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Aspergillus flavus Blast2GO Gene Ontology Database: Elevated Growth Temperature Alters Amino Acid Metabolism

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Abstract

The availability of a representative Gene Ontology (GO) database is a prerequisite for a successful functional genomics study. Using the online Blast2GO tool we constructed a GO database of *Aspergillus flavus*, a plant and human pathogen. Of the predicted total 13,485 *A. flavus* genes 8,987 were annotated with GO terms. The mean GO level was 5.64. Using a low stringency setting of a sequence cut-off number of 10 and a node score of 20, we obtained 1,177 GO terms associated with biological process, 388 GO terms associated with molecular function and 200 GO terms associated with cellular component. Of the 8,987 annotated genes 4,232 were mapped to 129 reference pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The utility of the GO database was evaluated with published transcriptomic data by assessing physiological states in relation to the metabolic capacity of *A. flavus* growing at 30°C and 37°C. Results showed that growth at 30°C favored degradation of amino acids with nonpolar side chains, such as valine, leucine, isoleucine, phenylalanine and tryptophan. In contrast, growth at 37°C favored the degradation of arginine and histidine (basic), aspartate and glutamate (acidic), and serine and threonine (uncharged but polar) but biosynthesis of the aforementioned nonpolar-side-chain amino acids. KEGG pathways of amino acid degradation contributing to acetyl-CoA production, (saturated) fatty acid biosynthesis and degradation as well as biosynthesis of unsaturated fatty acids were active at 30°C, which suggests a requirement that *A. flavus* maintain a high content of unsaturated fatty acids at the optimal growth temperature. The proposition was supported by the finding that the GO cellular component involved at 30°C growth was mainly a fatty acid synthase complex. The constructed *A. flavus* GO database was proven to be useful in our functional genomics study. We also outlined the procedures for future update and refinement of the current *A. flavus* GO database, which can be achieved by concerted efforts of the *Aspergillus* research community.

Keywords

Aspergillus, Gene Ontology, Transcriptome, Blast2GO, KEGG, Temperature, Amino acid metabolism

Introduction

Aspergillus flavus is predominately a saprophytic fungus in the soil that grows on dead plant, agricultural debris, and animal tissue. It can infect many crops such as corn, cotton, peanuts, and nut trees and the infection often leads to contamination with aflatoxin B_1, a toxic and potent carcinogenic compound. Because of its ability to grow at 37°C, it also is an opportunistic pathogen for humans and animals and is known to be the second leading cause of invasive aspergillosis in humans [1]. The genome of *A. flavus* NRRL3357, which consists of eight chromosomes, has been sequenced and its size determined to be about 37 Mb [2]. The genome sequence of another *A. flavus* strain AF70S (ATCC MYA-384), which produces small sclerotia, has also been annotated and assembled but the data are not yet available to the public (Yu, personal communication). Seven additional *A. flavus* genome sequences not yet assembled also are available [3]. Comparative genome studies have been performed to decipher evolutionary relationship among *A. flavus* and related species [2-4]. Genome-wide investigations using EST or whole-genome microarray assays have been performed to examine genes differentially expressed in degenerated *A. flavus* strains after serial transfers [5], in a 70S-derived strain that is defective in the *veA* regulatory gene [6], in NRRL3357 that examined tryptophan supplementation on aflatoxin biosynthesis [7], in the simulated conditions of *A. flavus*-host interactions [8] and in the process of *A. flavus*-seed colonization [9]. However, interpretations of the research results, more often than not, have not provided meaningful conclusions to the studied conditions. The inability to pertinently interpret the results is mainly due to the lack of knowledge in network interactions among the differentially expressed genes examined.

