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Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*)

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Abstract

Gibberella zeae is a broad host range pathogen that infects many crop plants, including wheat and barley, and causes head blight and rot diseases throughout the world. To better understand fungal development and pathogenicity, we have generated 7996 ESTs from three cDNA libraries. Two libraries were generated from carbon-(C-) and nitrogen- (N-) starved mycelia and one library was generated from cultures of maturing perithecia (P). In other fungal pathogens, starvation conditions have been shown to act as cues to induce infection-related gene expression. To assign putative function to cDNAs, sequences were initially assembled using StackPack. The estimated total number of genes identified from the three EST databases was 2110: 1088 contigs and 1022 singleton sequences. These 2110 sequences were compared to a yeast protein sequence reference set and to the GenBank nonredundant database using BLASTX. Based on presumptive gene function identified by this process, we found that the two starved cultures had similar, but not identical, patterns of gene expression, whereas the developmental cultures were distinct in their pattern of expression. Of the three libraries, the perithecium library had the greatest percentage (46%) of ESTS falling into the ''unclassified'' category. Homologues of some known fungal virulence or pathogenicity factors were found primarily in the N- and C-libraries. Comparisons also were made with ESTs from the related fungi, *Neurospora crassa* and *Magnaporthe grisea* and the genomic sequence of *N. crassa*.

Keywords: *Gibberella zeae*; *Fusarium graminearum*; Mycotoxin; Deoxynivalenol; Zearalenone; Perithecia; Ascus; Pathogenicity; Virulence; Expressed sequence tags

1. Introduction

In the past decade, Fusarium head blight of wheat and barley, caused by the fungal pathogen *Fusarium graminearum* (teleomorph *Gibberella zeae*), has reached epidemic proportions in the United States causing yield losses and price discounts resulting from reduced seed quality (Windels, 2000). Increasingly, the disease is becoming a threat to the world's grain supply due to recent head blight outbreaks on several continents (Dubin et al., 1997). The pathogen can significantly reduce seed quality and yield due to discolored, shriveled ''tombstone'' kernels. Scabby grain is also often contaminated with trichothecene and zearalenone, an estrogenic mycotoxin (McMullen et al., 1997), making it unsuitable for food or feed. Furthermore, highly resistant plant cultivars are not available and use of effective fungicides for controlling the disease is limited by cost, difficulty in efficient application to wheat heads and an incomplete understanding of factors that influence disease development (McMullen et al., 1997).

Gibberella zeae overwinters on infected plant debris as mycelia and produces ephemeral perithecia when temperature and moisture conditions are favorable. Ascospores, along with asexual conidia, are believed to

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be the major inoculum sources for infection of flowering wheat and barley heads (Fernando et al., 2000). The fungus, a homothallic ascomycete, is composed of eight phylogenetically defined lineages; lineage 7 is the predominant lineage in North America and Europe (O'Donnell et al., 2000; Ward et al., 2002). Cultures of *G. zeae* can be out-crossed (Bowden and Leslie, 1992) and classical and molecular genetic techniques such as fungal transformation and gene disruption are readily accomplished (Proctor et al., 1995). Several recent studies have begun to elucidate sexual development and pathogenicity in this fungus. To date, the *TRI5* gene involved in trichothecene production is the only virulence gene characterized in this pathogen (Desjardins et al., 1996; Proctor et al., 1995). Infection and host response have been investigated in wheat (Pritsch et al., 2000). Development and function of perithecia have been characterized in vitro (Trail and Common, 2000; Trail et al., 2002), and the molecular biology of mating type has been explored (Yun et al., 2000).

Recent advances in genomics studies have provided efficient approaches to identify pathogenicity or virulence genes in plant pathogenic fungi and oomycetes. Several expressed sequence tag (EST) databases have been generated with a limited number of phytopathogenic fungi. In *Phytophthora infestans*, 760 unique sequences were identified out of a total of 1000 random ESTs from a mycelial cDNA library (Kamoun et al., 1999). Four novel elicitin-like proteins were identified and may be involved in the recognition between *P. infestans* and *Nicotiana* plants. Following the sequencing of 986 clones from a cDNA library constructed with RNA isolated from 'budding' conidia of *Mycosphaerella graminicola*, 704 unigenes were identified and 407 of them had moderate homology to known genes (Keon et al., 2000). In the obligate pathogen *Blumeria graminis*, 4908 ESTs were sequenced from cDNA libraries constructed from ungerminated and germinating conidia (Thomas et al., 2001). Among 1669 putative genes identified in these ESTs, 65 had homologues in non-pathogenic fungi but only 29 displayed homology to genes from fungal pathogens, including *M. grisea* and *M. graminicola* (Thomas et al., 2001). EST projects for two basidiomycetes, *Agaricus bisporus* and *Pleurotus ostreatus*, compare expression of basidiocarp developmental stages (Ospina-Giraldo et al., 2000; Lee et al., 2002). The construction of a relational database of 3 phytopathogenic fungi has been described (Soanes et al., 2002). The authors also present a reference of all searchable, publicly accessible pathogenic fungal and oomycete genomic resources on the web. The list includes sites for 12 phytopathogenic fungi and oomycetes.

