Analysis of Expressed Sequence Tag Data and Gene Expression Profiles Involved in Conidial Germination of *Fusarium oxysporum*

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We obtained 3,372 tentative unique transcripts (TUTs) from a cDNA library of *Fusarium oxysporum*. A cDNA array with 3,158 TUTs was produced to analyze gene expression profiles in conidial germination. It seems that ras and other signaling genes, e.g., ceg, cooperatively initiate conidial germination in *Fusarium* by increasing protein synthesis.

*Fusarium oxysporum*, one of the most important phytopathogens, is widely distributed in every type of soil worldwide. Some strains tend to penetrate the host roots directly without producing fully differentiated infection structures, such as the appressoria of *Magnaporthe grisea* (11). Unlike *Fusarium graminearum* (Gibberella zeae), it has no known sexual stage (7, 8). The molecular mechanisms of pathogenicity and symptom induction caused by *F. oxysporum* are poorly understood thus far (15). Some fungal species, such as *F. oxysporum*, reproduce asexually through the production of spores called conidia, which is the most common means of dispersion for the filamentous fungi. Environmental factors are required to trigger their germination (14).

For the study presented herein, we performed the first large-scale expressed sequence tag (EST) sequencing of *F. oxysporum* and used cDNA arrays to analyze gene expression profiles during the conidial germination process in order to elucidate this asexual filamentous fungus at the molecular level during fungal development.

Fungal culture conditions. Strain ATCC 16416 is *F. oxysporum* f. sp. *cucumerinum*, and strain AFu68 is *F. oxysporum* f. sp. *radicis-cucumerinum* (18). To obtain conidia, the fungal strains were grown on potato dextrose agar plates at 27°C for 8 days, harvested by washing the surfaces of the plates with distilled water and filtering the water through four layers of filter paper, and then collected in the filtrate. For both strains, >90% of the collected conidia were microconidia and the others were macroconidia, as determined by visible microscopy (Nikon, Japan). For the induction of germ tubes, the harvested conidia were resuspended in germination water (0.5 g MgSO4·7H2O, 1 g NH4NO3, 1 g KH2PO4, and 15 g glucose in 1 liter of water) at a concentration of about 10⁶ conidia per milliliter and shaken slowly at 27°C for 6 h. More than 80% of the conidia from both strains germinated synchronously into germ tubes. To obtain mycelia that were growing vigorously before conidiation, the germ tubes were kept in the germination water with gentle shaking at 27°C for 30 h. The total RNAs were extracted from all samples of both strains.

**cDNA library construction and EST sequencing.** We constructed a cDNA library using mixed RNA samples from the three contiguous conidial development stages of strain ATCC 16416 and sequenced reconstructed plasmids from the 5’ ends of insert genes. After trimming off the vector and poor-quality DNA segments, we retained 6,448 ESTs with sequence reading lengths of >100 nucleotides (nt). Among these ESTs, the average reading length was 362 nt, while >43% were longer than 400 nt. After assembling the sequences, 2,551 singletons (only one EST in a cluster) and 821 contigs (two or more ESTs in a cluster) were generated. A total of 3,372 unique transcripts (TUTs) were found. About half of these TUTs (52.4%) exhibited homology to known genes, proteins, and ESTs (Table 1). The ratios of these TUTs are shown in Table 1. The remaining 47.6% of the TUTs did not have significant homology to any known sequences.

| Parameter | Value | % of total TUTs |
|-----------|-------|------------------|
| Total no. of EST clones | 6,448 | 100 |
| Avg EST length (nt) | 362 | 50.8 |
| G + C content (%) | 50.8 | 50.8 |
| Total no. of TUTs | 3,372 | 100 |
| Singletons (one EST) | 2,755 | 81.7 |
| Contigs (two or more ESTs) | 617 | 18.3 |
| TUTs exhibiting homology to encoded protein sequences (UniProt proteins of known function and hypothetical proteins) | 1,769 | 52.46 |
| TUTs exhibiting homology to encoded protein sequences (UniProt proteins of known function) | 924 | 27.40 |

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**TABLE 1. Summary statistics of ESTs obtained from *Fusarium oxysporum* cDNA library**