With the advent of the next generation sequencing of RNA (RNA-Seq) [10], functional genomics research in many organisms has expanded enormously in recent years. The application of RNA-Seq routinely generates unprecedented amounts of data on gene expression. To understand the transcriptional landscape and associated biological meanings, researchers have embraced bioinformatic technologies that categorize genes that are differentially expressed under studied conditions. One technique widely used to highlight the biological implications is to apply Gene Ontology (GO) enrichment analysis. This approach groups differentially expressed genes into categories based on predefined biological properties. It then statistically tests each category against the non-differentially expressed gene population to determine whether a category is over represented among the differentially expressed genes. GO is a

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collection of defined terms that represent gene product properties. It covers three main domains: 1) biological process, which represents operations or molecular events with a defined beginning and end; 2) molecular function, which describes gene product activities at the molecular level; and 3) cellular component, which indicates parts of a cell or its extracellular environment (http://www.geneontology.org). Many web-based tools are available for performing GO analysis, for example, Blast2GO [11], DAVID [12], ClueGO [13], AmiGO (http://www.geneontology.org), AgriGO [14], and FungiFun [15]. Among these, FungiFun is designed specifically for functional characterization of fungal genes, and Blast2GO has great versatility with statistically robust tests that aim to eliminate false-positive hits.

Although transcriptomic profiling by RNA-Seq has been carried out in *A. flavus* [16,17], functional genomics analyses have been lacking due to the unavailability of a suitable *A. flavus* GO database. To establish a GO database for *A. flavus* that can be freely shared by research groups in the form of an annotation file, we analyzed all predicted 13,485 genes of *A. flavus* NRRL3357 and obtained GO terms of the annotated genes using the free online resource of BLAST2GO (http://www.blast2go.com). The annotation file can be requested from the corresponding author. The utility of the database was evaluated with available RNA-Seq data previously used to investigate temperature effects on aflatoxin biosynthesis of *A. flavus* [17]. We used these data to further assess the metabolic states of *A. flavus* growing at 30°C and 37°C. The results showed that different groups of amino acids were utilized at different growth temperatures. The higher pathway activities of amino acid metabolism that produces acetyl-CoA, (saturated) fatty acid biosynthesis and degradation, and biosynthesis of unsaturated fatty acids at 30°C than at 37°C may account for the high content of unsaturated fatty acids in *A. flavus*, which comprises 70 to 75% of the total fatty acids [18]. The constructed *A. flavus* GO database although proven to be useful is still far from perfect. Procedures for future update and refinement of the current database for use in *A. flavus* and closely related aspergilli were outlined.

Materials and Methods

Construction of the *A. flavus* GO database

The *A. flavus* NRRL3357 genome sequence and annotations were acquired from NCBI (http://www.ncbi.nlm.nih.gov). The online resource Blast2GO (http://www.blast2go.com/b2ghome) was used to assign GO terms to *A. flavus* NRRL3357 gene products [11,19]. All 13,485 genes of *A. flavus* NRRL3357 were analyzed by a BlastX search against the NCBI non-redundant (nr) database with an Expect (E) value ≥1.0E-3 and a maximum of 20 hits for each gene. In the mapping step default weights of the evidence codes were used. In the annotation step only the gene hits with an E value ≥1.0E-6 were further analyzed. An annotation score of 55 was used as the cutoff value after a GO-Weight of 5 was given to mapped children terms. An InterProScan search for conserved domains and motifs was performed next followed by the use of the Annex function to augment the GO terms.