Recently Kruger et al. (2002) described an EST database from wheat heads infected with the *G. zeae*. While this library contained many sequences apparently unique to wheat scab interaction, only a small fraction of ESTs were attributable to the fungus. To better characterize genes unambiguously expressed by the fungus itself, three cDNA libraries were generated to identify genes important to disease production: one library was generated from cultures of maturing perithecia, and one library was generated each from carbon- (C-) and nitrogen- (N-) starved mycelial cultures. Because nutritional starvation, particularly nitrogen-starvation, may act as the environmental cue for disease development in fungal pathogens (Pieterse et al., 1994; Talbot et al., 1997; van den Ackerveken et al., 1994), the C- and N-starved libraries may contain many genes expressed during plant infection. The full complement of ESTs generated in the course of this study have been submitted to dbEST (Id 13375065-13384819) and are accessible on a publicly available searchable website (www.scabusa.org).

2. Materials and methods

2.1. Culture conditions and library preparation

Gibberella zeae lineage 7 strain PH-1 (NRRL 31084; Trail and Common, 2000) was used for construction of all cDNA libraries and maintained as soil stocks at −20 °C. To construct the library from sexually sporulating cultures, nearly synchronous lawns of perithecia were produced on carrot agar as previously described (Klittich and Leslie, 1988). After 2–4 days of growth on carrot agar in 60 mm Petri dishes, the mycelium reached the perimeter of the plate. Perithecia were induced from vegetative hyphae at this stage with the addition of 1 ml of 2.5% aqueous Tween 60 (Sigma, St. Louis, Missouri). Developing perithecia, 6 days postinduction, were gently scraped from the surface of carrot agar cultures with a sterile scalpel and immediately frozen at −80 °C. Tissue was subsequently lyophilized and total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) following manufacturer's directions. From total RNA, mRNA was prepared using Poly(A) Tract mRNA Isolation System II (Promega, Madison, WI) according to manufacturers directions. The perithecium cDNA library was constructed using the SuperScript Lambda System with ZipLox, *Not*I–*Sal*I Arms (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The plasmid was excised in the host strain DH10B(ZIP).

For libraries from C- and N-starved cultures, PH-1 mycelia were collected from 1 L of 2-day-old culture grown in complete medium (CM, Correll et al., 1987), rinsed with sterile distilled water, and transferred into 1 L liquid minimal medium (MM, Correll et al., 1987) lacking a carbon- or nitrogen-source, respectively. After incubation for an additional 24 h with shaking at 150 rpm, mycelia starved for carbon or nitrogen were collected by filtration through 1 layer of Miracloth

(Calbiochem, La Jolla, CA) and frozen at −80 °C. Total RNA was extracted as above and mRNA was purified with the Oligotex mRNA isolation kit (Qiagen, La Jolla, CA). The cDNA libraries were synthesized in the vector Uni-ZapII with a cDNA synthesis kit (Stratagene, La-Jolla, CA). The cDNA inserts were directionally cloned between the *Eco*RI and *Xho*I sites of pBluescript (with the 3′ poly(A) tails close to the *Xho*I site). Both C- and N-cDNA libraries were excised according to manufacturer's directions. For all libraries, individual white colonies were transferred by a Q-Pix robotic workstation (Genetix, UK) to 384-well microplates and preserved at −80 °C.

2.2. Sequence and functional analysis

Individual cDNA clones were purified with the Qiagen plasmid preparation kit (Qiagen, Valencia, CA) and sequenced with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA). EST sequences were clustered with StackPACK v2.1.1 (Burke et al., 1999; Miller et al., 1999). StackPACK compares sequences and groups them into clusters and singletons. The clusters are further divided into contigs and consensus sequences. We will use this terminology in describing our analysis. The contig and singletons resulting from the StackPACK analysis were compared to the yeast protein sequence reference set from MIPS (Mewes et al., 1997, 2002) and against GenBank number with BLAST programs (Altschul et al., 1997). The MIPS reference set has MIPS functional category associations encoded within the FASTA description line for each predicted open reading frame (ORF) in *Saccharomyces cerevisiae*. A match was considered to be significant if the expected (E) value was less than $1e-5$, and for these analyses only the most significant hit was examined, unless otherwise indicated. Clusters and singletons assigned to more than one MIPS category were individually examined and reassigned to a single appropriate functional category. Those designated to MIPS categories 98 and 99 (classification not yet clearcut and unclassified) were also individually examined and reassigned to a single appropriate functional category based on the BLAST search results against GenBank. Singletons and clusters which could not be reassigned were combined into the MIPS 99 category. Many cDNAs were sequenced from both the 3′ and 5′ ends, but were counted only once.

