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Gene expression shifts during perithecium development in *Gibberella zeae* (anamorph *Fusarium graminearum*), with particular emphasis on ion transport proteins

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Abstract

Gibberella zeae, the causal agent of Fusarium head blight, is a devastating pathogen of small grains worldwide. The sexual cycle is a crucial component of head blight epidemiology, as forcibly discharged ascospores serve as the primary inoculum. The recent development of an Affymetrix GeneChip containing probesets representative of all predicted genes of *G. zeae* has opened the door to studies of differential gene expression during sexual development. Using GeneChips, a developmental time course was performed in culture, from vegetative hyphae to mature perithecia with multiseptate ascospores. Time-points represent the development of the major cell types comprising the mature perithecium. The majority of the 17,830 *G. zeae* probesets, 78%, were expressed during at least one of the developmental stages; 12% of these appear to be specific to sexual development. Analysis of the 162 predicted ion transporter genes is reported in detail, due to their association with perithecium function. Expression patterns of the MirA-type siderophores, chloride channels, P-type ATPases and potassium transporters show some specialization in regard to developmental stage. This is the first whole-genome analysis of differential transcript accumulation during sexual development in a filamentous fungus.
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1. Introduction

Gibberella zeae (Schwein) Petch (anamorph *Fusarium graminearum* Schwabe), a filamentous ascomycete, is the causal agent of head blight of wheat, and, additionally, is capable of causing serious disease in barley, maize and other cereal crops. Infection is difficult to control because of a lack both of cost-effective fungicides and of resistant cereal cultivars. While fully capable of asexual growth and reproduction, *G. zeae* relies on forcible ejection of sexually derived ascospores from field debris to infect grain flowers. As the grain develops, the fungus reduces seed weight and produces mycotoxins which contribute to the devastating economic effects of this disease (Windels, 2000).

Gibberella zeae is homothallic and the sexual development process has been described in detail both in culture (Trail and Common, 2000) and in wheat plants (Guenther and Trail, 2005). Perithecia, the sexual fruiting bodies of *G. zeae*, are generated on the surface of colonized senesced host plants and crop residues. Synchronously developing cultures can be initiated and a summary of the developmental stages is presented in Fig. 1. Four tissue types are

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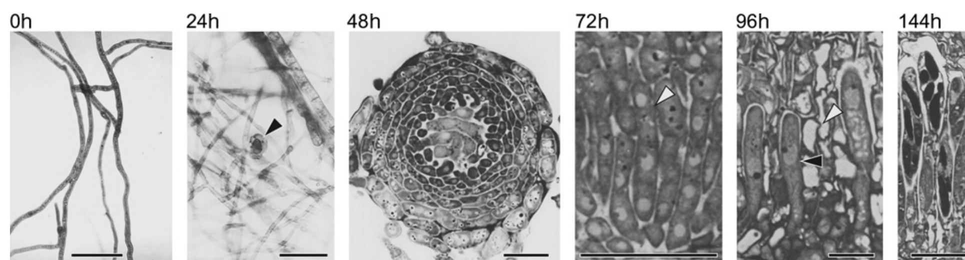

Fig. 1. Sexual development in *G. zeae* in culture: induction of haploid hyphae (time 0 h); dikaryotic cells and perithecium initials (24 h; initial shown at arrowhead); young perithecia, comprised of central ascogenous cells and the developing wall (48 h). In the last three stages, the center matures: paraphyses (white arrows) differentiate (72 h), and as they senesce, asci (arrowhead) develop (96 h) and finally, mature spores form (stained black, 144 h). Bars: 0, 24, 144 h = 20 μm; 48, 72, 96 h =10 μm.

formed during development: the perithecial wall, ascogenous hyphae, paraphyses, and asci, which produce the spores. As the tissues of the perithecium are produced in succession, a developmental time course to determine tissue-specific gene expression is possible. Development occurs rapidly, and is complete within 7 days from initiation in culture. In the field, dormancy can occur after formation of the perithecium initial and once further development is initiated, the perithecium will complete maturation and release spores uninterrupted. *G. zeae* has been a model for the study of the mechanism of forcible ascospore discharge. Pharmacological studies implicate both potassium and calcium transport in the discharge mechanism (Trail et al., 2002). The levels of potassium and chloride ions accumulating in the ascus epiplasmic fluid were shown to be sufficient to drive ascospore discharge (Trail et al., 2005).

Transport proteins maintain the intracellular environment of all cells and provide the front for cellular interactions with the environment. In microorganisms, the surface of most cells is directly exposed, and thus must be equipped to monitor and react to external conditions. With the availability of many microbial genome sequences comes the ability to study these membrane components as a group. In fungi, an inventory of transport proteins has been limited to *Saccharomyces cerevisiae*, for which several studies have been done to identify all transporters (André, 1995; Paulsen et al., 1998; De Hertogh et al., 2002). In addition, a whole-genome analysis provided phylogenetic clustering based on transporter profiles of 141 organisms, including *S. cerevisiae, Schizosaccharomyces pombe*, and *Neurospora crassa* (Ren and Paulsen, 2005). However, despite the current availability of genome sequences from numerous filamentous fungi, similar studies on the "transportome" of these important microbes have not been forthcoming. Both functional and phylogenetic classification of these genes in filamentous fungi is a priority for understanding their role in fungal life-styles from saprotrophic to pathogenic to mutualistic.

Fruiting bodies present a unique problem in fungal biology because of the presence of different cell types. With the advent of genomic sequencing, it is now possible to begin to understand how this development occurs. A *G. zeae* whole-genome Affymetrix GeneChip expression array has been generated based on the predicted gene set (Güldener et al., 2006). We have used this GeneChip to examine shifts in gene expression during perithecium formation. Several studies of gene expression during sexual development of filamentous fungi have been published (Nowrousian et al., 2005; Nowrousian and Kück, 2006; Qi et al., 2006). Prior to the availability of the whole genome array, we performed a limited study of three developmental time-points on the expression of approximately 2000 genes in a cDNA microarray (Qi et al., 2006).

The present study is the first whole genome analysis encompassing the development of all cell types in a fungal fruiting body. The analysis will provide information on gene expression that will be useful for other perithecium-producing fungi. We focus on identifying genes that, based on their expression patterns, might play a specialized role in sexual development. Because ion transport and regulation are known to be vital in all organisms and cation fluxes have been shown to be important to ascospore discharge in this fungus (Trail et al., 2002, 2005), we conclude with a comprehensive analysis of ion transport proteins.

