fungal origin were subtracted from the *F. graminearum* gene list. Detection of RMA-normalized probe set signals

was further validated using the Affymetrix MAS5.0 statistical algorithm (Hubbell et al., 2002) at a detection *P*-value < 0.04. A probe was called present when it was detected in at least two of the three replications at a detection *P*-value < 0.04 and was considered absent when it was not detected at *P* < 0.04 in any of the three replicates. Probe sets that yielded a present call at any time point of *Fusarium* treated samples were selected and from them, ones that did not also satisfy an absent call in water inoculated control plants were subtracted. For DNA labeling and cross-species hybridization assays, probe set signals were condensed and normalized by the RMA algorithm. A present/absent test was conducted at a detection *P* value < 0.04 to validate detection calls. A given probe set was called present only if it was detected in two GeneChips. Data from microarray experiments are stored at PLEXdb www.plexdb.org under accession numbers as follows: *Fusarium* transcript detection in barley, accession number FG1; *Fusarium* expression profiles in complete medium and carbon and nitrogen starvation conditions, accession number FG2; *Fusarium* cross-species hybridization, accession number FG3; Fusarium/barley RNA dilution series, accession number FG4.

#### 2.2.6. RNA dilution experiment

To test for signal sensitivity of *F. graminearum* probe sets in planta, RNA samples isolated from the fungus grown in CM and mock inoculated barley plants (described above) were mixed for labeling and hybridization. A total of 10 μg RNA was used to process each chip; samples consisted either 100% fungal RNA or fungal RNA diluted $10^1$-, $10^2$-, $10^3$-, or $10^4$-fold with barley RNA. A single chip was used for each dilution. Signals were normalized among chips using MAS5.0 and the level for positive probe set detection was arbitrarily set at 100.

## 3. Results

### 3.1. Experimental validation of the F. graminearum DNA chip

Four sets of experiments were conducted to validate and test the performance of the *F. graminearum* GeneChip. The first test was conducted to assay for potential design and manufacturing flaws in the chip by DNA hybridization. The second test was to determine the reproducibility of chip experiments and usefulness for gene expression analysis in vitro. The third experiment was to determine the usefulness of the *F. graminearum* GeneChip to detect fungal gene expression during plant infection. The final experiment was to test for sensitivity limits for detecting fungal RNA in planta and the potential for cross-hybridization between fungal probe sets and plant RNAs.

#### 3.1.1. DNA hybridization experiments

To determine the number and potential sources of errors in detecting gene expression using the *Fusarium* microarray, the DNAs from *F. graminearum* strain NRRL31084, four strains of closely related *Fusarium* species (*F. asiaticum*, *F. boothii*, *F. culmorum*, and *F. pseudograminearum*) or two more distantly related species (*F. verticillioides* and *F. oxysporum*) were used to interrogate the chip. A total of 17,572 of the 17,809 *Fusarium* probe sets (98.67%) were detected at least once in two repeats of hybridization with DNA from strain NRRL31084 (Table 2). This suggests that for *F. graminearum* treatments, there is a potential false negative rate of 1.33%.

To determine the usefulness of the array for studies on related *Fusarium* species capable of causing FHB or root rot disease on wheat (Goswami and Kistler, 2005; O'Donnell et al., 2004), DNAs from four additional species were used to interrogate the *Fusarium* GeneChip. The percentage of probe sets detected using DNA from strains of these species ranged from 92.33% for *F. boothii* to 84.51% for *F. pseudograminearum*. However, only 9.17 and 8.13% of the *F. graminearum* probe sets, respectively, were detected when using DNA from the more distantly related species *F. verticillioides* and *F. oxysporum*. These data indicate that the *F. graminearum* GeneChip is suitable for detecting the majority of genes in *F. graminearum* and closely related species.