The *N. crassa* genome sequence was available from the Whitehead Research Institute (www-genome.wi.mit.edu/annotation/fungi/neurospora/). EST sequences from the *Neurospora* perithecia specific library (Nelson et al., 1997) were downloaded from GenBank (Accession Nos: BG278041–BG280722). The *M. grisea* ESTs from five cDNA libraries constructed with RNAs isolated from appressoria, conidia, and mycelia grown on minimal, complete, and nitrogen-deficient media were available at the URL: plpalinux.tamu.edu maintained by D. Ebbole at Texas A&M University.

3. Results

3.1. Construction of cDNA libraries and single-pass sequencing of 7996 cDNAs

Three cDNA libraries were constructed with RNAs isolated from perithecia (P) and mycelia starved for N and C sources. The P-, N-, and C-libraries contained 5×10^5, 1×10^6, and 2×10^6 primary plaques and had the average insert size of 0.75, 0.8, and 0.8 kb, respectively. A total of 7996 ESTs were generated and the number of clones sequenced for each library was: 2567 from the P-library, 1218 from the C-library, and 4211 from the N-library.

At day 6 after induction, both immature and mature perithecia were present but the majority of perithecia were mature and actively discharging ascospores 6 days after induction. Within a mature perithecium at day 6, up to 50% of the asci may be immature (Trail et al., 2002). Therefore, the P-library represented those genes involved in perithecium and ascospore maturation. Vegetative mycelium and conidia were minimal in these cultures.

3.2. Distribution of ESTs to clusters, contigs, and singletons

StackPack was used to distribute ESTs into loose clusters. Within these clusters, one or more contigs were then established from which consensus sequences were derived. From an input of 7996 EST sequences, 1088

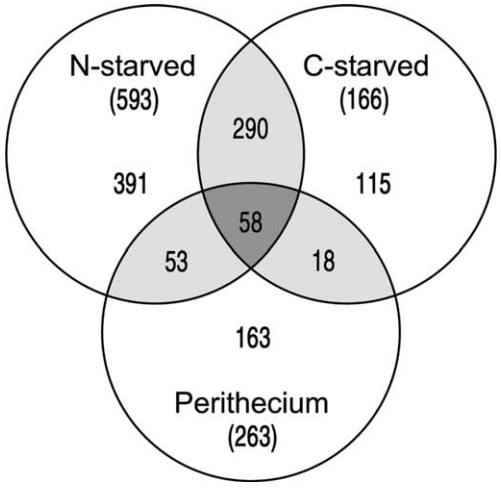

Fig. 1. Distribution of contigs among the three EST libraries. Numbers in shaded areas indicate contigs composed of ESTs from multiple libraries. Numbers in parentheses represent the singletons derived from each library.

Table 1
Distribution of ESTs functional classes categories as defined by MIPS

MIPS No.	Category	N-starved	C-starved	Perithecium	Total
1	Metabolism	413	151	133	697
2	Energy	175	47	27	249
3	Cell growth, division, and DNA synthesis	79	23	57	159
4	Transcription	288	73	48	409
5	Protein synthesis	984	237	156	1377
6	Protein destination	387	94	658	1139
7	Transport facilitation	56	26	27	109
8	Cellular transport and transport mechanisms	43	11	17	71
9	Cellular biogenesis	17	10	7	34
10	Cellular communication/signal transduction	52	18	10	80
11	Cell rescue, defense, cell death, and aging	103	40	15	158
13	Ionic homeostasis	87	22	26	135
30	Cellular organization	279	76	202	557
99	Unclassified	1248	390	1184	2822
	Total	4211	1218	2567	7996

contigs were generated and 1022 singleton sequences were identified. Therefore, the estimated number of genes identified by the EST database was 2110. Fig. 1 shows the distribution of contigs and singletons among the three libraries. The N- and C-libraries share the largest proportion of the contigs, 290.

3.3. Functional annotation and analysis and highly expressed genes

ESTs were given a putative functional assignment according to the classification developed by MIPS (Table 1). It should be noted that functional categories

Table 2
Major functional categories and representative subcategories that differ significantly between libraries[a,b]

MIPS No.	Functional category	% ESTs in library		
		Nitrogen-starved	Carbon-starved	Perithecia
1	Metabolism	9.81a	12.40b	5.18c
1.01	Amino acid	2.92a	3.20a	0.39b
1.02	Nitrogen and sulfur	0.47a	0.33a	0b
1.03	Nucleotide	0.71ab	1.15a	0.55b
1.04	Phosphate	0.21a	3.12b	0c
1.05	C-compound and carbohydrate	2.37a	2.71a	1.60b
1.06	Lipid, fatty acid	1.85a	2.71a	0.70b
2	Energy	4.16a	3.86a	1.05b
4	Transcription	6.84a	6.00a	1.87b
4.01	rRNA transcription	0.78a	0.08b	0.04c
4.03	tRNA transcription	0.17a	0.08a	0b
4.05	mRNA transcription	4.32a	4.93a	1.25b
4.07	RNA transport	0.19a	0.08a	0b
4.99	Other transc. activities	0.19a	0.16a	0b
5	Protein synthesis	23.37a	19.46b	6.08c
5.01	Ribosomal proteins	20.47a	17.73a	5.77b
5.04	Translation	2.78a	1.48b	0.23c
6	Protein destination	9.19a	7.72a	25.63b
6.04	Protein targeting, sorting, and translocation	0.66a	0.90a	18.08b
6.07	Protein modification	2.04a	1.64a	1.36b
6.10	Assembly of protein complexes	0.28a	0.49a	0.74b
7	Transport facilitation	1.33a	2.13b	1.05a
9	Cellular biogenesis	0.40ab	0.82a	0.27b
10	Cellular communication/signal transduction	1.23a	1.48a	0.39b
11	Cell Rescue, defense, cell death, and aging	2.45a	3.28a	0.58b
13	Ionic homeostasis	2.07a	1.81a	1.01b
99	Unclassified	29.64a	32.02a	46.12b