2. Materials and methods

2.1. Time course

Ten microliters of a spore stock of *G. zeae* strain PH-1 (FGSC 9075, NRRL 31084) at 10^6 conidia ml^{-1} were spread across the surface of carrot agar (Klittich and Leslie, 1988) in a 60-mm diam Petri plate and incubated at room temperature (rt; 22–24 °C) under continuous light, to suppress circadian rhythm, until the mycelia reached the edge of the plate (4 days). Sexual development was induced by removing the surface mycelium and applying 1 ml 2.5% Tween 60 to the surface (Bowden and Leslie, 1999) and continuing the incubation. The mycelium removed at the time of induction was considered the 0 h (0h), vegetative mycelium. Surface mycelium and, in subsequent time points, developing perithecia were similarly removed from the plates, lyophilized and frozen at −80 °C, according to the developmental time course shown in Fig. 1. As the timing of perithecium development and ascus maturation may vary slightly from experiment to experiment, material from representative plates was exam-

ined for developmental stage prior to collection, and samples were harvested only when the appropriate developmental stage was present (i.e., wide dikaryotic hyphae for the 24h samples). For the sake of convenience, sexual developmental stages were labeled by the approximate time at which each stage occurs, as shown in Fig. 1. Five replicates were harvested and analyzed from 0h, three replicates of 24h, four replicates of 48h, three replicates of 72h, five replicates of 96h, and three replicates of 144h.

2.2. RNA extraction

RNA was extracted from lyophilized samples using the Trizol reagent (Invitrogen). Later stages in the time course, particularly the 48–96 h stages, were polysaccharide-rich and required the addition of a CTAB[1]-chloroform step, which was incorporated into all RNA preparations. Lyophilized samples were ground in liquid nitrogen, and Trizol was added to obtain approximately 1 ml Trizol 30 mg^{-1} mycelia (dry weight). The resulting Trizol slurry was incubated 5 min at rt. For each ml Trizol, 200 μl chloroform was added and the sample was incubated an additional 2–3 min rt, then centrifuged 15 min at 12,000*g*, 4 °C. The upper aqueous layer was recovered, and 500 μl CTAB solution was added (2% CTAB, 2% polyvinylpyrrolidone K30, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 mg spermidine ml^{-1}, 2% β-mercaptoethanol (Chang et al., 1993). The sample was incubated at 65 °C for 25 min, then 500 μl chloroform:isoamyl alcohol (24:1) was added. The sample was centrifuged for 10 min at 12,000*g*, rt. The chloroform:isoamyl alcohol extraction was repeated. RNA was precipitated overnight with the addition of sodium acetate and isopropyl alcohol at −20 °C. The sample was resuspended in RNAse-free water. The sample was then purified using an RNeasy Mini Kit (Qiagen) following manufacturer's instructions.

2.3. Affymetrix labeling

Purified RNA was processed using the Affymetrix One-Cycle Target Labeling procedure, following the Affymetrix manual (Affymetrix, 2004). cDNA was generated from genomic RNA using the One-Cycle cDNA Synthesis kit and the resulting double-stranded DNA was cleaned up using the GeneChip Sample Cleanup Module. Complementary RNA (cRNA) with a biotin label was synthesized with the GeneChip Expression 3′-Amplification Reagents for IVT Labeling, and the resulting cRNA was put through the GeneChip Sample Cleanup Module and quantified. Hybridization, washing and chip reading buffers and procedures followed Affymetrix guidelines (Affymetrix, 2004) and were performed at the Research Technology Support Facility at Michigan State University.

[1] *Abbreviations used:* CTAB, hexadecyltrimethylammonium bromide.

The hybridization signals were scanned with a GeneChip GCS 3000 scanner (Affymetrix) and the cell intensity (CEL) files were obtained from GCOS 2.1 software (Affymetrix). CEL files are available at PLEXdb <http://www.plexdb.org/>, Accession No. FG5. CEL files were normalized in the Bioconductor package of R version 2.3.0rc (Gentleman et al., 2004; R Development Core Team, 2006) using RMA, an expression measure that accounts for background correction, quantile normalization and variation between arrays (Irizarry et al., 2003a,b).

2.4. Genes specific to sexual development

The Affymetrix Fusariuma520094 GeneChip contains 18,069 probesets, of which 17,830 are specific to predicted proteins, ESTs, short ORFs and ribosomal RNA of *G. zeae*, while the remaining 239 represent Affymetrix internal controls, and control sequences from host plants such as barley, wheat and maize (Güldener et al., 2006). The mas5-calls function of the affy package in Bioconductor (Gentleman et al., 2004) was used to generate Affymetrix present/marginal/absent calls for all replicates of all probe sets. Each "present" call was assigned a value of 1.0, "marginal" was assigned a value of 0.5, and "absent" a value of 0.0. The calls were averaged over the 3–5 replicates for each treatment, to determine a treatment-wide value between 0.0 and 1.0. For averages <0.4, the probe set was considered absent for that treatment. Values between 0.4 and 0.6 were classified as marginal, and those greater than 0.6 were considered present. For a conservative estimate of the number of probe sets expressed, and to simplify analyses, marginal probe sets were grouped with absent probe sets. The program mas5calls was also used to generate present/marginal/absent calls on data from an experiment in complete media (CM) and carbon- and nitrogen-starvation minimal media (C- and N-, respectively) (Güldener et al., 2006; PlexDB Accession No. FG2); these data, along with our 0h data, represent vegetative growth conditions.

2.5. Statistical analyses of expression data

All gene expression data from the sexual development time-points (24, 48, 72, 96 and 144h) were compared with the data from the 0h vegetative time-point using the Limma package in Bioconductor (Smyth 2005). The list of differentially expressed genes was ranked based on the moderated *t*-statistic introduced by Smyth (2004). In this approach a gene-wise linear model is fitted based on the experimental design with six time-points and varying numbers of replications at each time-point. Then estimators for model parameters are calculated. These estimators exhibit robust behavior even for a small number of arrays and allow for incomplete data arising from spot filtering or spot quality weights. Finally, for each gene a moderated *t*-statistic is calculated using posterior standard deviations to extract those with maximally differential expression.

Significantly up-regulated ion channel data (Fig. 4) were clustered using the Hopach package in Bioconductor (van der Laan and Pollard 2003; Pollard and van der Laan 2005). The expression profiles in Figs. 4 and 5 were assembled in Microsoft Office Excel 2003. Detailed expression profiles for each gene can be found in Supplementary Fig. S1, available in the online version of this paper. A multi-genome comparison of fungal ion transport protein genes was conducted following the methods of Shiu et al. (2005; Fig. 3).

