Expression of Biomass-Degrading Enzymes Is a Major Event during Conidium Development in *Trichoderma reesei*\(^*\)†

Benjamin Metz,\(^*\)‡ Verena Seidl-Seiboth,\(^1\) Thomas Haarmann,\(^2\) Alexej Kopchinskiy,\(^*\)‡ Patrick Lorenz,\(^2\) Bernhard Seiboth,\(^1\) and Christian P. Kubicek\(^1\)†

Institute of Chemical Engineering, University of Technology of Vienna, Gumpendorferstraße 1a, A-1060 Vienna, Austria, and AB Enzymes GmbH, Feldbergstrasse 78, D-64293 Darmstadt, Germany\(^2\)

Received 24 February 2011/Accepted 22 August 2011

The conidium plays a critical role in the life cycle of many filamentous fungi, being the primary means for survival under unfavorable conditions. To investigate the transcriptional changes taking place during the transition from growing hyphae to conidia in *Trichoderma reesei*, microarray experiments were performed. A total of 900 distinct genes were classified as differentially expressed, relative to their expression at time zero of conidiation, at least at one of the time points analyzed. The main functional categories (FunCat) overrepresented among the upregulated genes were those involving solute transport, metabolism, transcriptional regulation, secondary metabolite synthesis, lipases, proteases, and, particularly, cellulases and hemicellulases. Categories overrepresented among the downregulated genes were especially those associated with ribosomal and mitochondrial functions. The upregulation of cellulase and hemicellulase genes was dependent on the function of the positive transcriptional regulator XYR1, but XYR1 exerted no influence on conidiation itself. At least 20% of the significantly regulated genes were nonrandomly distributed within the *T. reesei* genome, suggesting an epigenetic component in the regulation of conidiation. The significant upregulation of cellulases and hemicellulases during this process, and thus cellulase and hemicellulase content in the spores of *T. reesei*, contributes to the hypothesis that the ability to hydrolyze plant biomass is a major trait of this fungus enabling it to break dormancy and reinstate vegetative growth after a period of facing unfavorable conditions.

Determination into a dormant resting stage (most frequently called “spores”) has been developed by a broad variety of prokaryotic and eukaryotic organisms to survive long periods of environmentally unfavorable conditions (19, 38). For a diverse group of fungi that includes many medically, industrially, and agriculturally important species, conidiation is also a common asexual reproductive mode and primary means for dispersion in the environment. It involves major rearrangements of many fundamental growth and cell cycle processes (including temporal and spatial regulation of gene expression, cell specialization, and intercellular communication) (13, 17, 37).

The genetic mechanisms controlling these processes in fungi have been addressed in some detail for only two well-studied ascomycetes, *Aspergillus nidulans* and *Neurospora crassa*, which, despite displaying similar general mechanisms, revealed significant differences (2, 10, 13, 47). A common feature of fungal spores from both species, however, is their content of a high number of RNAs that can be rapidly translated at the very beginning of germination, thus minimizing the time and energy needed to initiate growth once a suitable substrate becomes available.

The filamentous fungus *Trichoderma reesei* (the anamorph of the pantropical ascomycete *Hypocrea jecorina* [28]) is intensively investigated because of its ability to produce plant biomass-hydrolyzing enzyme mixtures suitable for biofuel production (26). Besides the development of high-producer strains by traditional and recombinant techniques (45), the propagation and maintenance of conidia of respective producer strains are of essential importance for strain application. Several aspects of the biochemistry and genetics of conidiation of *Trichoderma* spp. are well documented (for a review, see reference 48), but the events accompanying transition of the mycelia into the dormant conidia have not been studied in detail.

Here we report results of a genome-wide transcriptional analysis of spore formation in *T. reesei*. Among other findings, we will show that conidiation in *T. reesei* leads to massive upregulation of all kinds of cellulase and hemicellulase genes, suggesting that its conidia are preconditioned for germination in a habitat rich in plant biomass carbohydrates.

**MATERIALS AND METHODS**

**Strains.** *T. reesei* QM9414 (ATCC 26921), an early cellulase-producing mutant, and a *T. reesei* **Δxyr1** derivative of it (49) were used throughout this work and kept on potato dextrose agar (Sigma, St. Louis, MO).