[a] Values within a row followed by a letter in common are not significantly different, $P < 0.05$. Based on the significance tests of Audic and Claverie (1997).

[b] Subcategories are shown only for those categories with significant differences between libraries and containing more than 100 ESTs in a single library.

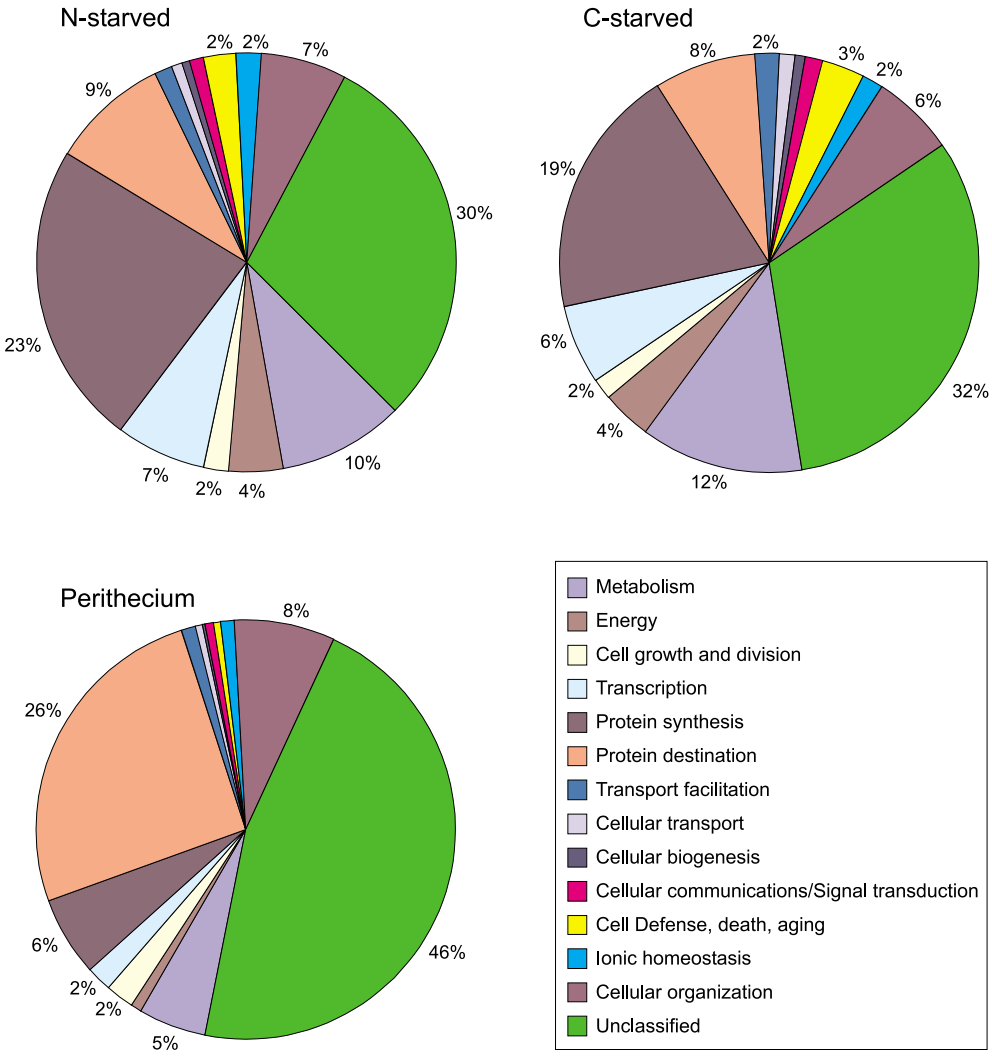

Fig. 2. Distribution of ESTs into MIPS categories for each of the libraries. Although initial comparisons were made using contigs and singletons, percentages are calculated from the total numbers of ESTs, not contigs, in each functional category to reflect the relative levels of expression of genes in each category.

for sequences were based entirely on inference from BLASTX reports; tests for gene function have not yet been conducted in the organism. All MIPS categories were compared among the 3 libraries. Table 2 shows the results for functional categories (and subcategories) that differ significantly among the libraries. The pattern of gene expression in the C- and N-cultures was remarkably similar (Fig. 2). Three categories show large differences in expression patterns between the C- and N-libraries and the P-library. Unclassified ESTs and ESTs encoding genes related to protein destination are significantly higher in the P-library (Table 2). Genes related to protein synthesis were more highly expressed in the N-and C-libraries.

