The first fifty microarray studies in filamentous fungi

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Microarray studies have examined global gene expression in over 20 species of filamentous fungi encompassing a wide variety of research areas. The majority have addressed aspects of metabolism or pathogenicity. Metabolic studies have revealed important differences in the transcriptional regulation of genes for primary metabolic pathways between filamentous fungi and yeast. Transcriptional profiles for genes involved in secondary metabolism have also been established. Genes required for the biosynthesis of both useful and detrimental secondary metabolites have been identified. Due to the economic, ecological and medical implications, it is not surprising that many studies have used microarray analysis to examine gene expression in pathogenic filamentous fungi. Genes involved in various stages of pathogenicity have been identified, including those thought to be important for adaptation to the host environment. While most of the studies have simulated pathogenic conditions in vitro, a small number have also reported fungal gene expression within their plant hosts. This review summarizes the first 50 microarray studies in filamentous fungi and highlights areas for future investigation.

Introduction

Microarray analysis allows the identification of differentially regulated genes from multiple distinct samples differing in environmental condition, developmental stage or genetic background. The first fungal microarray studies were reported in the budding yeast Saccharomyces cerevisiae 10 years ago (DeRisi et al., 1997; Lashkari et al., 1997). Since then microarrays have been fabricated for more than 20 species of filamentous fungi. The majority have been constructed using PCR products amplified from expressed sequence tag (EST) libraries. More recently, arrays including all predicted ORFs have enabled more comprehensive genome-wide expression studies. This review describes the earliest 50 microarray studies retrieved by a search of the Web of Science database using the query ‘microarray* AND fungus’. The first 50 microarray studies in filamentous fungi have covered a broad spectrum of research areas, including metabolism, development, pathogenesis, symbiosis and industrial applications (Table 1).

Metabolism

Microarray studies of primary metabolism have revealed striking differences between filamentous fungi and budding yeast. Bonaccorsi et al. (2006) examined the effects of altering oxygen availability on cultures of the obligatory aerobe Trichoderma reesei. The majority of genes for the glycolytic pathway and TCA cycle were strongly repressed under oxygen-free conditions. Such a reduced expression of glycolytic genes would be insufficient to maintain anaerobic metabolism for prolonged periods without oxygen. In contrast, S. cerevisiae has been previously shown to up-regulate glycolytic genes in oxygen-free conditions, allowing metabolism to continue anaerobically (Kwast et al., 2002). Several studies have examined the effects of glucose starvation on primary metabolic genes (Chambergo et al., 2002; Maeda et al., 2004; Xie et al., 2004). In S. cerevisiae strong glucose repression of TCA cycle genes means that glucose is preferentially metabolized anaerobically, and all TCA cycle genes are strongly up-regulated during glucose starvation (DeRisi et al., 1997). In T. reesei several TCA cycle genes were only partially up-regulated while others were unaffected by glucose starvation (Chambergo et al., 2002). In Neurospora crassa and Aspergillus oryzae some TCA cycle genes were even down-regulated by glucose starvation (Maeda et al., 2004; Xie et al., 2004). It is clear that the same degree of glucose repression does not operate in filamentous fungi and that aerobic respiration has a greater role in glucose metabolism by filamentous fungi than by S. cerevisiae.

Glucose starvation experiments have also revealed differences in ethanol utilization among filamentous fungi. Similar to the case in S. cerevisiae, the alcohol dehydrogenase responsible for ethanol assimilation in T. reesei was found to be constitutively expressed before and during glucose starvation (DeRisi et al., 1997; Chambergo et al., 2002). This is in contrast to A. oryzae and N. crassa, where it was strongly induced during glucose starvation (Maeda et al., 2004; Xie et al., 2004). Glucose repression of alcohol dehydrogenase is well characterized in Aspergillus nidulans and is mediated through the transcription factor CreA (Flipphi et al., 2002). CreA is also known to repress many other metabolic genes under glucose-rich conditions.
Table 1. The first fifty microarray studies in filamentous fungi