**Induction of asexual sporulation.** To induce asexual sporulation in *T. reesei*, the fungus was grown on malt extract (MEX; Merck, Darmstadt, Germany) agar plates (11-cm diameter) on a layer of cellophane to facilitate removal of mycelia. The formation of spores was followed microscopically and by measuring the activity of a marker enzyme of conidiation, the D-mannitol dehydrogenase LXR1 (3, 35). Samples of at least two biological replicates, consisting of at least 3 independent plates, were taken at appropriate time points (see Results).

**Biochemical assays.** Cellulase enzyme activities were determined using carboxymethyl cellulose (1%, wt/vol) at 50°C (53). Enzyme concentration and reaction time were chosen such that a difference in *A*\(_{540}\) between 0.15 and 0.60 and linearity over the reaction time was obtained. To detect cellulase activity on...
conidia, 0.5 ml of a spore suspension (containing 5 x 10^6 conidia) was used, and this assay thus measured the surface-located cellulase activity (27).

Transcriptional analysis of sporulation. Sporulating mycelia were scraped off the cellophane, washed with distilled cold water, and frozen and ground under liquid nitrogen. Total RNAs were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions and then purified using the RNeasy MiniElute cleanup kit (Qiagen, Hilden, Germany). The RNA quality and quantity were determined using a NanoDrop spectrophotometer. High-quality purified RNAs were submitted to Roche-NimbleGen (40 µg per 3-microarray set), where CDNAS were synthesized, amplified, and labeled and then used for subsequent hybridization.

A high-density oligonucleotide (HDO) microarray (Roche-NimbleGen, Inc., Madison, WI) was constructed, using 60-mer probes representing the 9,129 genes of *T. reesei*. Microarray scanning, data acquisition, and identification of probe sets showing a significant difference (P = 0.05) in expression levels between different conditions were performed by Roche-NimbleGen. Values were normalized by quantile normalization (8) and the robust multichip average (RMA) algorithm (21), which had already been performed by NimbleGen. After elimination of those transcripts, which exhibited a standard deviation of >20% within replicates, the false discovery rate (FDR) (Benjamini-Hochberg) method (7) was used to assess the significance of values. Transcripts showing significantly different expression compared to that of the 18 h control (at least 2-fold changes and a P of <0.05) were grouped by k-means clustering as implemented in ArrayStar 3.0.1 (DNAStar, Inc., Madison, WI). Gene accession numbers were annotated according to version 2 of the *T. reesei* genome assembly (http://genome.jgi-psf.org/Trire2/Trire2.home.html), and ambiguous cases were annotated manually.

Genes were then classified according to their major annotation in the Munich Information Center for Protein Sequences (MIPS) functional catalogue (FunCat) (43). To determine whether there were differences in the functional categories in each cluster, the distribution within each cluster was compared to the total distribution of all the annotated genes using independent chi-square tests.

Analysis of genomic clustering of transcripts. *T. reesei* genes have not yet been mapped to chromosomes, but their appearance on genomic scaffolds is known. To identify whether the transcripts would be clustered to particular areas on these scaffolds, we algorithms were used to generate lists of genes on the individual scaffolds. We tested the presence of clusters of expressed genes by two methods. The first method employed REEF, software that identifies genomic regions enriched in specific features. REEF applies a statistical test based on the hypergeometric distribution by using a sliding-window approach and adopting the false discovery rate for controlling multiplicity (11). The software, source code, and documentation were downloaded from http://telenthon.bio.unipd.it/boinio/reel. To use it for *T. reesei*, the scaffolds were treated as chromosomes. A window width of 100 kb, a shift of 10 kb, and an FDR-corrected P value of <0.05 were used. A minimum number of 5 clustered genes was used as a threshold for the analysis. In the second approach, we used a hierarchical clustering method, model-averaged clustering by maximum likelihood (MACML) under the AIC criterion (57). The software, source code, and documentation were downloaded from http://www.yale.edu/townsend/software.html. MACML adopts a divide-and-conquer approach to hierarchically detect heterogeneous regions and iterates similar analyses within each identified region. As we show, both methods yielded largely similar regions.