The highly expressed genes (as represented by contigs composed of 20 or more ESTs) from each library, are listed in Table 3. Of the 29 contigs having ESTs from the C-library (a smaller EST set), 27 also had ESTs in the N-library (the larger EST set). Contigs 161 and 176 contained ESTs from the C- and P-libraries, although both contigs consisted predominantly of P ESTs (contig 161: 47 P ESTs, 1 C-EST; contig 176: 25 P ESTs, 1 C-EST). Of the 41 highly expressed ESTs presented in Table 3, 17 were of unknown function and 11 were ribosomal components.

3.4. Comparison with N. crassa perithecial ESTs and predicted proteins

The clusters and singletons assembled from the P-library ESTs with StackPack were used to search against ESTs from the *N. crassa* perithecial library (Nelson et al., 1997) using Blastn and Tblastx. Only 5.2% of the *G. zeae* sequences had high levels of similarity ($P < e - 10$) to *N. crassa* perithecia ESTs (Table 4). Contig 278 shows homology to *Aspergillus nidulans, DIGA* and is highly

Table 3
The most prevalent mRNAs as measured by EST redundancy

Contig[a]	No. ESTs	Best match; GenBank Accession No.	E value[b]	Libraries[c]
140	31	Unknown	N/A[d]	N,P
144	50	Rps30ap [ribosomal protein]; NP013390	1.00E-30	N,P
147	35	Unknown	N/A	C,N,P
152	26	Unknown	N/A	P
155	21	Diacylglycerol acyltransferase type 2A [*Mortierella ramanniana*]; AAK84179	2.00E-62	C,N,P
156	61	Unknown	N/A	P
161	48	Unknown	N/A	C,P
166	27	Polyubiquitin; AJ223328	3.39E-124	C,N,P
167	95	Unknown	N/A	P
176	27	Unknown	N/A	C,P
179[e]	51	Translationally controlled tumor-associated protein P23fyp; NC003424	9.00E-33	P
180[e]	113	Translationally controlled tumor-associated protein P23fyp; NC003424	3E-46	C,N,P
264	21	60S Ribosomal protein L37; Q9C0T1	8.00E-34	C,N,P
267	43	Unknown	N/A	P
278	443	DigA protein; CAC27452	2.00E-44	P
282	27	Unknown	9.00E-11	P
292	27	40S Ribosomal protein S6; NC003424	2.00E-97	C,N,P
296	38	Unknown	N/A	P
298	21	Glutamine synthetase; AJ310443	3.00E-78	C,N,P
316	32	60S Ribosomal protein L18; NC003423	1.00E-51	N,P
330	21	60S Ribosomal protein L33-A; Q9USX4	1.00E-37	C,N,P
353	20	40S Ribosomal protein S26E; P21772	1.00E-108	C,N,P
360	28	Unknown	N/A	C,N,P
362	23	Yor088wp [*Saccharomyces cerevisiae*]; NC001147	3.00E-21	P
365	52	(U39049) Hmp1 [*Ustilago maydis*]; U39049	1.00E-08	C,N,P
474	23	60S Ribosomal protein L27-A; NC001136	4.00E-49	C,N,P
491	46	Unknown	N/A	C,N
493	30	Unknown	N/A	C,N
502	43	60S Ribosomal protein; Q9HFR7	4.00E-27	C,N
526	139	30 KD Heat shock protein; P40920	7.00E-55	C,N
547	22	Rpl6ap [*Saccharomyces cerevisiae* I.e., ribosomal gene]; NC001145	2.00E-41	C,N
548	39	Unknown	N/A	C,N
569	34	Unknown	N/A	C,N
574	22	5S rRNA binding ribosomal protein; O59953	2.00E-77	C,N
592	20	Putative cell wall protein [*Emericella nidulans*]; AH011296	8.00E-27	C,N
604	75	Unknown	N/A	C,N
632	27	60S Ribosomal protein L10; NC003423	1.00E-95	C,N
690	23	Ribosomal protein L15; AL669999	1.00E-135	C,N
692	28	Unknown	N/A	C,N
708	23	60S Ribosomal protein L13; NC001136	4.00E-41	C,N
783	22	Conserved hypothetical protein [*Neurospora crassa*]; AL513410	3.38E-12	C,N

[a] Contigs were generated by StackPack. NCBI was used for the BLAST searches against GenBank number initially, if no significant match was found, BLASTX was used. Only contigs composed of 20 or more ESTs are listed.

[b] E values represent matches expected merely by chance according to the model of Karlin and Altschul (1990). E values of greater than E-05 were considered to be insignificant and were listed as UNKNOWN.

[c] Libraries abbreviated as follows: P = perithecium, N = nitrogen starved, C = carbon starved.

[d] N/A = not applicable.

[e] Contigs 179 and 180 match to slightly different regions of the translationally controlled tumor-associated protein, indicating these contigs may represent the same gene.