2.6. Comparison with prior studies

Data from two cDNA studies of sexual development in *G. zeae* (Qi et al., 2006; Lee et al., 2006), examining the development of strain PH-1 over a developmental time-course and examining differential gene expression during sexual development between wild type and a *mat1-2* mating type deletion mutant, were compared with the data from the current study. We ran limma to determine whether the genes identified as showing significant differential transcript accumulation at 96 or 144 h compared with 0 h (Qi et al., 2006) also showed differential accumulation in the Affymetrix dataset, and whether this was statistically significant. We compared the Lee et al. (2006) data to the dataset we generated using mas5calls, to determine whether we identified the same genes as sexual development-specific.

2.7. Identification and annotation of ion channel genes

Identification of putative genes encoding transporters was compiled during the early stages of *G. zeae* sequence assembly and annotation, to identify and assign function to transporters in the genome using the TC system (Busch and Saier, 2002). These identifications were based on automated hydropathy analyses on the predicted proteins using the programs HMMTOP (Tusnady and Simon, 1998) and MEMSAT (Jones et al., 1994). Hidden Markov models were built from members of each transporter family within the TC classification system (Saier, 2000, http://www.biology.ucsd.edu/~msaier/transport/) using the programs CLUSTALW (Thompson et al., 1994) and HMMER (Eddy, 1998). Then the models were used to search all proteins with at least one predicted transmembrane sequence using the program HMMER with a cut off e-value of 10^{-5}. This analysis was used to aid the annotation effort for the genome. Some genes that had not been predicted by either analysis were discovered by their similarity to predicted or known ion transport genes in *G. zeae* or in other fungi. The subset of genes encoding putative ion transport proteins, including these new predictions, was examined in this paper. A complete listing of the genes used, together with the corresponding Affymetrix probe sets, may be found in Supplementary Table S1, in the online version of this paper.

3. Results

3.1. Transcripts detected during sexual development

The majority of *G. zeae* probe sets, 13,916 of 17,830 (78%), were expressed during at least one of the stages of sexual development and differentiation in culture. While 3739 were not expressed in any of the *in vitro* time-points we examined, 8279 probe sets were constitutively expressed. A total of 3845 probe sets were expressed during one or more stages of sexual development, but not during vegetative growth on carrot agar. When these data are compared with data taken from vegetative cultures grown in complete medium (CM), or in nitrogen-starved (N-) or carbon-starved (C-) conditions (Güldener et al., 2006; PlexDB Accession No. FG1, PlexDB Accession No. FG2), the number of probe sets detected during sexual development, but not during vegetative growth, drops to 2068. The expression profiles of 1862 of these probe sets are summarized in Fig. 2 (the profiles for all 2068 probe sets are given in Supplementary Table S2, along with the corresponding probe names).

3.2. Comparison with prior studies

Two other studies relate to the current expression data. Qi et al. (2006), using a *G. zeae* cDNA microarray, found differential accumulation of transcripts representing 572 genes at 96, 120 or 144 h post-induction compared with vegetative growth. As our current study does not have data for 120 h post-induction, comparisons were only made between datasets at 96 and 144 h. At 96 h, 246 genes

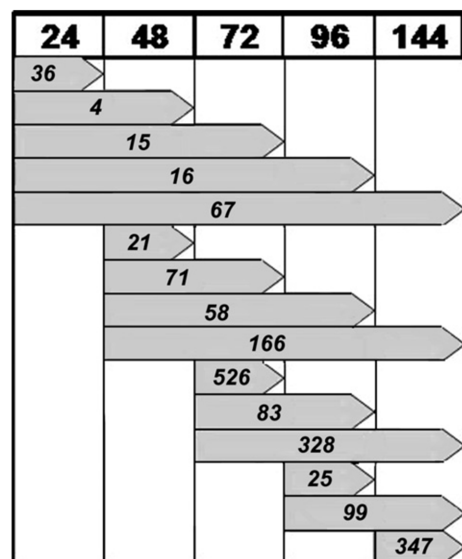

Fig. 2. Probe sets unique to sexual development. Times at which the probe sets were detected are in hours, and are given across the top of the figure. Numerals within the arrows give the number of probe sets detected at the given period of time. Probe sets showing disjunct expression (i.e., present at 24 h and 144 h, but not at 48, 72 or 96 h) are listed in Supplementary Table 2.

showed the same trend, 92 showed no significant change in either data set, 160 genes showed significant change in one data set, but not the other, and 29 genes showed opposite trends. At 144 h, 169 genes showed the same trends in both datasets, 158 showed no change, 204 genes showed significant change in just one set, and 11 showed opposite trends. Lee et al. (2006) reported 105 genes down-regulated at least two-fold in a *G. zeae* mating type *mat1-2* deletion mutant during sexual development (compared with wild-type), with a coefficient of variation less than 0.5. They considered these genes to be specific to *MAT*-mediated sexual development. Our analyses identified 15 of these genes as potentially exclusive to sexual development (fg02805, fg03109, fg03696, fg04331, fg05818, fg07575, fg07912, fg08413, fg08414, fg08512, fg09006, fg09151, fg09184, fg10478 and fg10818); transcripts for five genes were not detected in any of the developmental stages we examined (fg01090, fg02266, fg07522, fg09120 and fg10366), and the remaining 85 were constitutively expressed or nearly so in our analyses.

We compared data from an infection time course in barley (Güldener et al., 2006; PlexDB Accession No. FG1) with the probe sets unique to sexual development in *in vitro* studies to date. Of the 2068 probe sets that appear to pertain to sexual development, 135 were found to be expressed during at least one stage of an infection time course in barley (data not shown). It has recently been shown that dikaryotic hyphae are found in the vessels and pith of infected wheat and appear to be involved in spread of the infection (Guenther and Trail, 2005). If a similar invasive pattern occurs in barley, the expression in barley of the 135 genes that appear, in culture, to be specific to sexual development may reflect their role in formation of these specialized hyphae during infection and colonization.

3.3. Transcript profiles of genes encoding ion transporters

Our analysis showed that 919 predicted proteins fall into a known transporter class. Of these, 162 were predicted ion transport proteins, represented by 178 probe sets on the GeneChip (Supplementary Table S1). Table 1 gives a complete breakdown of these genes into functional categories, while Fig. 3 presents a comparison with other fungi for which genome sequences are available.

Forty-four genes for ion transport proteins showed an increase in transcript abundance of at least two-fold during sexual development, significant at $p < 0.05$ (Fig. 4). Six of these genes showed both a significant two-fold or greater increase and decrease in transcript abundance during successive stages of sexual development compared with 0h. Additionally, transcript abundance of nine genes increased and ten genes decreased less than two-fold with a *p*-value <0.05. Sixty-two genes for ion transport proteins showed at least a two-fold decrease in transcript abundance during sexual development compared with vegetative growth, significant at $p < 0.05$.