Genomic synteny with *Trichoderma virens* and *Trichoderma atroviride* was analyzed with the synteny browser at the *T. reesei* genome sequence website (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

Real-time PCR. cDNA was reverse transcribed with the RevertAid first-strand cDNA kit (Fermentas) according to the manufacturer’s protocol with a combination of 1:1 of the provided oligo(dT) and random hexamer primers. All real-time (RT)-PCR experiments were performed on a Bio-Rad (Hercules, CA) iCycler IQ. For the reaction, the iQ SYBR green supermix (Bio-Rad, Hercules, CA) was prepared for 25-µl assays with the standard MgCl₂ concentration (3 mM) and a final primer concentration of 100 nM each. All assays were carried out in 96-well plates, which were covered with optical tape. Primers, amplification efficiency, and R² values are given in Table S1 in the supplemental material. The amplification protocol for genes 123989 and 72567 (numbers based on the Trire2 gene accession numbers) consisted of an initial denaturation step (3 min at 95°C), followed by 40 cycles of denaturation (15 s at 95°C), annealing (20 s at 57°C), and elongation (10 s at 72°C). The protocol for genes 120229, 73638, 120312, 123232, 120901, 121418, 76210, 69597, 69944, 56840, 112520, 56684, 104690, and 70197 was changed as follows: the annealing temperature was raised to 59°C, and the elongation step was terminated from the protocol. tef1 measurement was performed with both protocols for reference calculation. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1, 0.1, 0.01, and 0.001). Amplification efficiency was then calculated from the given slopes using iQ5 Optical System software version 2.0. Expression ratios were calculated using REST software (41). All samples were analyzed in two independent experiments with three replicates in each run.

Microscopical analysis. Samples were examined on an inverted Nikon TE300 microscope by using differential interference contrast optics and imaged with a Nikon D5XM1200F digital camera.

Microarray data accession number. The microarray data and the related protocols are available at the GEO website (www.ncbi.nlm.nih.gov/geo/) under accession number GSE27471.

RESULTS

Transcriptional analysis of conidiation: experimental design and properties. For determination of the earliest time point of sporulation on agar plates overlaid with cellophane, the mannitol dehydrogenase LXR1 was chosen as a sporulation marker enzyme. Expression of an lxr1 orthologue in *Aspergillus niger* mtdA occurs only during the formation of conidiospores (3). Its expression is also limited to spores, and we reported that *T. reesei* LXR1 accumulated in conidiospores of *T. reesei* (35). When grown on malt extract agar plates covered with cellophane, *T. reesei* showed a sharp increase in lxr1 expression from 18 to 21 h, with the highest level at 27 h (Fig. 1a). Visual inspection showed that at 18 h, patches of white mycelium could be observed, which were at later time points the first areas that turned light green, indicating the progressing maturation of conidia. Thus, we concluded that the white patches observed at 21 to 24 h are differentiated aerial hyphae, thus indicating the onset of conidiation. A microscopical analysis of the respective time points is shown in Fig. 1b to e. However, due to the dense mycelial mat on the cellophane sheet, already differentiated hyphae are difficult to discern under the microscope. In comparison to hyphae on agar plates without cellophane, the aerial hyphae were relatively short and the mycelium appeared, in general, less fluffy. However, when respective cultures from later time points (21 and 24 h) were suspended, dispatched conidia could be observed, indicating normal conidiation.

Consequently, we used four time points between 18 and 27 h to obtain a representative sample of RNAs for the process. Figure 1b illustrates the accumulation of spores during this time period. These time points were then used to identify transcripts that are significantly regulated (greater than ±2-fold; P < 0.05) using whole-genome oligonucleotide arrays. Exactly 900 transcripts were identified (see Table S2 in the supplemental material) and subjected to k-means clustering, which grouped the transcripts according to their temporal expression patterns and hybridization intensities. A number of 8 clusters (A to H) was chosen (Fig. 2a). Clusters A to F (541 genes) all showed an upregulation during sporulation that was already visible after 21 h (equal to 3 h of induction of spor formation). Cluster A contained genes that continued being upregulated until 27 h (9 h of induction of spor formation), whereas expression in clusters D and F remained roughly at the 21-h level. Genes in clusters B and C were again down-regulated after 24 h (5 h of spor formation) and remained at higher levels than before the onset of sporulation (18 h, t = 0) in cluster B, whereas they approximated the level before sporulation in cluster C. Clusters G and H (359 genes) exhibited genes that were downregulated during sporulation, and this pattern was more pronounced in cluster G.
growth of *T. reesei* values were calculated by REST software (41) (*P*lxr1 normalized transcription ratio is defined as the ratio of abundance of *lated* genes (*n*) to that of *tfl1* at a given time point to that at 15 h after inoculation. *P* values were calculated by REST software (41) (***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05). (b) Microscopical analysis of *T. reesei* grown on agar plates covered with cellophane. Images were taken after 18 h (b and c), 21 h (d), and 24 h (e). Arrows in panel b point to swollen, germinated conidia. Germ tubes are relatively thick (ca. 5 to 7 μm) but differentiate after ca. 50 μm of growth into mature hyphae, which are considerably thinner (ca. 2 to 3 μm). Arrows in panels d and e point to potential development of conidiophores and conidia.