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TABLE 2. Differently expressed genes during conidial development of *Fusarium oxysporum* strains ATCC 16416 and AFu68

| Clone no. and class | Mean fold change in global normalized array data | Annotation<sup>a</sup> | Uniprot no. | E value |
|---------------------|-----------------------------------------------|------------------------|------------|---------|
|                     | Conidia | Germ tubes | Mycelia |                     |          |
| Class I: high expression in conidia |       |            |         |                      |          |
| FU022b10            | 4.94    | 4.93      | 2.43    | 1.90                | 1.23    | 1.95    | Phosphate permease PHO89 (*Saccharomyces cerevisiae*) | P38361 | 3E−17 |
| FU030f08            | 3.65    | 5.46      | 0.95    | 0.31                | 0.76    | 0.21    | Hypothetical 30.7-kDa protein in RVS161-ADP1 intergenic region (*S. cerevisiae*) | P25613 | 4E−35 |
| FU032e11            | 1.65    | 1.99      | 0.16    | 0.11                | 0.15    | 0.17    | Phosphate-repressible phosphate permease (*Neurospora crassa*) | P15710 | 2E−51 |
| FU042d01            | 2.14    | 1.17      | 0.44    | 0.31                | 0.28    | 0.26    | Similar to hypothetical 17.2-kDa protein in PCT1-AD5 intergenic region (*S. cerevisiae*) | P42937 | 4E−07 |
| FU054d09            | 4.59    | 12.6      | 2.28    | 1.77                | 2.19    | 0.48    | Nitrite reductase (*N. crassa*) | P38681 | 1E−33 |
| FU098e01            | 1.84    | 0.75      | 0.68    | 0.17                | 0.62    | 0.29    | Similar to maleylacetate reductase (*Burkholderia cepacia*) | P45072 | 0.001 |
| FU101d09            | 8.17    | 8.35      | 2.79    | 3.39                | 0.79    | 2.22    | PI6-like protein (*Schizosaccharomyces pombe*) | P24782 | 8E−55 |
| FU112e10            | 4.29    | 5.62      | 1.66    | 2.00                | 0.93    | 1.49    | Similar to hypothetical 25.3-kDa protein in TIM23-ARE2 intergenic region (*S. cerevisiae*) | P53721 | 7E−06 |
| Class II: low expression in conidia |       |            |         |                      |          |
| FU058f02            | 0.13    | 0.28      | 0.66    | 1.94                | 0.64    | 2.4     | ATP synthase beta chain, mitochondrial precursor (*N. crassa*) | P23704 | 1E−20 |
| FU067h12            | 0.71    | 0.17      | 1.58    | 1.42                | 1.85    | 1.63    | O-Acetylhomoserine (*Emericella nidulans*) | P50125 | 3E−14 |
| FU077a03            | 1.10    | 0.79      | 3.30    | 3.22                | 2.58    | 2.84    | Similar to clock-controlled protein 6 (*N. crassa*) | P41302 | 3E−5  |
| Class III: high expression in germ tubes |       |            |         |                      |          |
| FU017d03            | 0.33    | 0.68      | 0.96    | 2.14                | 0.44    | 0.34    | 60S ribosomal protein L37 (*E. nidulans*) | Q9C0T1 | 2E−31 |
| FU037g10            | 1.07    | 0.76      | 2.65    | 3.04                | 0.82    | 1.22    | 40S ribosomal protein S29 (*N. crassa*) | Q9C2P2 | 4E−24 |
| FU044h11            | 1.26    | 1.35      | 2.9     | 6.89                | 0.64    | 2.05    | Probable 5-methyltetrahydropteroylglutamate-homocysteine methyltransferase (*S. pombe*) | Q9UT19 | 1E−40 |
| FU048e10            | 0.34    | 0.45      | 1.1     | 1.14                | 0.25    | 0.41    | Similar to 60S ribosomal protein L36 (*Trichoderma hamatum*) | Q9HFR7 | 2E−08 |
| FU066a05            | 1.41    | 1.44      | 3.87    | 6.96                | 1.2     | 3.06    | Similar to hypothetical protein (*N. crassa*) | Q7S2C2 | 6E−06 |
| FU079a07            | 2.44    | 2.12      | 6.07    | 4.27                | 0.48    | 0.95    | 60S acidic ribosomal protein P2 (*Fusarium culmorum*) | Q8TFM9 | 3E−18 |
| FU082e01            | 0.7     | 0.49      | 1.94    | 1.53                | 0.41    | 0.46    | 60S ribosomal protein L44 (*Pichia jadinii*) | P52809 | 1E−52 |
| FU084a11            | 3.29    | 5.57      | 7.96    | 12.7                | 1.06    | 3.75    | 60S ribosomal protein P2 (*Alternaria alternata*) | P42037 | 9E−23 |
| FU110d07            | 0.65    | 0.58      | 1.85    | 1.19                | 0.22    | 0.21    | 60S ribosomal protein L38 (*N. crassa*) | Q9C2B9 | 2E−14 |
| FU117f01            | 1.05    | 1.28      | 2.94    | 7.99                | 1.22    | 2.41    | Anucleate primary sterigmata protein A (*E. nidulans*) | Q00883 | 3E−21 |
| Class IV: low expression in germ tubes |       |            |         |                      |          |
| FU026c01            | 1.74    | 2.15      | 0.65    | 0.82                | 1.83    | 1.89    | Leptomycin B resistance protein Pmd1 (*S. pombe*) | P36619 | 2E−26 |
| FU090g08            | 1.26    | 3.02      | 0.2     | 0.12                | 0.59    | 0.26    | Similar to hypothetical 30.7-kDa protein in RVS161-ADP1 intergenic region (*S. cerevisiae*) | P25613 | 3E−09 |
| Class V: high expression in mycelia |       |            |         |                      |          |
| FU055h10            | 4.70    | 6.09      | 2.72    | 5.32                | 16.1    | 12.7    | Ras-related protein Rab-11B (*Rattus norvegicus*) | Q35509 | 9E−16 |
| FU063c02            | 0.59    | 0.36      | 0.48    | 0.51                | 12.6    | 1.43    | Similar to protein FDD123 (*Cordyceps versicolor*) | Q74631 | 7E−04 |
| Class VI: low expression in mycelia |       |            |         |                      |          |
| FU002a03            | 4.81    | 2.89      | 4.26    | 0.82                | 0.87    | 0.37    | Similar to nitrate reductase (NADPH) (*Fusarium oxysporum*) | P39863 | 1E−08 |
| FU007c04            | 0.85    | 0.92      | 2.04    | 1.49                | 0.25    | 0.25    | 60S ribosomal protein L38 (*N. crassa*) | Q9C2B9 | 4E−27 |