Genes for twelve of the predicted ion transport proteins show no measurable expression during any of the time-points examined; eight of these twelve genes are expressed at some point during *in planta* growth (Hallen and Trail, unpublished results). A total of 32 transporter genes are expressed, but show no significant change in transcript level during sexual development as compared with vegetative growth.

4. Discussion

Fruiting body development in the Ascomycota has long formed the basis of systematics, and the perithecium-forming fungi have been particularly well-studied. A major revision of the systematics of pyrenomycetous fungi by Luttrell (1951) emphasized the relationship between the ascus and the ascocarp components and came to be known as the Luttrellian concept (Reynolds, 1981). Although the Lutrellian concept in systematics has been essentially replaced by molecular phylogenetics, the genetic basis for fruiting body development has remained a largely unexplored area of fungal biology. In this study, we have identified a set of genes which are expressed uniquely during one or more stages of sexual development: 2068 probesets were designated "present" by Mas5, and at the same time were "absent" in four discrete vegetative growth conditions. While this distribution does not prove that the genes corresponding to these probesets are unique to sexual development (some genes may be expressed in vegetative growth conditions not yet studied), it does provide the first group of genes associated with perithecium development on which to focus further studies.

The 72 h time-point was associated with the highest number of unique genes of any of the time-points, with 526 probe sets detected at 72 h alone. At 72 h, the perithecia have attained their full size and the paraphyses (sterile hyphae separating the asci) have filled the central cavity (centrum). Croziers will be forming within the next 24 h from the ascogenous hyphae at the base of the centrum and karyogamy and meiosis will be taking place. We used the FunCatDB feature at MIPS <http://mips.gsf.de/genre/proj/fusarium/Search/Catalogs/catalog.jsp>, to provide a functional breakdown of the 72 h specific genes. The majority of these probesets, 385 out of 526, are placed in Functional Category 99: unclassified proteins. Of genes to which predicted functions could be assigned, the majority were in FunCats 1 (metabolism), 11 (transcription), 16 (protein with binding function or cofactor requirement), 20 (cellular transport, transport facilitation and transport routes), and 32 (cell rescue, defense and virulence) with 32, 11, 11, 12 and 12 genes, respectively. FunCat 01.05 (C-compound and carbohydrate metabolism), with 16 genes, and 11.02.03 (mRNA synthesis), with 10 genes, were particularly highly represented. These categories reflect the period of cell development and nuclear division that is imminent.

Table 1
Functional categories of predicted transporter genes

Category	Pfam ID (Fig. 3)	Probes (probesets)
Ammonia permeases (COG0004)	Ammonium_transp	4
NhaP-type Na^+/H^+ and K^+/H^+ antiporters (COG0025)	Na_H_Exchanger	5
ClC-type Chloride channels (COG0038)	Voltage_CLC	4
Predicted Co/Zn/Cd cation transporters (COG0053)	Cation_efflux	4
K^+ transport (COG0168, 0475, 0667, 1226, 3158)	K transp; TrkH	12(14)
Related to PHO89-Na^+/phosphate cotransporter (COG0306)	PHO4	5
Related to iron transport protein (COG0316)		1
Predicted Na^+-dependent transporter/Arsenite efflux pump ACR3 and related permeases (COG0385, 0798)	SBF	1
Ca^{2+}/H^+ and Ca^{2+}/Na^+ antiporters (COG0387, 0530)	Na_Ca_ex	9
Related to MIDI stretch activated Ca^{2+} channel		1
Related to calcium channels (PF00520)	Ion_trans	2
Predicted divalent heavy-metal cations transporter (COG0428)		2
Related to PHO87 protein (COG0471)		1
Cation transport ATPases (COG0474, 2217)	Cation_ATPase_C, Cation ATPase N, E1-E2_ATPase	27(29)
Permeases of the major facilitator superfamily: phosphate permeases, MirA and SIT1 siderophore iron transporters (COG0477)		17
Mg^{2+} and Co^{2+} transporters (COG0598)	CorA	4
Sulfate permeases (COG0659)	Sulfate_transp	6
High-affinity iron permease (COG0672)	FTR1	2
Predicted permeases (COG0697)		1
Related to arsenite transporter ARR3 (COG0798)		1(2)
Na^+/H^+ antiporter NhaD and related arsenite permeases (COG1055)		1
Related to Na^+/K^+/2Cl-cotransporter (COG1113)		1
Related to heavy metal tolerance protein HMT1; related to Ycf1p, Yor1p anion transporters (COG1132)		5
Co/Zn/Cd efflux system component (COG1230)	Cation_efflux	3(5)
Tellurite resistance protein and related permeases; C4-dicarboxylate transporter/malic acid transport protein (COG1275, PF03595)	C4dic_mal_tran	9
Related to proposed vacuolar iron transport protein (COG1814)		1
Mn^{2+} and Fe^{2+} transporters of the NRAMP family (COG1914)	Nramp	1(2)
Protein implicated in iron transport, frataxin homolog (COG1965)	Frataxin_Cyay	1
Formate/nitrite family of transporters (COG2116)	Form_nir_trans	2
High-affinity nickel permease (COG3376)	NicO	1(2)
Mitochondrial phosphate transport proteins (PF00153)		4
Sodium:solute symporter family (PF00474)	SSF	1
HCO_3-transporter family (PF00955)	HCO3_cotransp	3
ZIP Zinc transporter (PF02535)	Zip	8
EXS family (PF03124)	EXS	2
V-ATPase subunit C (PF03223)	V-ATPase_C	1
Cadmium resistance transporter (PF03596)	Cad	1
Tricarboxylate carrier (PF03820)	Mtc	1
Ctr copper transporter family (PF04145)	Ctr	5(7)
ChaC-like protein (PF04752)	ChaC	1
Copper resistance protein D; Voltage gated chloride channel (PF05425, PF00654)		1(2)
Cytochrome C oxidase copper chaperone (PF05051)	Cox 17	1(2)