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<td><em>Gibberella zaece</em></td>
<td>Evaluated subtraction library enriched with sexual reproduction genes (N)</td>
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<td><em>Fusarium oxysporum f. sp. vasinfectum</em>*‡‡</td>
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<td><em>Gibberella zaece</em></td>
<td>Identified peritheicum development genes (RT)</td>
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<td><em>Blumeria graminis</em></td>
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<td><em>Blumeria graminis</em></td>
<td>Obtained profiles of metabolic genes during infection (RT)</td>
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<td><em>Fusarium oxysporum f. sp. lycopersici</em>††</td>
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<td><em>Metarhizium anisopliae</em></td>
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<td><em>Cryphonectria parasitica</em></td>
<td>Identified specific hypovirus-responsive genes (RT)</td>
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<td><em>Cryphonectria parasitica</em></td>
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playing a major role in carbon catabolite repression (Flippin et al., 2003). Sims et al. (2004) evaluated an A. nidulans microarray by examining the expression of previously characterized metabolic genes. CreA-mediated glucose repression was observed in cells which were shifted from ethanol to glucose. Aign & Hoheisel (2003) also explored the effects of carbon source, comparing cultures grown on ethanol to glucose. Aign & Hoheisel (2003) also explored the effects of carbon source, comparing cultures grown on ethanol to glucose.

Secondary metabolite genes are often clustered together in the genome and regulated by a common transcription factor. The nuclear protein LaeA is a global regulator of secondary metabolite clusters in A. nidulans. Bok et al. (2006) compared expression in an laeA deletion (ΔlaeA) mutant and a wild-type strain to identify clusters controlled by LaeA. Genome mining identified one particular cluster in which all five genes were down-regulated in the ΔlaeA strain. Analysis of the secondary metabolite produced by the cluster revealed the antitumour compound terrequinone A, a metabolite not previously described in A. nidulans.

Aflatoxins are highly carcinogenic secondary metabolites which contaminate a wide variety of crops, including maize.

### Table 1. cont.

<table>
<thead>
<tr>
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<tr>
<td><em>Neotyphodium</em> species/Epichloë festucae†‡</td>
<td>Identified rapidly evolving genes (N/A)</td>
<td>2161 (cDNA)</td>
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<td><em>Paxillus involutus</em>§§</td>
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<td>2161 (cDNA)</td>
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<td><em>Paxillus involutus</em>§§</td>
<td>Identified host-specific symbiosis-regulated genes (N)</td>
<td>2161 (cDNA)</td>
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<td><em>Paxillus involutus</em>§§</td>
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<td><strong>Aspergillus niger</strong></td>
<td>Evaluated subtraction library enriched with dithiothreitol stress genes (SL)</td>
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<td><em>Aspergillus nidulans</em></td>
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<td>5131 (oligo)</td>
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*Further details can be found in the text.
†Microarray studies are often validated by comparing expression data for a small number of genes (5–10) with results obtained from another analytical method. In general, the results obtained from the microarray studies in this review correlated well with Northern or real-time quantitative PCR data. The method used for validation is given in parentheses: N, Northern analysis; PN, compared to previously published Northern; PM, compared to previously published microarray; RT, real-time quantitative PCR; SL, compared to genes isolated from subtraction library; N/A, not applicable; NV, no validation reported.
‡Affy, Affymetrix GeneChips; DNA, short oligonucleotides based on genomic DNA; cDNA, PCR amplicons on coated glass slides; Nim, Nimblegen arrays; oligo, long oligonucleotides.
§Microarray based on whole genome.
‖Aspergillus flavus cDNA microarray hybridized with *Aspergillus parasiticus* probes.
¶*Phanerochaete chrysosporium* cDNA microarray hybridized with Trametes gallica probes.
#Neurospora crassa cDNA microarray hybridized with Sordaria macrospora probes.
**Microarray represents ESTs from both *Fusarium oxysporum* f. sp. *vasinfectum* and its host *Gossypium hirsutum*.
††Microarray represents a variety of *Fusarium* species that infect cereal grain.
‡‡Two microarrays were used, one representing 3806 *Neotyphodium coenophialum* and *Neotyphodium lolii* genes and the other representing 4195 *Neotyphodium* and 920 *Epichloë festucae* genes.
§§Microarray represents ESTs from both *Paxillus involutus* and its ectomycorrhizal partner *Betula pendula*.
‖‖Further information is available to academic groups and non-profit organizations (hans.roubos@dsm.com).
AflR regulates transcription of genes required for the biosynthesis of aflatoxin in Aspergillus parasiticus (Yu et al., 1996). Price et al. (2006) compared expression of an aflR deletion mutant (ΔaflR) with its parental wild-type. Microarray analysis revealed 23 genes up-regulated in the wild-type. These included three genes outside the aflatoxin pathway cluster which were previously not associated with aflatoxin biosynthesis. Earlier studies investigated the influence of fungal development (O’Brian et al., 2003) and culture conditions (Price et al., 2005) on aflatoxin production. Price et al. (2005) compared expression in culture conditions conducive and not conducive to aflatoxin production. Cluster analysis was used to identify genes with similar expression profiles to known aflatoxin biosynthesis genes. A similar strategy was employed to identify genes associated with fumonisin biosynthesis in the plant pathogen Fusarium verticillioides (Pirttilä et al., 2004). Nineteen genes had similar expression profiles to known fumonisin genes. Six of these were homologous to genes with characterized functions including the high-affinity (Zrt1p) and low-affinity (Msc2p) zinc transporters in S. cerevisiae.