Table 4
Comparison of *G. zeae* ESTs with *N. crassa* and *M. grisea* sequences

	E value < 1e − 5 (%)	E value < 1e − 10 (%)
Perithecial ESTs vs. *N. crassa* perithecial ESTs	6.5	5.2
Perithecial ESTs vs. *N. crassa* predicted proteins	31.9	26.1
Total ESTs vs. *N. crassa* predicted proteins	53.8	47.0
G. zeae N-ESTs vs. *M. grisea* N-ESTs	21.4	17.8

Table 5
ESTs from *Fusarium graminearum* with homologues associated with pathogenicity and virulence in other fungi

G. zeae EST	Library[a]	Homologue in GenBank	BLASTX	Reference
Fgr_4_F06_T3	N-	AF006827, *Magnaporthe grisea MAC1*	7e – 25	Adachi and Hamer (1998)
Fgr-C_1_G16_T3	C-	U70134, *M. grisea PMK1*	1e – 124	Xu and Hamer (1996)
Contig 530	C-(1) N-(1)	AF247189, *Candida albicans PHR1*	7e – 92	Saporito-Irwin et al. (1995)
Contig 781	C-(2)	AF091042, *Cercospora kikuchii CFP*	3e – 52	Callahan et al. (1999)
Contig 1163	N-(3)	P30574, *C. albicans CPY1*	6e – 59	Mukhtar et al. (1992)
Contigs 809 and 851	C-(1) N-(5)	P87090, *Cryphonectria parasitica CPC1*	2e – 32	Wang et al. (1998)
Contig 1183	N-(3)	U18061, *Glomerella cingulata CAP20*	1e – 41	Hwang et al. (1995)
Contig 882	N-(3)	P56092, *C. albicans EPD1*	1e – 27	Nakazawa et al. (1998)
Fgr-C_0_P06_T3	C-	AB052108, *C. albicans CaNAG3*	1e – 11	Yamada et al. (2001)
Contig 1032	N-(1)	AJ249387, *Stagonospora nodorum ODC1*	e – 112	Bailey et al. (2000)
Fgr-C_2_N12_T3	C-	Q12645, *Nectria haematococca PDA1*	4e – 13	Maloney and VanEtten (1994)
Fgr_1_F24_T3	N-	AF027979, *M. grisea PTH2*	5e – 50	Sweigard et al. (1998)
Fgr_2_K14_T3	N-	AB078975, *Fusarium oxysporum FOW1*	7e – 82	Inoue et al. (2002)
1610_B01_C02ZT	P	O42622, *M. grisea PTH9* or *TREB*	4e – 62	Sweigard et al. (1998)
Contigs 991 and 992	N-(4, 5 resp.)	AF115320, *C. albicans. SAP2*	7e – 19	Zaugg et al. (2001)
Contig 693	C-(1) N-(6)	AF254144, *C. albicans RBT4*	1e – 15	Braun et al. (2000)

[a] Numbers of ESTs within the contigs are provided in parentheses.

abundant in the P-library (Table 3). Contigs 179/180, probably corresponding to one gene (see footnote e, Table 3) is also highly abundant in the P-library. Surprisingly, the *N. crassa* homologues of these two genes were present but were not abundant among *N. crassa* perithecial ESTs.

We also compared *G. zeae* perithecia ESTs with the *N. crassa* genome sequence. A total of 26.1% (at $< e - 10$) or 31.9% (at $< e - 5$) of the P-library clusters and singletons had homologous sequences in the *N. crassa* genome (Table 4). Overall, 53.8% of *G. zeae* ESTs (combined from all three libraries) had homologues in the *N. crassa* genome at $P < e - 5$ (47.0% at $P < e - 10$). Interestingly, that is very similar to the percentages of *M. grisea* ESTs that have hits in the *N. crassa* genome (D.J. Ebbole, personal communication).

3.5. Genes homologous to known fungal virulence or pathogenicity factors

ESTs from all three libraries were examined for genes homologous to known fungal virulence or pathogenicity factors. Among 16 virulence/pathogenicity genes identified (Table 5), all were from the N- and C-libraries except one from the P-library. Six of them are homologous to known virulence factors in the human pathogen *Candida albicans*, including *PHR1*, *EPD1* and *CPY1* that are involved in yeast-to-hyphae or yeast-to-pseudohyphae transition (Mukhtar et al., 1992; Nakazawa et al., 1998; Saporito-Irwin et al., 1995). Both *PHR1* and *EPD1* encode glycosylphosphatidylinositol-anchored proteins. *G. zeae* genes homologous to *MAC1*, *PMK1*, *PTH2*, and *PTH9* genes of *M. grisea* were also found (Table 5). *PTH2* and *PTH9* encoding a carnitine acetyl transferase and a neutral trehalase, respectively, are pathogenicity genes identified by insertional mutagenesis (Sweigard et al., 1998). In the N-library, ESTs homologous to *ODC1* and *FOW1* were also found (Table 5). *ODC1* encodes an ornithine decarboxylase gene that is necessary for wheat infection in *Stagonospora nodorum* (Bailey et al., 2000). *FOW1* encodes a mitochondrial carrier protein, which is required specifically for colonization in the plant tissue by *F. oxysporum* (Inoue et al., 2002). Mutants disrupted in *FOW1* have normal growth and conidiation in culture but are defective in colonizing plant tissues and causing diseases.