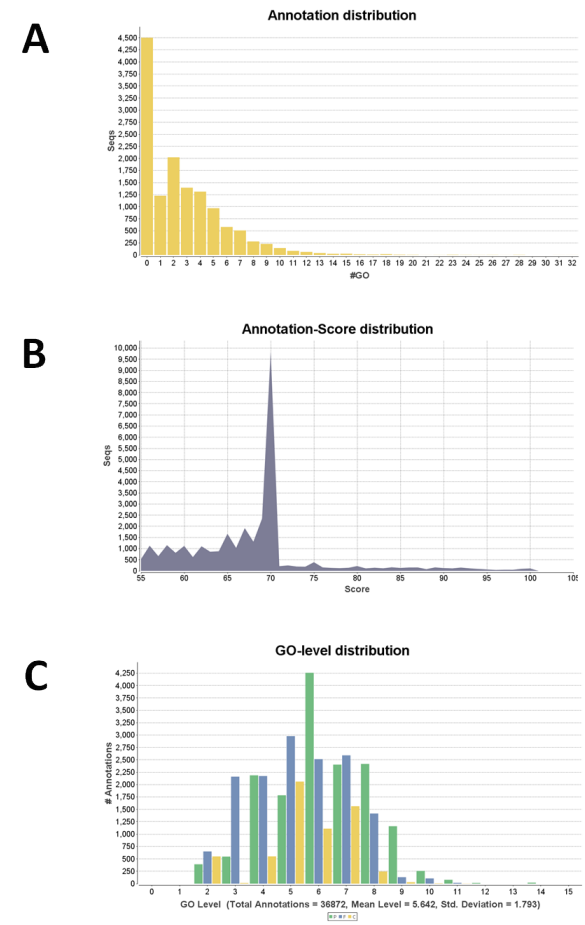
GO analysis and KEGG pathway assignments

The RNA-Seq data from the study of temperature effects on genome-wide gene expression of *A. flavus* NRRL3357 [17] were obtained from the NCBI's GEO database, which is under the accession number, GSE30031. Functional GO enrichment analyses were performed on annotations of up-regulated (≥ four-fold) genes at 30°C or 37°C using Fisher's Exact Test with Multiple Test Correction of False Discovery Rate (FDR) at the significance threshold of 0.05. The setting of FDR <0.05 is in general 1,000 times more stringent than the *p*-valve <0.05 [20]. Both (i) the differentially expressed genes, that is, those up-regulated ≥four-folded at 30°C or 37°C, and (ii) the GO-enriched and annotated genes with FDR <0.05 at 30°C or 37°C were analyzed using the reference metabolic pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [21].

Results

GO annotation of *A. flavus* genes

We first performed a BlastX search against the NCBI non-redundant (nr) database with an Expect (E) value ≥1.0E-3 and a 20 hit maximum. Of the 13,485 predicted genes 12,742 (94.5%) met the selection criteria. Those genes that did not meet the selection criteria included 141 with no homology (HSP length cutoff=33) to proteins in the nr database. The majority of the hits had E values ≥1.0E-25 with degrees of homology ranging from 65% to 100%. Of the 12,742 selected genes 12,194 (95.7%) were mapped sequences. GO annotation, which applies a specific annotation rule on obtained ontology terms, resulted in 31,194 annotations and a mean GO level of 5.66 from a pool of 8,519 annotated sequences. In this step, the number of sequences with an enzyme code (EC) was 2,952 and 3,712 ECs were obtained. The next InterProScan step, which retrieves functional domains or motifs from amino acid sequences, increased the total annotations to 32,714. Among these 28,206 were confirmed annotations, and 4,508 were too general and were removed. The further Annex step, which increases the annotation density but does not increase the number of annotated sequences, enriched the annotations by 4,445 (13.6%). The overall analysis resulted in 8,987 *A. flavus* sequences annotated with 37,139 GO terms. Figure 1 shows the distribution of annotation, i.e., number of GO term in relation to the total number of the *A. flavus* sequences, annotation score, and GO level.