![Figure 1](http://dx.doi.org/10.1128/9781555814571.ch11f1)

FIG. 1. (a) Expression of the sporulation marker gene *brl1* during growth of *T. reesei* conidiospores on cellophane MEX plates. The normalized transcription ratio is defined as the ratio of abundance of *brl1* to that of *tfl1* at a given time point to that at 15 h after inoculation. *P* values were calculated by REST software (41) (***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05). (b) Microscopical analysis of *T. reesei* grown on agar plates covered with cellophane. Images were taken after 18 h (b and c), 21 h (d), and 24 h (e). Arrows in panel b point to swollen, germinated conidia. Germ tubes are relatively thick (ca. 5 to 7 μm) but differentiate after ca. 50 μm of growth into mature hyphae, which are considerably thinner (ca. 2 to 3 μm). Arrows in panels d and e point to potential development of conidiophores and conidia.

Annotation and FunCat (43) categorization of the respective genes revealed that 39.2% and 28.2% of all up- and downregulated transcripts, respectively, encoded conserved but unknown and unique proteins. Genes involved in intermediary metabolism (i.e., of carbohydrates, amino acids, and lipids) comprised a significant portion within both the up- and the downregulated genes (*n* = 130; *P* value [n/n − 1] < 0.05). Sixty-five of them encoded unknown short-chain reductases/dehydrogenases, cytochrome P450-monoxygenases, and putative iron-dependent dioxygenases. Most of the remaining 75 genes were categorized as involved in carbohydrate, amino acid, and lipid metabolism. The genes of lipid metabolism accounted for 50% of the genes downregulated during sporulation (Fig. 2b).

The percentage of genes encoding transcription factors, carbohydrate-active enzymes (CAZyme) and lipolytic enzymes, and secondary metabolite synthesis enzymes (mainly polyketide synthases and nonribosomal peptide synthases) was significantly increased (*P* < 0.05) in the upregulated gene clusters (Fig. 2c). The two downregulated clusters contained a high percentage of genes for ribosomal proteins and for mitochondrial translation and import, which were completely absent from the upregulated clusters.

**CAZome gene upregulation during conidiation.** The above-mentioned upregulated CAZome genes comprised 57 glycosyl hydrolases (GHs) involved in cellulose and hemicellulose degradation, 3 carbohydrate acetyl esterases, and 3 chitinases (Fig. 3a). They included 6 of the 9 cellulases (CEL5A, CEL6A, CEL7A, CEL7B, CEL12, and CEL45), two cellulase-enhancing proteins (CEL61A and CEL61B), swollenin (a protein carrying an expansin-like domain and that disrupts the crystalline cellulose structure) (44), CIP1, a protein that contains a signal peptide and a cellulose-binding domain (12), 7 β-glucosidases, and 4 of the 5 xylanases (XYN2, XYN3, XYN4, and XYN5). The remaining upregulated GHs (21 of 28) comprised glycosidases and glycanases active against various side chains in hemicelluloses, including two pectinases. All of these genes exhibited a strong peak of expression early during sporulation (at 3 h), after which most of them declined. However, with the exception of the four genes of cluster I (Fig. 3a), they all remained at the same level or at a level up to 2-fold higher at 9 h than at time zero.

Of the 32 GH families detected, 11 expressed all of their members (Fig. 3b), whereas only half or a third of the genes present in the remaining GH families were expressed. Four families (GH18, GH47, GH76, and GH92; comprising chitinases and various α-mannosidases) were only scarcely represented. We note that the GH families that were completely expressed during spore formation (indicated in red in Fig. 3b) were dominated by enzymes acting on cellulose.