The ESTs from the N-library were assembled into 480 contigs and 711 singletons with StackPack and compared with the *M. grisea* ESTs from a nitrogen starvation library (Table 4). Among these 1191 contigs and singletons of *G. zeae*, 17.8% had homologous ESTs in the *M. grisea* N-library at $< e - 10$ while 21.4% had homologous sequences at $< e - 5$. These results indicated that the majority of *G. zeae* ESTs in this library did not have homologues in the *M. grisea* nitrogen starvation library.

4. Discussion

EST sequencing has been widely used as an efficient approach for gene discovery. In this study, we sequenced 7996 clones from three cDNA libraries, representing different growth conditions and sexual reproduction, and identified 2110 putative genes of *G. zeae*. It has been estimated that there are 5900 genes encoded by the *S. cereviseae* genome, 8100 by the *A. nidulans* genome and 9200 in the *N. crassa* genome (Kelkar et al., 2001; Kupfer et al., 1997). *G. zeae*, a Pyrenomycete, is most closely related to the Pyrenomycete *N. crassa*, and assuming similar genome size and conservation of gene number, the ESTs generated in this

study represent approximately one quarter of the genes encoded by the *G. zeae* genome. Although closely related gene families may not be distinguished by separate contigs, numbers of genes predicted by contig and singleton analysis are usually overestimated due to nonoverlapping sections of genes that may be sequenced (Fernandes et al., 2002; Ohlrogge and Benning, 2000; White et al., 2000).

The profile of *G. zeae* ESTs from three different libraries is a first attempt to understand gene expression during sexual development and under nutritional conditions that induce expression of pathogenicity genes. The C- and N-starved libraries had similar patterns of gene expression as determined by functional analysis (Figs. 1 and 2; Tables 2 and 3). In the major categories Protein synthesis, Protein destination, and Unclassified, the fraction of ESTs from the P-library is significantly different than those of the C- and N-libraries. Based on developmental studies of the perithecium (Trail and Common, 2000), we can speculate that greater transcription of genes related to the category "Protein destination" would be necessary for development of asci during this stage. However, the reduced amounts of ribosomal proteins in the P-library as compared to the C- and N-libraries may be due to the presence of large numbers of cells in the mature perithecium wall that have begun to decline (Trail and Common, 2000). Other categories in Table 2 were also significantly different among the 3 EST libraries, but the differences are relatively small, and difficult to interpret.

Of the three libraries, the P-library had the greatest percentage (46%) of ESTs falling into the "unclassified" category. This may be explained by the lack of characterization of genes involved in sexual development in ascomycetous fungi. The genera *Gibberella* (in the Hypocreales) and *Neurospora* (in the Sordariales) belong to different orders within the *Pyrenomycetes*. There are differences in perithecium development which distinguish these orders. The low percentage of *G. zeae* perithecia ESTs that had homology in *N. crassa* perithecia ESTs may reflect these differences. For instance, *Gibberella* perithecia are formed in a stroma, whereas *Neurospora* develop independent perithecia. However, it is also likely that the low percentage of homologous perithecia ESTs between *G. zeae* and *N. crassa* resulted from differences in RNA samples used for cDNA library construction. The *N. crassa* perithecia cDNA library was constructed with RNA isolated from perithecia, 5 days after fertilization (Nelson et al., 1997). The *N. crassa* perithecia at five days were undeveloped and had not yet initiated ascospore development. The *G. zeae* P-library was isolated from cultures of mature perithecia that were discharging mature spores.

Do the most abundant contigs in each library (Table 3) tell us anything about gene expression under different conditions? Of the 10 highly expressed genes in the P-library, six have unknown functions, including contig 362 which has high homology to a putative gene from *S. cerevisiae*, but has an undescribed function. The most remarkable EST in the P-library is contig 278, which is a DigA homologue (*A. nidulans*) and is responsible for fully 17% of the ESTs from that library (functional subcategory 6.04; Table 3). DigA has been shown to be required for vescicle sorting and *digA* mutants show effects on nuclear migration, mitochondrial morphology and polarized growth. The associated homologue in *S. cerevisiae* is *PEP3* (GenBank Accession No. NP_013249; Goffeau et al., 1996) and in Drosophila is *DOR* (Shestopa et al., 1997). It is not surprising that this gene would be highly expressed in ascus formation, as numerous vescicles are visible in developing asci, necessary for the generation of ascus and spore membrane (Read and Beckett, 1996; Trail and Common, 2000). Further studies will focus on the function of this protein in perithecium development. Polyubiquitin (contig 166) is expressed moderately in all three libraries (17 ESTs in P, 1 in C-, 9 in N-), an indication of normal cell protein degradation (Funakoshi et al., 2002). Contig 298, encoding a putative glutamine synthetase, is composed of predominantly ESTs from the N-library (1 ESTs in P, 4 in C-, 16 in N-). In *N. crassa*, glutamine synthetase is synthesized at a higher rate in nitrogen-starved cultures, where it apparently facilitates protein recycling (Mora, 1990). Two contigs, 179 and 180 cluster together, and probably represent the same gene. Together they constitute 169 ESTs, primarily in the P-library. The best match for these contigs is to the so-called Translationally Controlled Tumor-Associated Protein (Thaw et al., 2001) that is highly conserved and abundantly expressed among eukaryotes. The exact function is unclear in any organism, but there is substantial support for a role in abundant cell growth (as is associated with cancerous growth). In particular, there is evidence that this protein binds to Rab proteins (of the *ras* gene family) and functions as a molecular chaperone (Thaw et al., 2001).