#### 3.1.2. RNA expression analysis in vitro

To determine the usefulness and reproducibility of the *Fusarium* GeneChip, RNA isolated from mycelia of *F. graminearum* NRRL31084 grown in liquid defined media were used to interrogate the microarray. The fungus was grown in either complete medium (CM), or minimal medium lacking any carbon (MMC) or nitrogen (MMN) source. Three replicate cultures were grown under each condition. To estimate source of error, GeneChip response using RNAs from separate cultures of the fungus grown on

Table 2
Detection of probe sets on the *Fusarium* GeneChip using DNA from *F. graminearum* and related *Fusarium* species

| *Fusarium* strains | Probe sets detected in 2 chips (% total[a]) | Probe sets detected in 1 chip (% total) | Probe sets detected in 0 chips (% total) |
|---|---|---|---|
| *F. graminearum* NRRL31084 | 17484 (98.18%) | 88 (0.49%) | 237 (1.33%) |
| *F. boothii* NRRL29020 | 16202 (90.98%) | 239 (1.34%) | 1368 (7.67%) |
| *F. asiaticum* NRRL26156 | 15778 (88.60%) | 324 (1.82%) | 1707 (9.59%) |
| *F. culmorum* NRRL25475 | 15120 (84.90%) | 485 (2.72%) | 2204 (12.38%) |
| *F. pseudograminearum* NRRL28338 | 14205 (79.76%) | 845 (4.74%) | 2750 (15.44%) |
| *F. verticillioides* NRRL20956 | 1633 (9.17%) | 1437 (8.07%) | 14739 (82.76%) |
| *F. oxysporum* NRRL34936 | 1447 (8.13%) | 1384 (7.77%) | 14978 (84.10%) |

[a] Percentage of the total 17809 *Fusarium* specific probe sets (categories 1–5 in Table 1).

complete medium (biological replicates) were compared to response of identical RNA samples to probe separate GeneChips (technical replications). Source of error was assessed by calculating the false change rate (FCR), which is the percentage of the probe sets showing ⩾2-fold change between any two replications. For the fungus grown on CM, the average FCR in biological replications was 5.03%, while the FCR for technical replications was 0.08%. For experiments with the fungus grown on MMC or MMN, the FCR between the biological replications was 3.84 and 3.08%, respectively.

Probe sets detected using RNA targets obtained from the fungus grown under different culture conditions were compared (Fig. 2A). In cultures grown in CM, or MMC or MMN, a common suite of 8754 probe sets were detectable. Another 1610 probe sets were detected in any two of these three cultural conditions. Among them, 768 probe sets were detected in both N- and C-starvation conditions and likely contain genes involved in generalized response to starvation including a large number of cytochrome P450s, membrane associated proteins, transcriptional activators and small molecule transporters (Supplementary Table A). Many of the 449 probe sets found only in N-starvation conditions were genes likely to be involved with acquisition and utilization of organic nitrogen sources including probe sets for likely proteases, ureases, and transporters for reduced nitrogen compounds such as peptides, amino acids, GABA, nucleosides, allantoate, and choline (Supplementary Table A).

### 3.1.3. Detection of fungal RNA in planta

A total of 7132 probe sets were detected using target sequences derived from infected barley in one or more time-points during the infection time-course. The timing of when the majority (6877) of these sequences were detected during the time course is illustrated in Fig. 3 and Supplementary Table B. The remaining 255 probe sets had more complex detection patterns, generally with signals found in two or more non-sequential time points (Supplementary Table B). To estimate source of error for the barley infection experi-