**Verification of the microarray results by quantitative expression analysis.** To confirm these microarray results, quantitative RT-PCR was performed on a subset of the genes belonging to different clusters. For this experiment, the RNAs were isolated from plates that were not covered with cellophane in order to rule out that expression of some genes (e.g., of cellulases) would be specifically triggered by it and not by conidiation. Under these conditions, sporulation was observed only after 48 h, and thus, the time point correlated morphologically to the 21-h point of the experiments with cellophane-covered agar plates (Fig. 4a and b). As shown in Fig. 4c and d, the genes that showed differential expression between two or more of the conditions used in the microarray study were, in general, also differentially expressed in the same direction upon RT-PCR analysis, although some minor differences (probably due to the different conditions for inducing sporulation) were noted (e.g., gene 76210 or 69944). We therefore conclude that the microarray expression ratios indeed reflect differences in the expression of these genes and that the cellulase genes also show the same trend in the absence of cellophane.

**XYR1 controls conidiation-associated cellulase transcription but not sporulation.** The expression of *T. reesei* cellulases is dependent on induction, which is positively controlled by the Zn(II) Cys6 transcriptional regulator XYR1 (49). Since our experimental system did not contain an inducer of cellulase formation, we wondered whether cellulase gene expression during sporulation would still be subject to regulation by XYR1. To this end, we investigated conidiation and cellulase gene expression in a Δxyr1 strain of *T. reesei* (in the absence of cellophane). Sporulation was virtually unaffected by the xyr1
deletion (data not shown), but cellulase gene expression (monitored by measuring expression of the major cellulase genes \(cbh1\) and \(cbh2\)) was eliminated (Fig. 5). We therefore conclude that sporulation-associated cellulase expression requires the positive regulator XYR1, but neither XYR1 nor any of the cellulases is functionally involved in the process of spore formation.

Conidia-associated cellulases are required for fast germination on cellulosic substrates. A possible reason for the triggering of cellulase gene transcription during conidiation could be to endow these structures with the ability to germinate faster than other competitors once a lignocellulosic substrate becomes available. To test this, we first examined whether spores still contained cellulase transcripts or proteins after a longer storage time (7 days). In fact, we did not find the transcripts of \(cel7A\) and \(cel6A\) anymore, but they exhibited clear cellulase activity, and CEL7A was also demonstrable by means of a monoclonal antibody (data already shown in reference 34 and thus not repeated here). The \(Δxyr1\) strain lacked both activity and the CEL7A protein. When strain QM 9414 and the \(Δxyr1\) strain were both germinated in liquid medium with carboxymethyl cellulose as the only carbon source, QM 9414 started to germinate after 10 to 12 h, whereas \(Δxyr1\) did not do so until 18 h. Both strains, however, started to germinate after 8 h on glucose under otherwise similar conditions. Thus, the conidia-located cellulases enable the fungus to germinate in the presence of a cellulosic carbon source.

The sporulation-associated transcriptome is nonrandomly distributed in the genome. We were further interested in whether the genes expressed during conidiation would be clustered in the genome. To test this, two different strategies were applied. In the first, we made use of REEF, which detected 179 of the 900 genes (19.9%) to be nonrandomly distributed within 20 clusters on 12 scaffolds (Fig. 6; see also Table S3 in the supplemental material). Eight of them occurred near one of the ends of the respective scaffolds, and 11 occurred in regions that lacked synten with other ascomycetes, notably two other Trichoderma spp. (\(T. virens\), \(T. atroviride\)). Basically, the same
pattern (201 genes, 22.3%) was obtained by the application of MACML (57), yet with a few important differences (Fig. 6). One of those differences was the finding of a large cluster of genes (including CAZyme) at the end of scaffold 2 and the detection of two clusters and one small cluster on scaffolds 6 and 15, respectively. The other difference was the lack of support for two clusters (on scaffolds 3 and 5) identified by REEF. Despite these differences, the high level of consistency of the data obtained by these two methods demonstrates that a significant portion of the genes expressed during sporulation are clustered in the genome.