Development

Filamentous fungi provide excellent models to study development in multicellular eukaryotic organisms. Kasuga et al. (2005) used microarrays to investigate the genetic mechanisms important for conidial germination in N. crassa. Expression was compared at distinct morphological events such as isotropic swelling, germ tube emergence and the formation of a mycelial mat. A total of 1287 genes were differentially regulated and the majority grouped into seven clusters with similar expression patterns. Transcriptional profiles were consistent with previously published data and corresponded with known biochemical processes associated with conidial germination. The results were also compared to previous microarray studies detailing spore germination in the dimorphic fungus Ustilago maydis (Zahiri et al., 2005) and the amoeba Dictyostelium discoideum (Xu et al., 2004). Of the 1287 genes differentially regulated in N. crassa, 25 were orthologous to those regulated in U. maydis and 16 orthologous to those regulated in D. discoideum. Kasuga et al. (2005) concluded that the transcriptional similarities between these diverse organisms suggest that mechanisms of spore germination could be evolutionarily conserved.

Nowrousian et al. (2005) examined the complex developmental process of fruiting body formation in Sordaria macrospora. Expression profiles of three developmental mutants (pro1, pro11 and pro22) unable to produce mature perithecia were compared to a wild-type strain. Genes encoding proteins associated with cell wall biogenesis and fungal development were significantly down-regulated in the mutant strains. Sexual development in S. macrospora was further examined by Pöggeler et al. (2006), who identified genes regulated by the mating type gene smta-1. Since smta-1 is also unable to produce mature perithecia it is not surprising that 10 of the genes identified were also differentially expressed in the three pro mutants (Nowrousian et al., 2005; Pöggeler et al., 2006). The N. crassa MAP kinase MAK-2 and its target PP-1 are also required for sexual development (Li et al., 2005). Li et al. (2005) compared expression in a wild-type to Δmak-2 and Δpp-1 deletion mutants. Interestingly, two of the genes up-regulated in both Δmak-2 and Δpp-1 are rhythmically expressed circadian-clock-controlled genes (Bell-Pedersen et al., 1996).