Group	Genome	Ammonium_transp	C4dic_mal_tran	COX17	Cad	Cation_ATPase_C	Cation_ATPase_N	Cation_efflux	ChaC	CorA	Ctr	E1-E2_ATPase	EXS	FTR1	Form_nir_trans	Frataxin_Cyay	HCO3_cotransp	Ion_trans	K_trans	Mid1	MirA/Sit1	Mtc	Na_Ca_ex	Na_H_Exchanger	NicO	Nramp	PHO4	SBF	SSF	Sulfate_transp	TrkH	V-ATPase_C	Voltage_CLC	Zip
AscoYeast	SCE	3	1	1	0	5	7	5	1	5	3	11	2	2	1	1	1	2	0	1	6	1	4	3	0	3	1	1	1	4	2	1	1	5
	SPO	3	6	1	0	3	5	3	1	3	3	9	3	1	0	1	1	1	0	1	3	1	3	4	1	1	0	0	3	4	2	1	2	3
	CGU	4	1	1	0	3	5	4	1	4	3	8	3	4	1	1	2	2	1	1	7	1	4	4	0	3	1	1	0	3	1	1	3	4
	CLU	6	1	1	0	3	4	5	1	5	3	7	2	4	1	1	1	2	1	1	1	1	4	2	0	3	1	1	2	4	1	1	2	4
	EGO	2	0	1	0	3	4	3	1	4	2	8	2	3	1	1	1	1	0	1	0	1	3	2	0	3	0	0	2	3	1	1	2	4
	PST	3	4	1	0	5	6	4	1	4	3	11	2	4	1	1	1	2	1	1	2	1	4	3	0	3	1	1	6	4	1	1	3	4
FilAsco	BCI	3	4	1	0	8	8	7	1	2	3	14	2	1	1	1	2	1	1	1	7	1	6	7	1	1	1	1	2	4	2	1	3	8
	CIM	2	3	1	0	7	7	7	1	3	2	13	2	0	0	1	2	2	1	1	6	1	5	5	1	1	1	1	1	4	2	1	3	8
	NHA	4	5	1	0	18	16	8	1	4	5	28	1	2	2	1	2	4	1	1	12	1	12	10	1	1	3	1	6	6	6	1	3	9
	CGL	2	2	0	0	8	10	6	1	3	2	13	1	1	1	1	2	2	0	1	6	1	9	5	0	1	2	1	1	5	4	1	3	9
	FGR	4	8	0	1	13	11	8	1	4	5	20	2	2	2	1	3	2	1	1	10	1	9	7	1	1	3	1	1	6	4	1	4	9
	TRE	2	5	0	0	10	12	6	1	3	4	16	2	2	1	1	1	4	0	1	7	1	9	6	1	1	1	1	4	5	1	1	3	6
	SNO	5	7	1	0	9	10	8	1	4	5	15	2	1	0	1	2	2	1	1	7	1	9	9	1	0	3	1	2	6	4	1	4	8
	NCR	4	3	0	0	7	6	8	1	4	2	13	2	1	1	1	2	3	1	1	2	1	8	5	1	2	1	2	1	4	2	1	3	8
	ANI	4	4	1	0	10	9	6	1	7	3	17	2	0	1	1	2	2	0	1	10	1	6	8	1	1	4	1	4	3	3	1	3	8
	MGR	3	3	1	1	10	12	9	1	4	6	18	2	1	0	1	0	3	1	1	5	1	7	7	2	0	3	1	3	5	4	1	3	8
	SSC	3	2	1	0	6	8	7	1	2	2	12	2	1	1	1	1	3	1	1	4	1	8	7	1	2	1	1	0	4	2	1	3	7
	ANR	4	5	1	0	9	14	7	1	5	4	18	2	3	1	1	3	2	1	1	5	1	9	7	1	1	1	1	4	3	4	1	3	8
Heterobasidio	UMA	2	3	0	0	7	7	5	1	3	3	13	2	1	1	1	1	3	0	1	3	1	5	7	1	1	1	1	4	2	1	1	2	2
Homobasidio	PCH	2	3	0	0	4	5	4	1	4	2	11	0	1	1	1	1	1	2	1	1	0	5	6	0	2	0	1	3	4	2	0	2	7
	CCI	4	1	0	0	5	7	4	1	5	2	11	2	0	0	1	1	2	0	1	1	0	6	7	1	1	3	1	1	3	2	1	3	4
	CNE	2	2	1	0	3	6	6	1	3	3	9	2	3	0	1	1	2	0	1	5	1	5	4	1	1	1	1	1	3	2	0	3	4
Micro	ECU	0	0	0	0	0	0	1	0	0	0	2	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
Zygo	ROR	5	1	0	0	11	22	9	1	5	3	30	2	1	0	0	0	3	0	1	4	1	12	6	1	3	0	3	1	9	3	2	2	8
Metazoan	MMU	4	0	1	0	15	15	10	2	1	2	23	1	0	0	2	10	107	0	0	0	5	9	14	0	2	2	8	14	11	0	2	9	15
Plant	ATH	6	4	2	0	14	25	12	3	11	6	35	13	0	0	1	7	32	13	0	0	0	13	40	2	7	1	6	1	12	1	1	7	18

Fig. 3. Distribution of PFAM categories pertaining to ion transport in fungal genomes, with mouse and *Arabidopsis* as outgroups. Ascomycetous yeasts: SCE, *Saccharomyces cerevisiae*; SPO, *Schizosaccharomyces pombe*; CGU, *Candida guilliermondii*; CLU, *Candida lusitaniae;* EGO, *Eremothecium gossypii*, PST, *Pichia stipitis*. Filamentous ascomycetes: BCI, *Botrytis cinerea*; CIM, *Coccidioides immitis*; NHA, *Nectria haematococca*; CGL, *Chaetomium globosum*; FGR, *Fusarium graminearum*; TRE, *Trichoderma reesei*; SNO, *Stagonospora nodorum*; NCR, *Neurospora crassa*; ANI, *Aspergillus nidulans*; MGR, *Magnaporthe grisea*; SSC, *Sclerotinia sclerotiorum*; ANR, *Aspergillus niger*. Heterobasidiomycetes: UMA, *Ustilago maydis*. Homobasidiomycetes: PCH, *Phanerochaete chrysosporium*; CCI, *Coprinopsis cinereus*; CNE, *Cryptococcus neoformans* serotype A. Microsporidian: ECU, *Encephalitozoon cuniculi*. Zygomycete: ROR, *Rhizopus oryzae*. Animal: MMU, *Mus musculus*. Plant: ATH, *Arabidopsis thaliana*.

We previously developed a cDNA microarray prior to the availability of the *G. zeae* Affymetrix GeneChip, and conducted studies on differential transcript accumulation during sexual development and differentiation (Qi et al., 2006). It is reassuring that similar trends were observed for the majority of genes in both the cDNA study and the current GeneChip study, despite the differences in platform. Genes showing significant expression shifts in either the cDNA experiment or the GeneChip experiment—but not both—usually showed similar trends in both experiments. Of some concern are the genes showing significant increases in transcript abundance in one experiment, and decreases in the other, for the same time point. However, few genes fell in to these categories (29 at 96 h, 11 at 144), and in no case did the fold change exceed approximately two-fold.