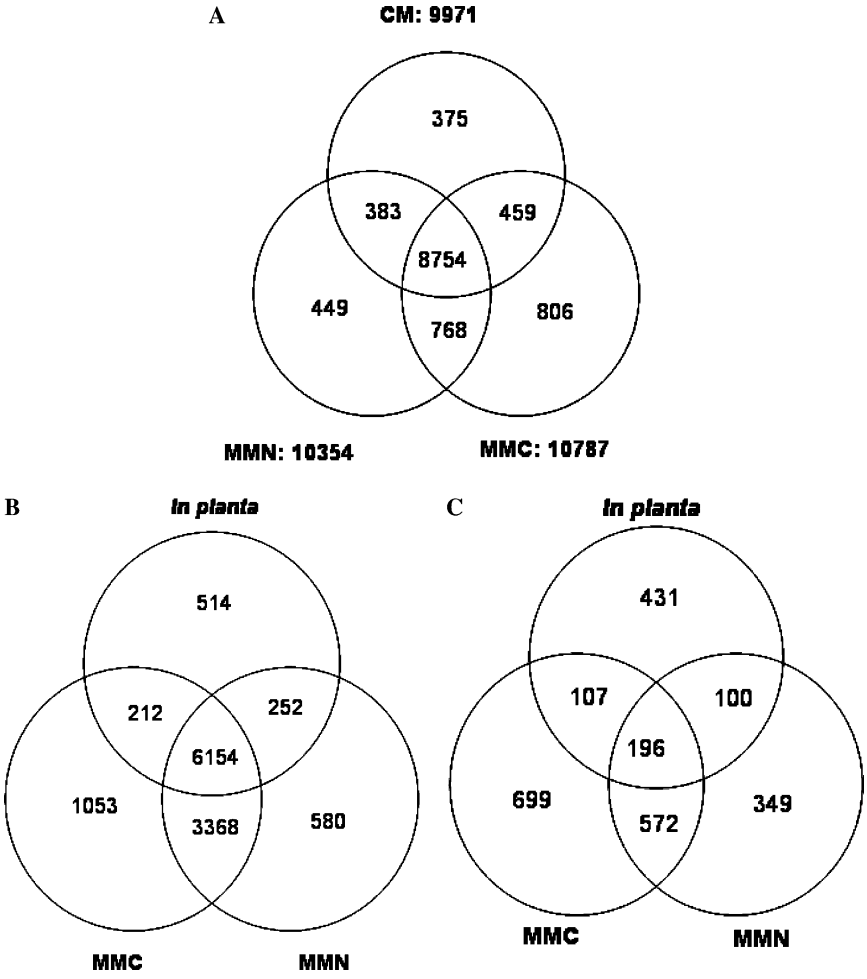


Fig. 2. Venn diagrams showing the number of probe sets detected using targets obtained from the fungus grown under different conditions. (A) From the fungus grown in complete medium (CM), under nitrogen starvation (MMN), or carbon starvation (MMC). (B) From the fungus grown in planta, nitrogen starvation (MMN), or carbon starvation (MMC). (C) From the fungus grown in planta, nitrogen starvation (MMN), or carbon starvation (MMC) after subtraction of probe sets that are also expressed in complete media from each cluster of Venn diagram B. The identities of probe sets in each cluster are given in the Supplementary Tables A, C, and D.

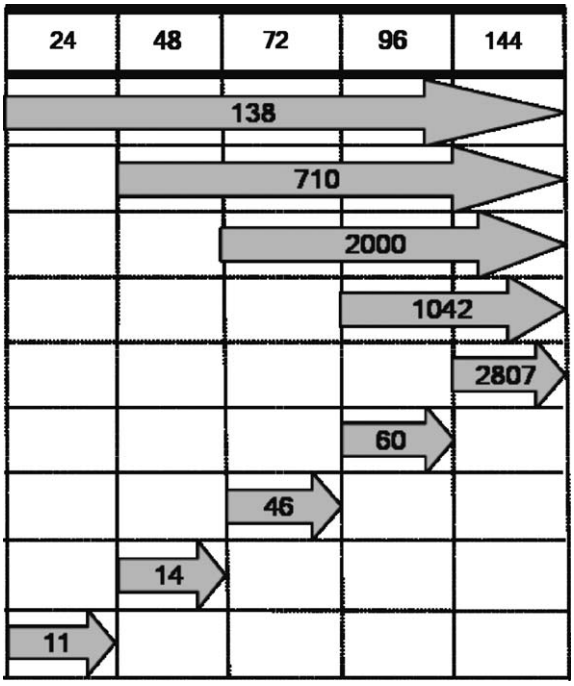


Fig. 3. Major patterns exhibited by the *F. graminearum* probe sets detected across time points tested. Numerals within the block arrows indicate the number of probe sets detected for particular periods of time. In addition to these, minor patterns of detection are shown in Supplementary Table B under the title of "miscellaneous patterns."

ment, GeneChip response using RNAs from biological replications within each time point or among controls were compared. Average FCR for all time points and controls was 1.6%. Correlation coefficient of probe set response among all biological replications was 0.949.