Circadian-clock-controlled genes have been characterized in cyanobacteria, fungi, plants and animals (Young & Kay, 2001). Sleep patterns, hormone cycles and body temperature in humans and photosynthesis in plants rely on the rhythmic expression of clock-controlled genes (Takahashi & Zatz, 1982). Temperature and light keep them in synchrony with the environment. Circadian rhythms are important for development in N. crassa (Sargent et al., 1966). Conidiation peaks at the transition from dark to light or from cold to warm, a time known as subjective dawn. Several studies have used microarrays to identify novel clock-controlled genes in N. crassa (Correa et al., 2003; Nowrousian et al., 2003). Expression was compared at various time points throughout the circadian cycle and clustering used to identify rhythmically expressed genes with similar expression patterns. The genes identified coded for proteins with diverse functions, including protein synthesis, metabolism and development (Correa et al., 2003; Nowrousian et al., 2003).

The products of frq, wc-1 and wc-2 form an auto-regulatory negative feedback cycle known as the FRQ/WCC oscillator that is required for circadian regulation in N. crassa (Nowrousian et al., 2003). Nowrousian et al. (2003) used microarray analysis to demonstrate that the FRQ/WCC oscillator is responsive to temperature. Of the 27 clock-controlled genes identified in the study, 14 were also temperature regulated. However, temperature regulation of all 14 genes was lost in an frq knockout mutant (Δfrq). This result suggests that the FRQ/WCC oscillator mediates temperature-induced synchrony of N. crassa clock-controlled genes. In addition to its role as a circadian regulator, WC-1 regulates all known blue-light-responsive clock-independent genes (Lewis et al., 2002). Lewis et al. (2002) used microarrays to identify genes that are induced by light and genes that are induced by overexpression of WC-1. Since only four of the 22 light-induced genes identified are also induced by WC-1 overexpression it was concluded that elevated levels of WC-1 alone are not sufficient to activate all light-responsive genes.

Pathogenesis

Microarray studies have addressed fungal pathogens of both plants and animals. Many plant-pathogenic fungi rely on the formation of appressoria (swollen hyphae required for anchorage and penetration) to infect their hosts. Turgor pressure generated by the appressorium combined with
enzyme degradation allows these fungi to penetrate the plant cuticle. Takano et al. (2003) aimed to identify genes important for appressorium formation in *Magnaporthe grisea* using cultures grown on appressorium-inducing GelBond medium. Sixty-seven genes were up-regulated in developing appressoria compared to vegetative mycelia. Interestingly, transcripts for 19 of these genes were present at higher levels in dormant conidia compared to vegetative mycelia. These included a homologue of a characterized virulence gene from *Collectotrichum gloeosporioides* (cap20) (Hwang et al., 1995) and $\Delta^{24}$-sterol C-methyl transferase. Sterol biosynthesis has also been linked to pathogenicity in *C. gloeosporioides* (Kim et al., 2002) and the insect pathogen *Metarhizium anisopliae* (Wang et al., 2005a). The results suggest that a pool of pre-existing conidial mRNA may contain transcripts important for pathogenesis.

The *Blumeria graminis* cap20 homologue was also significantly up-regulated in developing appressoria (Both et al., 2005a). Gene expression was examined in this plant pathogen as part of a wider study of fungal development during infection of barley (Both et al., 2005a, b). RNA was collected from eight stages of infection representing the complete asexual cycle of the fungus. Ten other potential pathogenicity genes were identified whose expression correlated with the cap20 homologue. Five were homologous to previously characterized virulence genes and five were homologous to genes with previously proposed pathogenicity roles. Microarray analysis of fungal genes expressed in planta has also revealed pathogenic genes in the rust fungus *Uromyces fabae* (Jakupovic et al., 2006) and the cotton pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (McFadden et al., 2006). McFadden et al. (2006) identified a putative oxidoreductase gene, with homologues in both pathogenic and non-pathogenic fungi, which was expressed 500-fold higher in planta compared to vegetative mycelia. Furthermore, in planta expression of the gene in pathogenic isolates was greater than that in nonpathogenic isolates. Expression was also positively correlated with vascular browning, a characteristic symptom of *Fusarium* wilt infection (McFadden et al., 2006).