Continued on facing page
cluster) were defined as tentative unique transcripts (TUTs) (Table 1). Compared with the 11,640 predicted genes in F. graminearum and the 11,109 predicted genes in M. grisea (Broad Institute), the 3,372 TUTs in our library represent roughly one-fourth of the F. oxysporum genes. After performing BLASTx searches (E values, \(10^{-10}\)), we found that 1,769 of these TUTs share homology with proteins in the UniProt databases. Of these, 924 TUTs have known protein functions. Twenty TUTs were the most abundantly expressed genes in our library because each of their clusters included >20 ESTs. Four of these were homologous to the ribosomal protein genes (S25, S26E, L39, and P2).

cDNA array detection of gene expression profiles during conidial germination. A total of 3,158 PCR products from TUT clones were printed on Immobilon-Ny+ transfer membranes (Millipore, Bedford, MA). The RNA samples of two strains, ATCC 16416 and AFu68, in the conidium, germ tube, and mycelium stages were labeled with \(^{33}\)PdCTP during first-strand reverse transcription (RT) reactions and then hybridized with the F. oxysporum cDNA arrays. The \(t\) test approach (2, 9) in the CyberT program was used to determine gene expression changes between samples. After the genes with significant differences (\(P < 0.01\); change of twofold or more) were selected for each of the strains, only those with the same expression pattern trends in both of the strains were considered to be specially expressed genes of a certain developmental stage. Some of them have been annotated are listed in Table 2, where they are categorized by class.