It has been suggested that growth of fungi under starvation conditions may promote in vitro expression of pathogenicity genes (Solomon et al., 2003; Talbot et al., 1997). To assess similarities in fungal RNA profiles specific for disease and nutrient stress-related responses in *F. graminearum*, probe sets detected during the barley infection time-course (7132) were compared to probe sets detected from the fungus grown in MMC (10787) or MMN (10354) (Fig. 2B; Supplementary Table C). To determine genes specifically expressed during plant infection or under nutrient limiting regimes, probe sets detected during growth in CM (9971) were subtracted. The number of probe sets detected in common between in planta, MMC or MMN experiments with the CM experiments were 6298, 9213, and 9137, respectively resulting in 834, 1574, and 1217 probe sets, respectively, related to in planta, MMC and MMN conditions. However, approximately 50% of the probe sets detected in planta were also detected under growth in MMC, MMN or both (Fig. 2C; Supplementary Table D). Only 431 probe sets were detected exclusively in planta. The largest portion of this group (293; 71.2%) corresponded to predicted genes with no known function. Among the probe sets corresponding to annotated genes, are members of major functional groups involved in carbohydrate metabolism, fatty acid metabolism, secondary metabolism, ionic homeostasis, disease, virulence, and defense functions. These probe sets include many corresponding to genes for potential plant cell wall degrading enzymes including xylanases, mannanases, pectinases, glucanases, galactosidases, and cutinases as well as many of the known genes involved in the biosynthesis of the trichothecene mycotoxin deoxynivalenol (Supplementary Table D).

### *3.1.4. RNA dilution experiments*

To test for limits of fungal probe set detection in planta and the potential for cross-hybridization between fungal probe sets and plant RNA, a dilution series was made between fungal and barley RNA. When fungal RNA made up 100, 10, 1 or 0.1% of the total RNA labeled and used for hybridization, the number of probe sets with detectable signals decreased from 6566 to 688, 76, and 9, respectively. With only two exceptions, relative signal for each probe set also decreased proportionally to the concentration of fungal RNA (Supplementary Figure 1). Probe sets having the lowest signal were not detected after even a 10-fold dilution while probe sets having the highest relative signal still were detected at a $10^4$- fold dilution. Fgd246-110_at and Fgr-S3-1-P04_at were the only two probe sets that had increased detection signals when fungal RNA was diluted with plant RNA, indicating that they cross-hybridize with barley sequences. These two probe sets correspond to *F. graminearum* genes of unknown function. Fgr-S3-1-P04 appears to be a fungal mitochondrial gene expressed during plant infection.

## 4. Discussion

The availability of the whole genome shotgun sequence assembly of *F. graminearum* has hastened progress on functional analysis of this important plant pathogen. Directed and random mutagenesis of the fungus are now more efficient and have accelerated forward and reverse genetic procedures. Candidate genes potentially involved in pathogenesis or toxin production can be identified and chosen for functional characterization by deletion or disruption. The availability of gene expression data also will be valuable for selecting target genes. In this study we used the *Fusarium* draft sequence assembly to construct an appropriate gene set for designing a custom Affymetrix GeneChip microarray, attempting to represent all predicted gene sequences from the assembly.