**DISCUSSION**

Genome-wide changes in the expression of genes that accompany the shift from mycelial growth to conidiation in *T. reesei* revealed a number of expression patterns that are consistent with results from *Aspergillus fumigatus* and *N. crassa* asexual conidiation (18, 29) but also revealed a number of unique findings. The most striking finding was the massive upregulation of cellulases and various hemicellulases during the early phase of sporulation, which has not been reported for *A. fumigatus* or *N. crassa* conidia (also, a manual inspection of the arrays from these studies [29, experiment ID64 from the Filamentous Fungal Gene Expression Database, http://bioinfo.townsend.yale.edu/browse.jsp] did not detect upregulation of cellulase genes [C. P. Kubicek, unpublished data]). When considered one gene category, these 63 genes accounted for the highest number of identifiable transcripts (chi-square test; \( P < 0.05 \)). The presence of cellulohydrolases on the surface of the conidia of *T. reesei* has been reported previously (27, 34) and has been interpreted as a means by which *T. reesei* can sense the presence of cellulose and form the inducer of expression of more cellulases for their degradation. The present findings, in combination with the detection of upregulation of carboxylate transporters and the simultaneous downregulation of proteases and amino acid transporters, indicate that *T. reesei* spores are preconditioned to reinitiate metabolism on cellulosic or hemicellulosic material. We speculate that this is a trait that has conveyed an ecological advantage to *T. reesei* as an endophyte of tropical flowers.

It will be interesting to look for *T. reesei* in the rhizosphere or as an endophyte of tropical flowers.

Another striking finding was the downregulation of a large number of genes associated with mitochondrial function. Although such a finding has not been reported for either *N. crassa* or *A. fumigatus* (18, 29), spores of the latter have been reported to perform fermentative metabolism (50), which is also reflected in an increased abundance of pyruvate decarboxylase and alcohol dehydrogenase in its conidia. We did not observe this for *T. reesei*, although it is capable of using pyruvate decarboxylase for fermentative metabolism at low oxygen concentrations (9). The downregulation of genes associated with mitochondrial functions may be a specific means to adapt to conditions of fermentative energy generation.

Reactive oxygen species (ROS) are believed to be the trigger for conidiation when a fungus comes into contact with air, and a hyperoxidant state has been demonstrated to occur at the start of conidium formation, eventually leading to the oxidation of several enzymes (4). It was thus interesting to learn that spores of *T. reesei* accumulate gene transcripts for two superoxide dismutases, two catalases, one bifunctional catalase/peroxidase, one peroxiredoxin, and two thioredoxin reductases. Proof of the involvement of these enzymes in an antioxidant response has been demonstrated in other fungi (23, 30, 36, 51), and we infer that conidia of *T. reesei* also face a hyperoxidant state. Six glutathione S-transferase genes were also upregulated. Glutathione is an important antioxidant molecule which reacts nonenzymatically with a series of reactive oxygen species (42). However, glutathione has several other functions as well that may also be important for sporulation: protein-glutathione mixed disulfides result in protection against desiccation-induced oxidative injuries in lichens (25), and glutathione is also involved in the detoxification of heavy metal ions that could be essential for the spores when settling down in a new environment (1).

The present study also reveals additional mechanisms that *T. reesei* uses for combating the hyperoxidant state which have not yet been emphasized in other fungi and which may be specific for this fungus. One is the upregulation of arsenate reductase, which is, together with thioredoxin reductase, part of a thio-disulfide exchange complex responsible for the production of reducing equivalents in bacteria (31). Another one appears to be the formation of D-erythroascorbate, the fungal equivalent of plant L-ascorbic acid, which is a crucial antioxidant for scavenging hydrogen peroxide. Its formation is suggested by the upregulation of the gene for a key enzyme in its biosynthesis, *D*-arabino-1,4-lactone oxidase. *D*-Erythroascorbate glucoside has also been reported to accumulate in the spores of *Phycomyces blakesleeanus* (6). We further identified potential additional sources for *H₂O₂* generation, i.e., *D*-aspartate race-
mase and D-aspartate oxidase. D-Aspartate is one of a few D-amino acids that are known to occur as a protein component in mammals, and the occurrence of free D-aspartate was also demonstrated (56). A role in H$_2$O$_2$ generation for ROS-dependent thyroid hormone signaling in mice has recently been suggested by Topo et al. (52). Finally, we also identified a peptide methionine sulfoxide reductase, capable of antagonizing the oxidation of proteins (46), and a fructosamine kinase, which removes nonenzymatically attached glucose residues from proteins (54). Both enzymes may act to reactivate proteins denatured by the hyperoxidative state. From these findings, we conclude that protection against oxidative stress is a major event during conidiation of \textit{T. reesei}. Interestingly, except for arsenate reductase in \textit{N. crassa}, we did not find up-regulation of these genes in \textit{N. crassa} and \textit{A. fumigatus} (C. P. Kubicek, unpublished data).