Through the EST and comparative cDNA array analyses, we found that ribosomal protein genes were highly redundant in our library and that some of them fluctuated during development. These genes included the 60S acidic ribosomal protein P2 gene (Table 2, class III) (FU084a11), which was expressed at greater levels in germ tubes than during the other two stages. Many studies have shown that protein synthesis becomes highly active during early germ tube stages. Some of them which have been annotated are listed in Table 2, where they are categorized by class.

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| Clone no. and class | Mean fold change in global normalized array data | Annotation\(^a\) | Uniprot no. | E value |
|---------------------|-------------------------------------------------|-----------------|------------|--------|
|                     | Conidia | Germ tubes | Mycelia |                     |                      |
| FU007c11            | 1.00    | 1.21       | 1.95    | 3.06 | 0.39 | 0.6 | 40S ribosomal protein S18 (S. cerevisiae) | P35271 8E—64 |
| FU009c09            | 1.45    | 0.79       | 1.38    | 0.78 | 0.50 | 0.33 | Similar to probable eukaryotic translation initiation factor 3 subunit 11 (Drosophila melanogaster) | Q9W2D9 2E—10 |
| FU011c11            | 3.52    | 15.8       | 4.66    | 7.69 | 0.61 | 1.55 | Flavohemoprotein (Bordetella pertussis) | Q7TTP0 5E—34 |
| FU013c02            | 2.70    | 2.13       | 1.69    | 2.22 | 0.53 | 1.04 | t-Lactate dehydrogenase A (Rhizopus oryzae) | Q9P4NB6 5E—30 |
| FU040f06            | 0.99    | 0.91       | 1.06    | 0.80 | 0.36 | 0.33 | Hypothetical UPF0327 protein NCU06495.1 (N. crassa) | Q7RYY1 2E—29 |
| FU040g03            | 3.26    | 2.25       | 1.98    | 1.40 | 0.8  | 0.7 | Similar to arsenical resistance protein ACR3 (S. cerevisiae) | Q65958 1E—06 |
| FU045g01            | 3.29    | 3.01       | 2.37    | 1.77 | 0.62 | 0.73 | 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase 1 (Candida albicans) | O13433 2E—16 |
| FU055a07            | 2.78    | 6.38       | 2.25    | 2.11 | 0.77 | 0.16 | Flavohemoprotein (Pseudomonas deragutina) | Q9J0H4 2E—14 |
| FU055e09            | 5.48    | 28.8       | 6.80    | 5.90 | 2.55 | 2.93 | Nitride reductase (Leptosphaeria maculans) | P43504 2E—31 |
| FU071b09            | 0.82    | 0.89       | 0.79    | 2.93 | 0.21 | 0.43 | 60S ribosomal protein L18-B (S. pombe) | Q8TFH1 4E—50 |
| FU073c12            | 0.38    | 0.43       | 0.83    | 0.53 | 0.17 | 0.07 | 40S ribosomal protein S24 (Rhizomucor racemosus) | P14249 2E—16 |
| FU079a05            | 2.92    | 3.19       | 5.34    | 2.12 | 0.68 | 0.72 | Ubiquitin (S. cerevisiae) | P61864 1E—36 |
| FU079a07            | 2.44    | 2.12       | 6.07    | 4.27 | 0.48 | 0.95 | 60S acidic ribosomal protein P2 | Q8TFM9 3E—18 |
| FU083h08            | 1.54    | 2.07       | 1.36    | 0.88 | 0.44 | 0.43 | Clathrin coat assembly protein AP17 (R. norvegicus) | P62744 3E—28 |
| FU085g01            | 1.72    | 1.21       | 3.02    | 1.07 | 0.22 | 0.13 | 60S ribosomal protein L3-A (S. pombe) | Q9UX4 2E—38 |
| FU088a11            | 2.30    | 1.16       | 4.59    | 1.56 | 0.55 | 0.35 | 60S acidic ribosomal protein P1 (A. alternata) | P49148 8E—28 |
| FU089a10            | 0.94    | 0.92       | 0.97    | 0.75 | 0.18 | 0.25 | 60S ribosomal protein L5 (N. crassa) | O59953 1E—110 |
| FU097e03            | 0.77    | 0.82       | 1.04    | 1.07 | 0.36 | 0.18 | 60S ribosomal protein L14-A (S. cerevisiae) | P36105 5E—13 |
| FU100c11            | 0.85    | 0.58       | 2.01    | 1.00 | 0.24 | 0.18 | 60S ribosomal protein L29 (S. cerevisiae) | P05747 7E—14 |
| FU107b04            | 1.15    | 1.37       | 1.49    | 0.65 | 0.33 | 0.22 | NHP2L7aE family protein YEL026W | Q21568 2E—30 |
| FU107d04            | 1.33    | 1.11       | 2.37    | 0.93 | 0.58 | 0.36 | 40S ribosomal protein S28 (N. crassa) | Q7SW55 1E—22 |