The gene set developed was an amalgamation of several different gene prediction processes. Beside the two automatically predicted gene sets (The Broad Institute and MIPS), manual annotation on putative pathogenicity related genes was performed using additional gene prediction programs, information on matching EST sequences and BLASTX analysis. This information was then integrated using manual analysis to decide the most reliable gene model. Our goal was to provide a comprehensive list of gene models based on the best informatics currently available. However, in many cases more than one conflicting gene model existed

for a given sequence (for example, see Fig. 1). When possible, probe sets were designed that could potentially discern conflicting gene predictions (e.g., fgd2-60_at and fg00016_at). However, this procedure was constrained by the necessity to design probe sets near the 3′ end of gene sequences for efficient labeling of target transcripts. Therefore, in most cases, additional informatics or functional analysis will be required to determine the validity of particular gene predictions or whether one gene model should be favored over another.

The *Fusarium* GeneChip is efficient at detecting sequences corresponding to genes from a variety of *Fusarium* species closely related to *F. graminearum* by DNA hybridization. In two repeats, 98.67% of the probe sets on the microarray were detected using *F. graminearum* DNA. Additionally, the chip is useful for detecting genes for three other closely related species capable of causing FHB (Goswami and Kistler, 2004) including *F. asiaticum*, a predominant FHB-causing species in portions of Asia (Gale, 2003; Gale et al., 2002), *F. boothii*, and *F. culmorum*, a FHB species common in Western Europe (Waalwijk et al., 2003). Additionally, gene sequences from *F. pseudograminearum*, the major causal agent of crown rot on small grains (Aoki and O'Donnell, 1999) also were readily detected. A total of 13,217 probe sets or 74.22% of the *F. graminearum* sequences on the *Fusarium* GeneChip were detected on both replicate experiments for all four FHB species as well as the *Fusarium* crown rot pathogen. These results indicate that the Affymetrix *Fusarium* GeneChip would be useful for studying these related species and the diseases they cause as well as similarities and differences in gene expression among the species.

Under ideal conditions, using DNA of the sequenced strain of *F. graminearum* to probe the *Fusarium* GeneChip should result in the detection of all *Fusarium* probe sets. However, in fact, 237 probe sets, slightly more than 1% of the total, were not detected by this method. There are several possible explanations for lack of signal with these genes: (1) several probes of the probe set may be designed across intron borders of a predicted transcript, so that oligonucleotides corresponding to these probes do not exist in genomic DNA, (2) sequencing errors exist in the region where the probe sets were designed, (3) manufacturing errors exist for these probe sets on the chip, (4) labeling reactions were inefficient for DNA corresponding to these probe sets or (5) hybridization was inefficient for the oligonucleotides corresponding to the probes. If the negative results were due to inefficient labeling or hybridization, further replication could potentially detect more probe sets. Indeed, of the 237 probe sets not detected with two replications with *F. graminearum* DNA, 12 probe sets were detected with *F. boothii* DNA, 8 probe sets were detected with *F. asiaticum* DNA and 17 probe sets were detected with both *F. boothii* and *F. asiaticum* DNA. Further analysis using hybridization results from *F. culmorum* and *F. pseudograminearum* indicate that there are only 190 probe sets that are not detected by one or more of the *Fusarium* species tested (Supplementary Table E). Thus, only 47 of the 17, 809 *Fusarium* probe sets (0.26%) may be considered false negatives caused by poor hybridization and/or labeling in two experiments with *F. graminearum* DNA. The remaining 190 probe sets may contain errors due to sequencing or manufacturing or contain probe sets that are not suitable for detection using genomic DNA rather than cDNA.