*Cryphonectria parasitica* is the causative agent of chestnut blight and infects chestnut trees worldwide. Hypoviruses have been shown to reduce virulence in *C. parasitica* and are important for its biological control (Allen et al., 2003). Allen et al. (2003) compared fungal expression in a strain of *C. parasitica* infected with the hypovirus CHV1-EP713 to a virus-free parent. A group of 295 differentially regulated genes belonged to a variety of functional groups including metabolism, development and cell wall growth. A homologue of *M. grisea* Mst12, a transcription factor regulating infectious hyphal growth, was amongst those down-regulated in the virus-infected strain. A major connection between viral hypovirulence and G-protein-mediated signal transduction was established when expression of two mutant strains (Δcpg-1 and Δcpgb-1) missing Gα and Gβ G-protein subunits was examined (Dawe et al., 2004). Almost half (45 %) of the genes previously shown to be regulated by hypovirus infection were also differentially expressed in at least one of the G-protein mutants compared to the parental wild-type.

Microarray analysis has also been used to investigate infection in animal pathogens. Perhaps the best-studied animal pathogens are dimorphic, not filamentous, and so are not described here (recent microarray studies in *Candida albicans* include Hromatka et al., 2005; Sandovsky-Losica et al., 2006; Lepak et al., 2006). The human pathogen *Aspergillus fumigatus* must adapt to its host temperature for successful infection. Nierman et al. (2005) examined the thermostolerance of the fungus by comparing expression at temperatures representing pathogenic (37°C) and non-pathogenic (30°C and 48°C) environments. Many genes encoding heat-shock proteins were differentially regulated, suggesting their importance for temperature adaptation. Voriconazole is used to treat patients infected with *A. fumigatus*. However, its use is often limited by drug resistance (Ferreira et al., 2006). Ferreira et al. (2006) examined the ability of the fungus to adapt to this antifungal agent. Cluster analysis revealed one group of rapidly up-regulated genes containing an ABC multidrug transporter and a glutathione S-transferase. Both genes are thought to have roles in voriconazole detoxification and are potentially required for adaptation to the drug.

Adaptation by the insect pathogen *Metarhizium anisopliae* during infection of its host has also been addressed using microarray analysis. *M. anisopliae* must adapt to both the cuticle and haemolymph of its host (Wang et al., 2005a). Microarray analysis revealed subsets of genes associated with its adaptation which included genes for cell wall reorganization and adaptors to osmotic stress (Wang et al., 2005a). *M. anisopliae* was also observed to adapt specifically to different insect hosts (Freimoser et al., 2005). One particular hydrophobin (a family of proteins which mediate fungal attachment to hydrophobic surfaces) was up-regulated on medium containing cockroach or beetle cuticle but unaffected on medium containing gypsy moth cuticle. The differential regulation of this and other hydrophobins (Freimoser et al., 2005) further links cell wall changes to fungal adaptation.

**Symbiosis**

Ectomycorrhizae (ECM) are ecologically important symbiotic associations consisting of both plant and fungal tissue. While the fungus gains a source of photosynthetic sugars, the plant benefits from improved nutrient uptake. Several studies have investigated global expression during the development of ECM between *Betula pendula* (birch) and *Paxillus involutus*. Both fungal and plant genes specifically regulated in the ectomycorrhizal symbiosis have been identified.

Fungal genes specifically regulated in the ECM have a variety of putative functions. A predicted fungal hexose transporter...
was up-regulated during the entire course of ECM development (Le Quéré et al., 2005). This is significant because the fungal partner in the symbiotic relationship receives sugars from the plant (Söderström et al., 1988). Le Quéré et al. (2005) speculated that the putative hexose transporter is a candidate for fungal hexose assimilation in the ECM tissue. Homologues of proteins in the electron transport chain were also highly up-regulated during ECM development (Le Quéré et al., 2005). However, the lowered expression of the TCA cycle enzyme malate dehydrogenase suggested that the potential increase in respiration was not connected to glucose metabolism. Fungal genes with putative functions in the secretory pathway were also differentially expressed in ECM tissue (Johansson et al., 2004; Wright et al., 2005). Interestingly, many were expressed more strongly in rhizomorphs, tubular hyphal aggregates that function in the transport of nutrients to the ECM root tissue, than in ECM root tissue itself (Wright et al., 2005).