Nutritional starvation has been shown in several fungal pathogens to induce the expression of genes expressed in planta (Pieterse et al., 1994; Talbot et al., 1997; van den Ackerveken et al., 1994). In the *G. zeae* C- and/or N-ESTs, 16 known pathogenicity-associated genes were identified. Three of the genes (*PHR1*, *cpCPC1*, and *RPT4*) were expressed in both C- and N-libraries, nine were only found in the N-library, three only in the C-library, and one was expressed only in the P-library (Table 5). The reason more known pathogenicity genes were identified in ESTs from N-library may be related with the fact that about three times more ESTs were sequenced from the N-library than from the C-library. We also identified several ESTs that are homologous to genes that are known to be highly or specifically expressed during plant infection, including homologues of ESTs from appressoria of *M. grisea* or

haustoria *Uromyces fabae* (such as the homologue of *U. fabae AAT1*; Hahn and Mendgen, 1997; Fgr_7_H06). However, the functions of these genes in pathogenesis have not been determined genetically and therefore, they have not been included in Table 5. Two of these genes, *PMK1* MAP kinase and *MAC1* adenylate cyclase of *M. grisea*, are related to two signaling pathways important for pathogenesis and other developmental processes in several pathogenic fungi (reviewed by Kronstad et al., 1998). In *Glomerella cingulata, CAP20* is a novel protein expressed in appressoria and important for plant infection (Hwang et al., 1995). Three ESTs homologous to *CAP20* were found in the N-library. It will be interesting to determine the function of *CAP20* homologue in *G. zeae* because it does not form appressoria for plant penetration. The *C. parasitica CPC1* (*cpCPC1*) is a general amino acid transcriptional activator homologous to *N. crassa CPC1* and *S. cerevisiae GCN4* (Wang et al., 1998). Mutants with a defective DNA binding domain of *cpCPC1* were reduced in virulence on chestnut tissues. ESTs homologous to *cpCPC1* were found in both C- and N-libraries, indicating that mycelia starved for nitrogen or carbon starvation may result in general amino acid starvation (Ebbole et al., 1991).

Contig 781 (Table 5) appears to be a homologue of *CFP*, a gene for a major facilitator superfamily (MFS) protein located within the toxin (cercosporin) biosynthetic gene cluster of *Cercospora kikuchii* (Callahan et al., 1999). This membrane associated protein results in cercosporin efflux and increased tolerance to the toxin (Upchurch et al., 2002) and is required by the fungus for normal levels of toxin production and maximum virulence toward its host (Callahan et al., 1999). The *CFP* homolog presumably may play a similar role in export of toxic compounds in *G. zeae* but does not correspond to the gene for the MFS protein associated with the trichothecene biosynthetic cluster, *Tri*12 (Alexander et al., 1999). Also found only in the C-library is an EST with similarity to *PDA1* from *Nectria haematococca*, another gene involved with tolerance to a toxic compound and fungal virulence. *PDA1* encodes a cytochrome P450 monooxygenase involved in the detox- ification of an antibiotic (phytoalexin) produced by the host plant during infection by the fungus (Maloney and VanEtten, 1994) and is a determinant of virulence of the fungus to pea (Wasmann and VanEtten, 1996). It remains to be determined if the corresponding gene in *G. zeae* may be involved in a detoxification mechanism and/or important for determining the outcome of a disease reaction.

The single EST listed in Table 5 that is expressed in the P-library encodes a neutral trehalase. It is unclear why this would be required for pathogenicity in *M. grisea*, where it was identified as a pathogenicity gene (Sweigard et al., 1998). However, trehalose is a common storage carbohydrate in fungi, and trehalase may be required to breakdown reserves within the fungus for disease development.

Individual genes of interest identified by sequencing projects need to be disrupted in the organism to confirm function. This work is in progress for many of the genes identified here. Sequencing of ESTs from additional cultural conditions and developmental stages is in progress. Our goal is to obtain a more global knowledge of genes expressed at various developmental stages of the fungus and to establish an EST database representing high percentage of *G. zeae* genes. Comparison of these additional libraries, and microarray analysis will provide a more comprehensive picture of gene expression in this fungus. In addition, the EST library will help greatly with the genome sequence annotation.

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