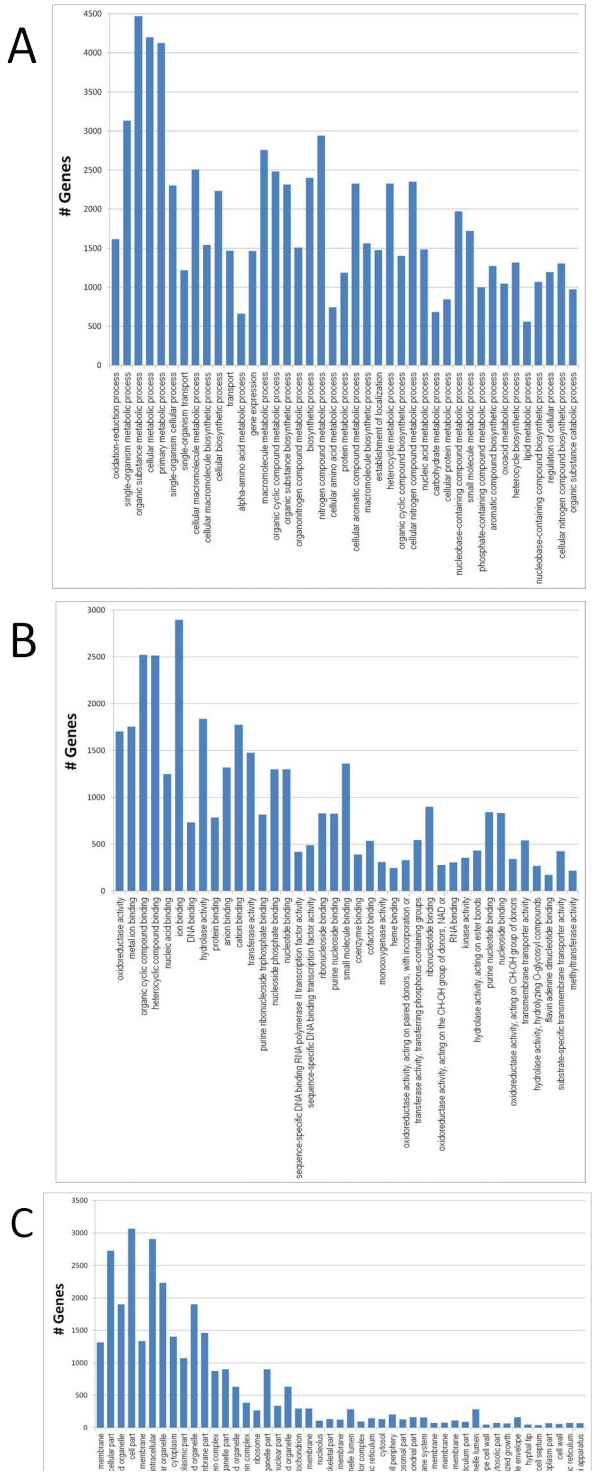


(A) GO term number in relation to the total number of the *A. flavus* sequences; (B) Annotation score in relation to the number of sequences; and (C) GO level in relation to the number of annotation.

Figure 1: Summary of the distribution of annotations of *A. flavus*.

GO functional categories and KEGG metabolic pathways of *A. flavus*

The graphical presentation of the three main GO functional categories: Biological process, molecular function and cellular component allows visualization of grouped annotated genes to reveal associated biological functions. Parameters in Blast2GO such as Sequence Number Filter and Node Score Filter can be set to control the graphic content, which in turn affects the numbers of GO terms tabulated for the three functional categories. Using a preliminary setting of a sequence number of 10 and a node score of



(A) Biological process: GO level was set at three to five, sequence cut-off number was 450 and node score cut-off was 200; (B) Molecular function: GO level was set at three to five, sequence cut-off number was 150 and node score cut-off was 150; and (C) Cellular component: GO level was set at three to six, sequence cut-off number was 15 and node score cut-off was 35.

Figure 2: GO functional categories of biological process, molecular function, and cellular component for *A. flavus*.