Two more distantly related *Fusarium* species also were tested by DNA hybridization to determine whether their gene sequences could be detected using the *F. graminearum* GeneChip. *F. verticillioides* is a potent mycotoxin-producing fungus on maize (Munkvold, 2003) and *F. oxysporum* is an important pathogenic fungus causing wilts on a variety of plant species (Kistler, 1997). The two representative strains of these species tested here recently have been approved for whole genome sequencing by the NSF/USDA microbial genome sequencing program. DNA from neither species hybridized efficiently with the *Fusarium* array, as less than 20% of the probe sets were reliably detected using DNA from these species. As anticipated, the degree of hybridization of probe sets to the *Fusarium* array corresponds to the relatedness of the species to *F. graminearum* (Fig. 4). Both *F. boothii* and *F. asiaticum* are members of the *F. graminearum* species complex (34) and are sister taxa of *F. graminearum*. Greater than 90% of probe sets on the array were detected using DNA from either species. *F. culmorum* and *F. pseudograminearum* are B-trichothecene producing species that are more genetically distant from *F. graminearum* based on phylogenetic inference from DNA sequence data (O'Donnell et al., 2000; O'Donnell et al., 2004). DNA from these species can be used to detect between 80 and 90% of the probe sets on the *F. graminearum* GeneChip. *F. verticillioides* and *F. oxysporum* are even more distantly related to *F. graminearum* than the others and only less than 20% of the probe sets from these species are detected using DNA from these species.

The *Fusarium* GeneChip design also allows for the detection of expression of thousands of genes corresponding to tar-

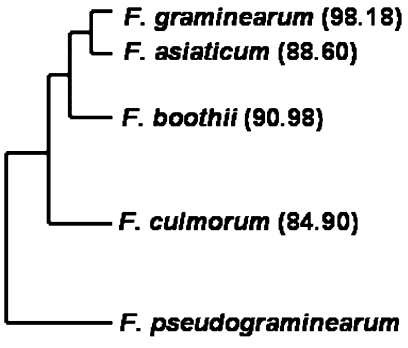


Fig. 4. Phylogenetic relationship among *Fusarium* species and number of corresponding probe sets on the *F. graminearum* GeneChip. Phylogenetic relationships between five different *Fusarium* species was aligned with the percentage of probe sets that were detected in two chips using targets obtained from DNA of those species. Previously determined phylogenetic relationships (O'Donnell et al., 2000) corresponded to percentage of probe sets detected (in parentheses), except for *F. asiaticum* and *F. boothii*.

gets from the fungus grown in culture or in infected plants. A total of 11,994 of a possible 17,809 *Fusarium* probe sets (67.35%) were detected under various conditions of the fungus grown in culture examined here and a total of 7132 probe sets (40.05%) were detected from the fungus during infection of barley. The reproducibility of the *Fusarium* Affymetrix GeneChip appears to be outstanding with the FCR for technical replications being 0.08%. This rate for most applications would be negligible so there would be no need for technical replication. By far, biological variation would be the greatest source of error for gene expression studies, resulting in 1.6–5.0% FCR for pairwise comparison of individual experiments. However, using three biological replications for treatment and controls, the number of probe set false positives repeatedly exhibiting 2-fold change expected would be less than 10, even at the highest FCR. While this level of error would be acceptable for most purposes, increasing replication levels or increasing the level of change required for significance would decrease the number of expected false positives to an even lower level.

Surprisingly, a large number of fungal probe sets (7132) were called present during the barley infection time course. The fraction of fungal transcripts in the total RNA from infected plants is likely very low, especially during the early stages of infection. No procedures were taken to enrich for fungal transcripts and the initial spore density used to spray inoculate plants was fairly low. Nevertheless even at the earliest time point, over 100 sequences from the fungus were detectable (Fig. 3). The fraction of fungal RNA in the total RNA pool doubtlessly increases over time as fungal biomass within the plant increases during the infection process. Therefore changes in "gene expression" implied in Fig. 3 actually also reflect increases in transcript abundance due to increased fungal biomass within the plant.

The list of sequences provided by in vitro and in planta GeneChip experiments provides a satisfying first look at genes likely involved in transcriptional response to environmental cues. For example, probe sets uniquely detected during nitrogen limiting conditions are greatly enriched for genes whose annotation implies they are involved in the acquisition and uptake of reduced nitrogen. Likewise, probe sets detected only during the barley infection process correspond to genes with annotation implying that they would be involved in breakdown of complex substrates likely to be found in plants, especially monocots. These results strongly indicate that the microarray will be extremely useful for fungal gene expression studies under a variety of experimental and environmental conditions including different stages of plant infection.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.01.005.

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