\(^a\) The differentially expressed genes were calculated by the program CyberT (http://visitor/ics.uci.edu/genex/cybert/), and the thresholds were \(P\) values (lnp) of <0.01 and changes of more than twofold.

\(^b\) The annotation “similar to” means that the E value was from \(10^{-3}\) to \(10^{-10}\), and annotations without these words means that the E value was \(<10^{-10}\).
normal circadian rhythm of asexual macroconidial development of *N. crassa* (16). In this study, a gene similar to *ccg6* (FU077a03) was expressed at higher levels in the germ tube and mycelium than in the conidium. This suggested that *ccg* could play dual regulatory roles in the circadian clock and during fungal spore development.

The normalized signal values for the *ras*-related gene encoding the protein Rab-11B (FU055h10) in our cDNA arrays were 4.70, 2.72, and 16.1, respectively, for the conidium, germ tube, and mycelium stages for strain ATCC 16416 and 6.09, 5.32, and 12.7, respectively, for strain AFu68. These data were far above the average expression level of all genes in the cDNA array (because of global normalization, the mean value of each cDNA array was 1). This shows that the *ras*-related gene is abundantly expressed during the entire conidial development process, but interestingly, it was most active in the mycelium.

In eukaryotes, from yeast to humans, the *ras* gene has been implicated in transducing growth and differentiation signals (1). However, whether *ras* is necessary for spore germination is still arguable. An activated mutant form of *ras* induced conidial germination of *A. nidulans* in the absence of a carbon source (13), whereas deletion of the *ras* homologue smco7 from *N. crassa* did not inhibit germination, although the hyphal growth rate of the mutants was about 1/10 that of wild-type cells (10). Through the results of our arrays, it can be deduced that the main function of the *ras*-related gene is to regulate hyphal growth, while this and other signal genes, such as *ccg*, can cooperatively initiate conidial germination by increasing protein synthesis.

Anucleate primary sterigmata protein A (ApsA) regulates nuclear migration during hyphal growth (17). The ApsA mutant form of *A. nidulans* almost completely blocks entry of the nuclei into primary buds during conidiophore development, which results in developmental arrest (6). Herein, we found that the *ApsA* gene (FU026c01) was highly expressed in the germ tubes of *F. oxysporum*, indicating that *ApsA* is expressed abundantly before rapid vegetative growth of the germ tube. The relationships of the genes discussed above to conidial development are illustrated in Fig. 1.

The other genes listed in Table 2, including those for which there are no annotations, were initially regarded as development-related genes whose relationships to the conidial development of *F. oxysporum* require additional studies.

Beckman and Roberts first described the time course of the major events during *F. oxysporum* pathogenesis (3). The overall development process is outlined clearly, but details of the major events, especially interactions with the host plant in vivo, remain unfocused. Since *F. oxysporum* does not produce fully differentiated infection structures upon infection, interpreting in vitro gene expression data must be conducted with caution, given that there is no known link with the in vivo situation. Nevertheless, the differences in gene expression patterns during fungal development do provide a framework for developing hypothesis-driven experiments to investigate specific aspects of host-pathogen interactions.

**Confirmation of cDNA array results by quantitative real-time RT-PCR.** Four TUTs (FU084a11, FU077a03, FU026c10, and FU055h10) which were specifically expressed during certain developmental stages, as revealed by cDNA arrays, were utilized to perform quantitative real-time RT-PCR experiments in order to validate the array results. In general, the relative mRNA levels determined by quantitative real-time
RT-PCR were in accordance with those obtained by cDNA arrays (Fig. 2).

All the details of methods, EST sequences, and original data for cDNA arrays can be found at www.estarray.org.

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