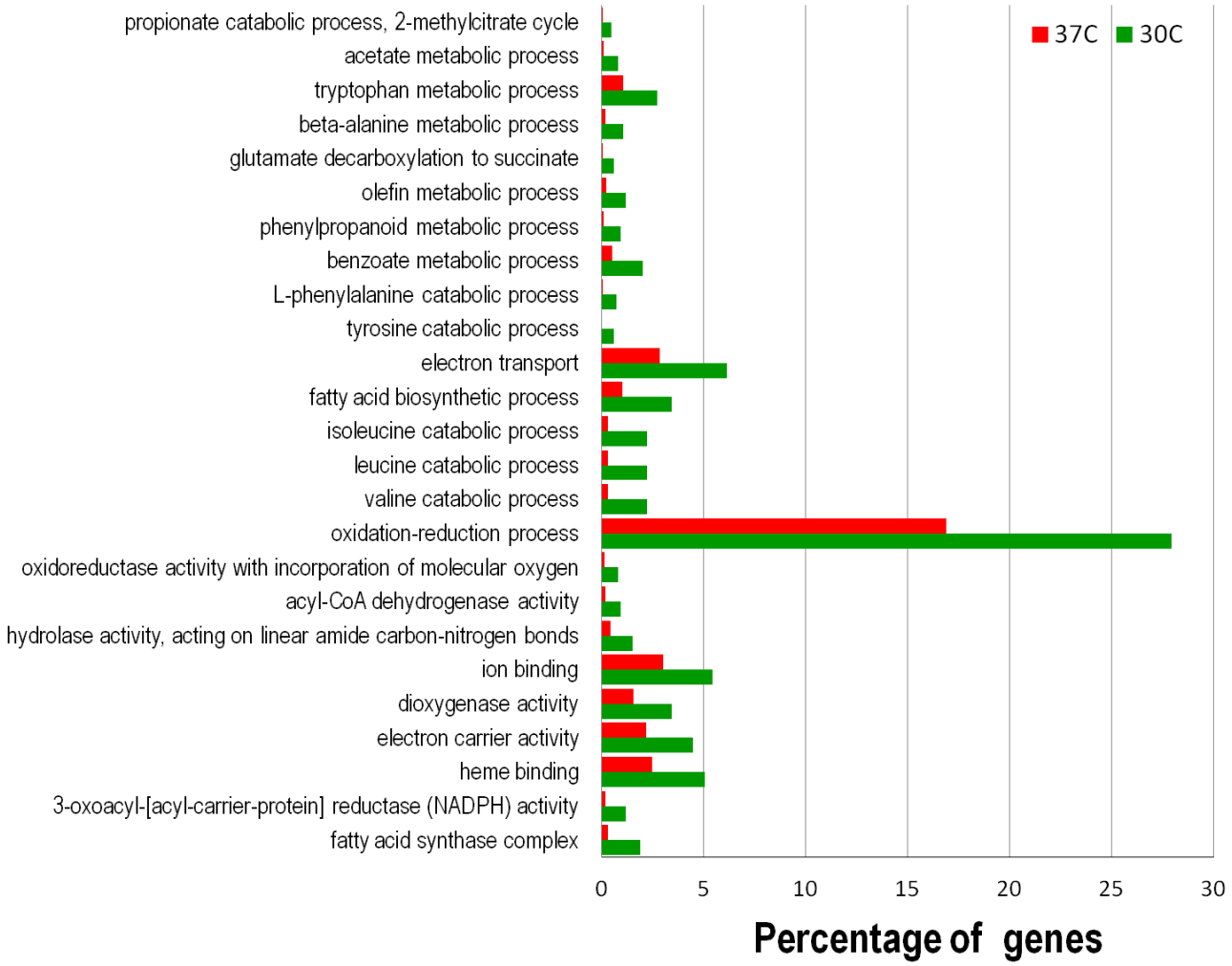
20, we obtained 1,177 GO terms associated with biological process, 388 GO terms associated with molecular function and 200 GO terms associated with cellular component. Biological process, molecular function, and cellular component are each the ultimate parent of its own functional category and are treated as "level one" GO terms. When the GO level, sequence number and node score were arbitrarily set to intermediate ranges (Figure 2), the number of GO terms in each functional category was substantially decreased. Under these specific criteria, organic substance metabolic process (GO:0071704), cellular metabolic process (GO:0044237), and primary metabolic process (GO:0044238) are the main categories of the biological process, each with over 4,000 genes. Organic cyclic compound binding (GO:0097159), heterocyclic compound binding (GO:1901363), and ion binding (GO:0043167) are the major binding activities of the molecular function, each with over 2,500 genes. For the cellular component, intracellular part (GO:0044424), cell part (GO:0044464), and intracellular (GO:0005622) form the main categories, each also with over 2,500 genes. To identify metabolic pathways of *A. flavus*, we mapped the 8,987 annotated genes to the reference pathways in the KEGG database. Of the annotated genes, 4,232 were assigned to 129 pathways. The pathways with over 100 genes annotated included those for metabolism of purine (222), starch and sucrose (138), and amino acids of glycine, serine and threonine (116) (Table S1).