The fact that the majority of MAT1-2p-controlled genes discussed by Lee et al. (2006) were expressed during vegetative growth in our study is of interest. We find *MAT1-2* (fg08893) expressed at all developmental time-points and in the CM, C- and N- vegetative growth conditions; it is possible that the MAT1-2p function is not exclusive to sexual development. Alternately, although in our view less likely, it is possible that differences in the strain of *G. zeae* examined in the two studies (Z03643 in Lee et al.; PH-1 in our study) may account for some differences in expression.

There are no striking novelties in the complement of ion transport genes in *G. zeae* compared with other organisms (see Fig. 3). The filamentous ascomycetes possess more genes encoding cation ATPases (Cation_ATPase_C, Cation_ATPase_N and Cation_efflux), sodium–calcium exchangers, and Zip-type zinc transporters than do any of the other fungi, except the zygomycetes. Functional studies will be essential to determining the importance of these in ascomycete-specific functions, such as development and performance of sexual and asexual reproductive structures. The similarities amongst members of this group of fungi indicate these finding may be applicable to other fil-

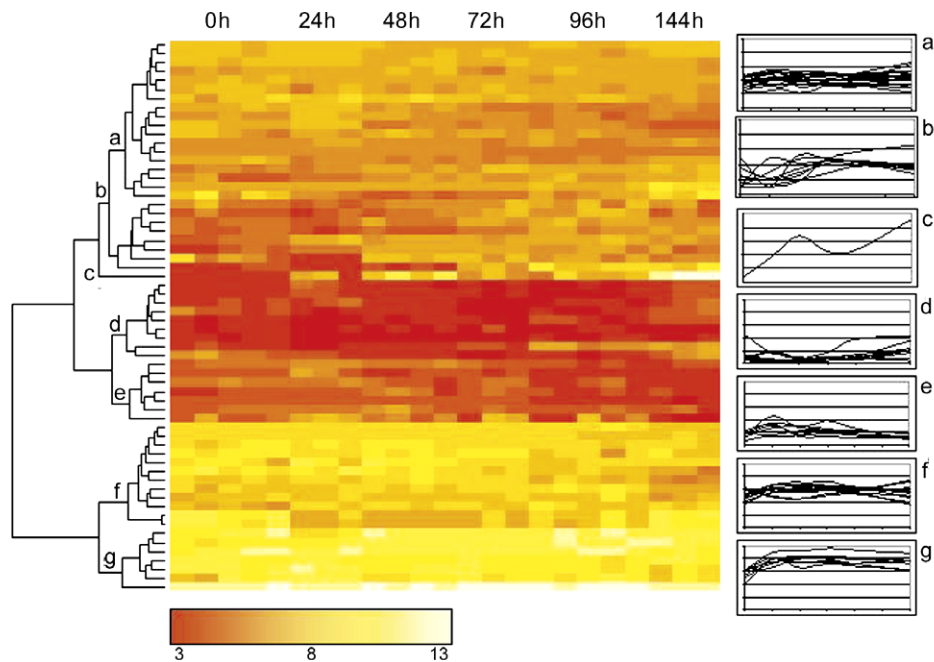

Fig. 4. Cluster analysis of ion transporters demonstrating an up-regulation of at least two-fold compared with 0 h vegetative growth during at least one sexual development time-point, significant at $p < 0.05$. Gene names can be seen in Supplementary Fig. S2. The expression profiles for the members of each cluster are plotted to the right; individual gene expression patterns for all ion transport genes can be viewed in Supplementary Fig. S1. Transcript abundance is indicated by log_2-normalized intensity values, ranging from 3 to 13.

amentous ascomycetes. With the possible exception of C4-dicarboxylate-malic acid transport proteins, *G. zeae* does not appear to have undergone radiation or contraction of ion transporting genes. C4 dicarboxylate-malic acid transport proteins function in the uptake of malate and other dicarboxylic acids (pfam.wustl.edu) and may be important in the *in planta* carbon uptake by *G. zeae*. The plant pathogens *G. zeae* and *Magnaporthe grisea* are the only fungi to possess Cad-type cadmium resistance transporters; however, since other plant pathogenic fungi appear to lack these transporters, it cannot be said at this time whether this is a reflection of ecology or of evolutionary history.

Of the 44 genes for ion transport proteins showing a two-fold or greater increase in transcript abundance, six genes also demonstrated a significant two-fold or greater decrease in transcript abundance during stages of sexual development. The fluctuation in expression of these genes likely reflects the need for rapid shifts in protein profiles during the profound developmental changes. The 12 ion transport genes showing no measurable expression in this study and the 32 genes lacking significant change in transcript level during sexual development, as compared with vegetative growth, will not be discussed further. The lack of measurable changes in transcript abundance does not preclude a role for the transporters encoded by these genes during sexual development. However, the GeneChip data alone are insufficient to determine whether or not these genes influence sexual development.

All four predicted ammonium transporter genes were constitutively expressed but significantly up-regulated during sexual development (Fig. 4 cluster G; Supplementary Figure S2). Nitrogen needs would be great for synthesis of a complex structure such as a perithecium. Three of the four transporter genes are annotated as encoding MEPA-type ammonium transporters, while the fourth, fg00529, encodes an MEAA-type transporter. In *Aspergillus nidulans*, MEPA is a high-affinity ammonium permease, and during asexual development the gene is expressed only during nitrogen starvation (Monahan et al., 2002), while MEAA is the ammonium permease responsible for growth in the presence of higher concentrations of ammonium. The three predicted MEPA-type ammonium transporter genes in *G. zeae* do indeed show increased transcript abundance in nitrogen-starvation conditions, compared with growth in complete media or under carbon-starvation conditions (Güldener et al., 2006, PlexDB Accession No. FG2). In addition, nitrogen starvation has been shown to serve as an inducer of sexual development in many ascomycetous fungi (Nelson and Metzenberg 1992; Okazaki et al., 1998). During perithecium development, increased MEPA mRNA may reflect a need to recover nitrogen from senescing cell types within the perithecium to use for ascus formation. It is not known how much nutrient influx from the surrounding environment into the developing perithecium occurs during perithecium development. However, in *G. zeae* perithecia form from initials (the dormancy stage) that contain large stores of nutrients (Guenther and Trail, 2005), so it is likely that nutrients are transported from within fungal tissue to be used for ascus and spore development.