In contrast to sporulation in \textit{A. fumigatus} and \textit{N. crassa}, we could draw only a few conclusions from the differential regulation of metabolic genes. About half of them comprised unknown genes involved in oxidation or oxidoreduction. It is possible that at least some of them could be involved in the biosynthesis of unknown secondary metabolites. The differentially regulated genes with known metabolic function did not

![Figure 4](image-url)  
**FIG. 4.** Experimental design for quantification of transcription of selected genes during conidiation in \textit{T. reesei} in the absence of cellophane. (a and b) Images of aerial hyphae after 48 and 72 h of growth on agar plates, showing conidiophores and patches of conidia of \textit{T. reesei}, which are oval, ca. 2- to 3-$\mu$m-long structures. Conidia are at first light-green pigmented (a) and turn dark green upon maturation (b). Scale bars = 20 $\mu$m. (c) Quantification of the transcription of selected genes during sporulation on plates not covered by cellophane. (d) Microarray results for the same genes. Numbers given indicate \textit{Tirim2} protein identification numbers: 120229 (GH10 candidate xylanase, XYN3), 73638 (CIP1), 120312 (CEL5A), 123232 (CEL12A), 120961 (CEL61B), 121418 (G-D-S-L lipase/acylase), 76210 (GH62 $\alpha$-L-arabinofuranosidase ABF2), 69957 (predicted transporter/major facilitator superfamily), 69944 (GH31 $\alpha$-glucosidase), 56840 (predicted oxidoreductase), 112520 (unique protein), 56684 (predicted transporter/major facilitator superfamily), 106490 (unknown membrane protein), 70197 (Zinc-dependent hydrolase/β-lactamase superfamily), 123989 (CEL7A/CBH2), and 72567 (CEL6A/CBH1). The normalized transcription ratio in panel c is defined as the ratio of abundance of the respective gene to that of \textit{tef1} at a given time point to that at 48 h ($t$ = 0 for the experiment). Light gray bars represent results at 3 h, open bars at 7 h, and dark gray bars at 24 h after time zero (48 h). $P$ values were calculated by REST software (41) (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (d) Gray bars represent results at 3 h, open bars at 6 h, and dark gray bars at 9 h after time zero (18 h).

![Figure 5](image-url)  
**FIG. 5.** Transcription of \textit{cbh1} (open bars/light gray bars) and \textit{cbh2} (medium gray bars/dark gray bars) during induction of conidiation of \textit{T. reesei} QM 9414 and its \textit{Δxyr1} strain. Normalized transcription ratios are defined as explained in the legend to Fig. 4. Conidiation time (at 0 h) relates to the control (48 h).

![Figure 6](image-url)  
**FIG. 6.** Location of clusters of conidiation-expressed genes on the genomic scaffolds of \textit{T. reesei} as determined by REEF (red boxes) and MACML (blue bars over scaffold) analysis. The scaffolds are drawn to scale. Areas of synteny with \textit{T. atroviride} and \textit{T. virens} are indicated in yellow.
lead to a clear insight into the changes in metabolism that accompany sporulation, with the exception of osmoregulation and trehalose biosynthesis. The latter occurs in fungi through two different pathways that use glycosyltransferases from the GT1 or the GT20 family (5). We have observed the accumulation of one gene from each family, including the GT1 orthologue of CCG9 that is highly expressed in N. crassa and A. fumigatus conidia (18, 29). In addition, and similar to what occurs in A. fumigatus, we also saw a high expression of choline oxidase, which produces the osmoprotectant glycine betaine (14), suggesting that this compound is also active in T. reesei.

Genes in the chromosomes of many eukaryotic genomes, like yeast, worm, fly, mouse, and human, display a high-order dispersal and exploration of new habitats.

Among these, more than half occurred either in regions without a specific chromosomal region (22). In ascomycetes, characterized by high recombination frequencies that seem to create a favorable environment for the rapid generation of novel genes needed for adaptation to new ecological niches (16). Such a view is also supported by the detection of quickly evolving genes, particularly those encoding secreted proteins and orphan genes in subtelomeric regions in Fusarium graminearum (12) and N. crassa (23). We consider it rational that such processes should be strongly reflected in the transcriptome of asexual spores, which are destined for withstanding long unfavorable periods and, in the case of the latter, dispersal and exploration of new habitats.

ACKNOWLEDGMENTS

This work was supported by grants from the Austrian Science Foundation to C.P.K. (FWF P-21266) and B.S. (FWF P-19421).

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