Consistent with the fungal partner, ECM-regulated plant genes also have a variety of putative functions. Many predicted metabolic genes, including several encoding TCA cycle and electron transport chain enzymes, were down-regulated in ECM tissue (Johansson et al., 2004). Johansson et al. (2004) suggested that the reduced expression of these enzymes indicated a lower rate of aerobic respiration in ECM compared to free-living roots. Genes with putative roles in plant defence were also differentially expressed in ECM tissue (Johansson et al., 2004; Le Quéré et al., 2005). While many were up-regulated in early stages of ECM development they were not significantly regulated in later stages (Le Quéré et al., 2005).

Recently microarrays have been fabricated for the grass fungal endophytes Neotyphodium coenophialum, Neotyphodium lolii and Epichloë festucae (Felitti et al., 2006). Several proof-of-concept hybridizations were reported comparing different saprophytic growth conditions (Felitti et al., 2006). The microarrays should allow further insight into the mutualistic associations between plants and fungi.

**Industrial applications**

Filamentous fungi are also used as hosts to produce commercially important heterologous proteins. Industrial-scale production of heterologous proteins often results in lower yields than those achieved for native proteins. A molecular understanding of the secretory system is important for improving efficiency of recombinant protein secretion. Sims et al. (2005) used microarray analysis to identify genes associated with recombinant protein production in A. nidulans. Expression was compared in a recombinant bovine-chymosin-producing strain and its wild-type parent. The study revealed a variety of secretion-related genes involved with the unfolded protein response (UPR). Manipulation of UPR genes in Aspergillus species has been shown to improve yields of heterologous proteins (Moraledo et al., 2001; Conesa et al., 2002). MacKenzie et al. (2005) aimed to identify further UPR genes in Aspergillus niger using the secretion blocker DTT, thought to mimic the effects of recombinant protein secretion. Subtractive hybridization and microarray analysis were used to identify genes regulated by DTT. While a number of stress-responsive genes were identified they did not appear to be related to the UPR, suggesting that DTT does not specifically induce the UPR.

**Future prospects: the next fifty**

Microarray analysis is still in its infancy and much potential remains for further study in all of the areas covered in this review. Of the studies investigating pathogenesis only a few have reported expression in plant hosts and none have reported expression in animal hosts. There is certainly much potential for further investigation of in-host expression, provided that complications of obtaining sufficient quantities of fungal RNA and interference from host RNA can be overcome. It would also be of great interest to compare genes induced during pathogenic interactions to those induced in symbiotic relationships. Such a comparison may distinguish genes associated with host adaptation from those related to pathogenicity. Of the 50 studies detailed in this report it is surprising that none have investigated the cell cycle. Microarray studies in both budding and fission yeast have identified many periodically expressed cell cycle genes not previously connected to the cell cycle (Spellman et al., 1998; Bähler, 2005; Peng et al., 2005). There is great potential for similar studies in filamentous fungi, particularly in A. nidulans, where many cell cycle mutants are available.

Comparing expression profiles of different species of filamentous fungi may provide insight into how this diverse group of fungi has evolved. Interestingly, microarrays have only been fabricated for three filamentous species of basidiomycetes (Paxillus involutus, Phanerochaete chrysosporium and Uromyces fabae) and none represent filamentous zygomycetes. The potential for comparative functional genomics within filamentous fungi would greatly benefit from more microarrays of filamentous members from these groups. Several studies have described successful cross-species hybridizations in closely related species of filamentous fungi. An Affymetrix GeneChip recently fabricated for
the plant pathogen *F. graminearum* has been shown to efficiently detect genes from four other closely related species of *Fusarium* (Güldener et al., 2006). Cross-species analysis could dramatically increase the number of studies available for comparative functional genomics.

The recent completion of several fungal genome projects has further fuelled the potential for global gene expression studies in filamentous fungi. The first 50 microarray studies in filamentous fungi were published over a four-year period. With the explosion of fungal microarray studies the next 50 will appear much faster.

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