The largest family of ion transporters in *G. zeae* is the cation transporting ATPases. While 27 cation transport

ATPases are predicted in *G. zeae*, only five show an increase in transcript abundance during sexual development; two of these five genes, fg01196 and fg02677, show a >8-fold increase during sexual development and are worth detailed examination. Fg01196 encodes a probable calcium P-type ATPase homolog and is likely to be involved in calcium signaling, either directly or indirectly. In addition to fg01196, a second calcium P-type ATPase gene, fg03202, shows a slight (less than two-fold) increase in transcript abundance at the 72 h stage compared with 0 h. Fg02677 encodes a probable ENA-1 sodium ion transporting ATPase—one of four predicted in the genome. One of the other three predicted sodium transport ATPase genes (fg04919) demonstrates a decrease in transcript abundance during sexual development; the others show no change. In *S. cerevisiae*, the *ena-1* mutant shows increased sodium sensitivity (Haro et al., 1991). These proteins are likely involved in maintenance of sodium homeostasis.

The Clc family of chloride channels, of which *G. zeae* possesses four members, exhibits considerable variation in regulation. Chloride channel gene fg05451 is up-regulated by 2.07–2.24-fold during the 24, 48 and 96h stages compared with vegetative growth, but shows no significant deviation from 0h at 72 or 144h (Supplementary Fig. S2). Gene fg05303 is weakly up-regulated at 24, 48 and 72h (1.57–1.84-fold increase compared with 0h, significant at $p < 0.05$), fg05555 is weakly down-regulated at 24h compared with 0h, and expression of fg04598 is not detected at any time-point examined. From this we can hypothesize that the chloride channel genes encoded by fg05451 and fg05303 play a role in chloride transport during the earlier stages of sexual development but, as all chloride channel genes with the exception of fg04598 are constitutively expressed, there may be considerable functional redundancy.

An interesting suite of expression profiles can be observed in the MirA-type siderophore iron transporters (Fig. 5). Only one MirA-type transporter gene, fg03737, shows any increase in transcript accumulation during sexual development. The expression of this gene, which is increased in transcript accumulation by two-fold at 72h, eight-fold at 96h and greater than 11-fold at 144h, is the opposite of that of fg00539, which shows a 3-, 5- and 7-fold reduction in transcript accumulation at 72, 96 and 144h, respectively. Genes fg08093 and fg09701 (not pictured) are not detected during any time-point examined. Gene fg11275 displays moderate expression at 0h, but is not detected during 24h or any of the later time-points. Genes fg00539, fg03744 and fg07665 are expressed during sexual development, but at equal or significantly lower levels than during vegetative growth. Finally, fg11029 is highly expressed throughout sexual development, but does not differ significantly from 0h. It is reasonable to suppose that fg03737, which is not expressed until after 48h, is specific to sexual development and may encode the primary siderophore iron transporter in the perithecium. Its high expression during the last stages of perithecium formation may indicate a role for this gene in spore development.

Due to the great variety of functions and substrates in ion transporters, this analysis gives a complex picture of ion transport gene regulation during sexual development in *G. zeae*. Adding to that complexity will be (1) the fact that several ion transporters are not transcriptionally regulated, but are regulated by pH or substrate availability once the proteins are already in place in the membranes (Peñalva and Arst, 2002; Yenush et al., 2005; Suzuki et al., 2006); (2) the potential time lag between transcription—the quality we are measuring on the Affymetrix GeneChip—and translation and the resulting availability of a functional protein; and (3) the potential for functional redundancy in ion transporters, many of which possess multiple predicted genes in a given fungus. In our continuing efforts to identify and characterize genes which play a role in sexual development, some of these questions can be addressed by targeted gene deletions.

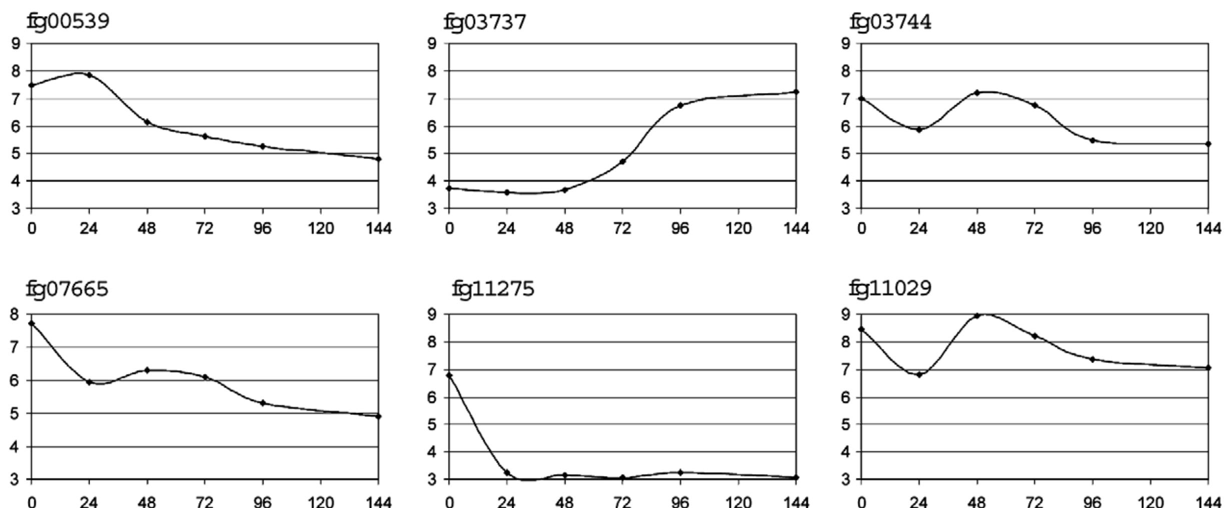

Fig. 5. Expression profiles of MirA-type iron siderophore transporters. The *x*-axis on the individual expression charts gives time in hours post-induction and the *y*-axis shows expression as $\log_2$-normalized intensity.

Of particular interest to us in our studies of the ascus discharge mechanism, are the potassium, chloride and calcium ion channels. Of the four potassium transport proteins identified in the genome, all are expressed during ascus formation—72–96 h—although fg03834 (*Trk-1*) transcript accumulation increases the most during this time period. Interestingly, fg01196, encoding a probable calcium-transporting P-type ATPase, has increased transcript accumulation during the final stages of perithecium maturation. Fg05451, encoding a chloride channel, also has higher transcript accumulation at 96 h. Finally, a gene for a stretch activated calcium ion channel, fg07418 (*Mid1*), known to function during sexual development in yeasts (Iida et al., 1994) and its associated voltage-gated calcium channel (encoded by fg01364; *Cch1*; Fischer et al., 1997; Locke et al., 2000) are constitutively expressed, but are likely candidates to have a role in a cell that functions by stretching. Functional analyses of these transport proteins and their role in both sexual development and forcible discharge of ascospores are in progress.

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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.2007.04.007.

References

Affymetrix, 2004. GeneChip Expression Analysis.

André, B., 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. Yeast 11, 1575–1611.

Bowden, R.L., Leslie, J.F., 1999. Sexual recombination in *Gibberella zeae*. Phytopathology 89, 182–188.

Busch, W., Saier, M.H., 2002. The transporter classification (TC) system. Crit. Rev. Biochem. Mol. 37, 287–337.

Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11, 113–116.

De Hertogh, B., Carvajal, E., Talla, E., Dujon, B., Baret, P., Goffeau, A., 2002. Phylogenetic classification of transporters and other membrane proteins from *Saccharomyces cerevisiae*. Funct. Integr. Genomics 2, 154–170.

Eddy, S.R., 1998. Profile hidden Markov models. Bioinformatics 14, 755–763.

Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G., Sanders, D., 1997. The *Saccharomyces cerevisiae* CCH1 gene is involved in calcium influx and mating. FEBS Lett. 419, 259–262.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J., 2004. Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Guenther, J.C., Trail, F., 2005. The development and differentiation of *Gibberella zeae* (anamorph: *Fusarium graminearum*) during colonization of wheat. Mycologia 97, 229–237.

Güldener, U., Seong, K.-Y., Boddu, J., Cho, S., Trail, F., Xu, J.-R., Adam, G., Mewes, H.-W., Muehlbauer, G.J., Kistler, H.C., 2006. Development of a *Fusarium graminearum* Affymetrix GeneChip for profiling fungal gene expression in vitro and in planta. Fungal. Genet. Biol. 43, 316–325.

Haro, R., Garciadeblas, B., Rodriguez-Navarro, A., 1991. A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. 291, 189–191.

Iida, H., Nakamura, H., Ono, T., Okumura, M.S., Anraku, Y., 1994. *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2+} influx and mating. Mol. Cell Biol. 14, 8259–8271.

Irizarry, R.A., Bolstad, B.M., Collin, S.F., Cope, L.M., Hobbs, B., Speed, T.P., 2003a. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31. Art. No. e15.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, F., Antonellis, K.J., Scherf, U., Speed, T.P., 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1994. A model recognition approach to the prediction of all-helical membrane protein structure and topology. Biochemistry 33, 3038–3049.

Klittich, C.J.R., Leslie, J.F., 1988. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi)*. Genetics 118, 417–423.

Lee, S.-H., Lee, S., Choi, D., Lee, Y.-W., Yun, S.-H., 2006. Identification of the down-regulated genes in a *mat1-2*- deleted strain of *Gibberella zeae*, using cDNA subtraction and microarray analysis. Fungal. Genet. Biol. 43, 295–310.

Locke, E.G., Bonilla, M., Liang, L., Takita, Y., Cunningham, K.W., 2000. A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. Mol. Cell Biol. 20, 6686–6694.

Luttrell, E.S., 1951. Taxonomy of the pyrenomycetes. Univ. Missouri Studies 24, 1–120.

Monahan, B.J., Fraser, J.A., Hynes, M.J., Davis, M.A., 2002. Isolation and characterization of two ammonium permease genes, *meaA and mepA*, from *Aspergillus nidulans*. Eukaryot Cell 1, 85–94.

Nelson, M.A., Metzenberg, R.N., 1992. Sexual development genes of *Neurospora crassa*. Genetics 132, 149–162.

Nowrousian, N., Ringelberg, C., Dunlap, J.C., Loros, J.J., Kück, U., 2005. Cross-species microarray hybridization to identify developmentally regulated genes in the filamentous fungus *Sordaria macrospora*. Mol. Gen. Genomics 273, 137–149.

Nowrousian, N., Kück, U., 2006. Comparative gene expression analysis of fruiting body development in two filamentous fungi. FEMS Microbiol. Lett. 257, 328–335.

Okazaki, N., Okazaki, K., Watanabe, Y., Kato-Hayashi, M., Yamamoto, M., Okayama, H., 1998. Novel factor highly conserved among eukaryotes controls sexual development in fission yeast. Mol. Cell Biol. 18, 887–895.

Paulsen, I.T., Sliwinshi, M.K., Nilissen, B., Goffeau, A., Saier, M.H., 1998. Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae*. FEBS Lett. 430, 116–125.

Peñalva, M.A., Arst Jr., H.N., 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol. Mol. Biol. Rev. 66, 426–446.

Pollard, K.S., van der Laan, M.J., 2005. Cluster analysis of genomic data. In: Gentleman, R., Carey, V., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and computational biology solutions using R and bioconductor. Springer, New York, pp. 209–228.

Qi, W., Kwon, C., Trail, F., 2006. Microarray analysis of transcript accumulation during perithecium development in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). Mol. Gen. Genomics 276, 87–100.

R Development Core Team, 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ren, Q., Paulsen, I.T., 2005. Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. PLoS Comput. Biol. 1, e27.

Reynolds, D.R., 1981. Ascomycete Systematics: The Luttrellian Concept. Springer-Verlag, New York, 242 pp..

Saier, M.H., 2000. A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol. Mol. Biol. Rev. 64, 354–411.

Shiu, S.-H., Shih, M.-C., Li, W.-H., 2005. Transcription factor families have much higher expansion rates in plants than in animals. Plant Physiol. 139, 18–26.

Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, article 3.

Smyth, G.K., 2005. Limma: linear models for microarray data. In: Gentleman, R., Carey, V., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and computational biology solutions using R and bioconductor. Springer, New York, pp. 397–420.

Suzuki, M., Morita, T., Iwamoto, T., 2006. Diversity of chloride channels. Cell Mol. Life Sci. 63, 12–24.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Trail, F., Common, R., 2000. Perithecial development by *Gibberella zeae*: a light microscopy study. Mycologia 92, 130–138.

Trail, F., Gaffoor, I., Vogel, S., 2005. Ejection mechanics and trajectory of the ascospores of *Gibberella zeae* (anamorph *Fusarium graminearum*). Fungal. Genet. Biol. 42, 528–533.

Trail, F., Xu, H., Loranger, R., Gadoury, D., 2002. Physiological and environmental aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). Mycologia 94, 181–189.

Tusnady, G.E., Simon, I., 1998. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J. Mol. Biol. 283, 489–506.

Van der Laan, M.J., Pollard, K.S., 2003. Hybrid clustering of gene expression data with visualization and the bootstrap. Biostatistics 2, 445–461.

Windels, C.E., 2000. Economic and social impacts of Fusarium head blight: Changing farms and rural communities in the Northern Great Plains. Phytopathology 90, 17–21.

Yenush, L., Merchan, S., Holmes, J., Serrano, R., 2005. pH-responsive, posttranslational regulation of the Trk1 potassium transporter by the type 1-related Ppz1 phosphatase. Mol. Cell Biol. 25, 8683–8692.