Effects of growth temperatures on *A. flavus* GO functional categories and KEGG pathways

The utility of the constructed *A. flavus* GO database was evaluated with RNA-Seq data that examined temperature effects on expression of genes of the aflatoxin gene cluster [17]. Using the dataset we calculated that 12,081 (89.6%) genes were expressed for all samples combined and 10,594 (78.6%) genes were expressed at both 30°C and 37°C. In this study, using fold-of-increase ≥four as the criterion, we found that a total of 1,973 genes were differentially expressed under these conditions with 1,259 genes up-regulated at 30°C and 714 genes up-regulated at 37°C. Among these 849 of the 1,259 genes and 473 of the 714 genes were annotated with GO terms. GO enrichment analyses indicated that many biological processes and molecular functions were negatively affected at 37°C. In other words, the activities of the corresponding processes and functions at 30°C were relatively enhanced. Figure 3 shows that catabolism for specific amino acids, such as valine, leucine, isoleucine, tyrosine, phenylalanine, alanine, and tryptophan, fatty acid biosynthesis, metabolism of simple carbon compounds such as acetate and propionate, and electron transport were elevated at 30°C. The cellular component involved was the fatty acid synthase complex. KEGG metabolic pathway analysis concluded that the annotated genes up-regulated at 30°C were associated with 104 pathways. At 37°C only the molecular function of transferase activity was enriched (data not shown). Nonetheless, 81 KEGG pathways were associated with the up-regulated annotated genes. To investigate how growth temperatures affected *A. flavus* metabolism, we arbitrarily compared the top 10 metabolic pathways associated with growth at 30°C and those with growth at 37°C, which contained approximately 5% and 1% of the annotated sequences, respectively (Table 1). We further analyzed KEGG pathways associated with GO-enriched, annotated sequences for growth at 30°C and at 37°C. The top 10 pathways enriched at 30°C were identical to and in the same order as those obtained from the analysis of the genes up-regulated ≥four-fold at 30°C (Table 1). At 37°C only seven out of the 10 pathways associated with the differentially expressed genes are identical to those from the GO enriched sequences. The three additional enriched pathways were histidine metabolism, butanoate metabolism, and phenylalanine metabolism.

Discussion

Our results suggest that the constructed *A. flavus* GO database is useful for providing reasonable interpretations of the test RNA-Seq dataset. The GO enrichment analysis implies that *A. flavus* growth at 30°C favors degradation of amino acids mainly with nonpolar side chains, such as valine, leucine, isoleucine, phenylalanine, and tryptophan (Figure 3). In contrast, growth at 37°C favors the degradation of arginine and histidine (basic amino acids), aspartate and glutamate (acidic), and serine and threonine (uncharged but polar) (Table 1). The KEGG pathway analyses also confirm that 30°C promotes the degradation of the aforementioned nonpolar amino acids, while the higher growth temperature of 37°C enhances the biosynthesis of nonpolar amino acids. Therefore, the growth temperatures influence catabolism and anabolism of amino acids in *A. flavus* differently. A higher activity of carbohydrate and sucrose metabolism is found to be associated with up-regulated genes at



Gene up-regulated ≥ four-fold was the selection criterion for differential expression. Fisher's Exact Test with the Benjamini and Hochberg FDR (False Discovery Rate) correction at the significance threshold of 0.05 was used to obtain the adjusted *p*-values between the up-regulated test gene group and the reference gene group.

Figure 3: Functional categories of differentially expressed genes at 30°C and 37°C.

37°C, but the GO enrichment result, which is based on the highly selective criterion of FDR < 0.05, shows otherwise. Carbohydrate and sucrose metabolism ranks second in the whole-genome reference pathways (Table S1); thus the higher pathway activity observed in the differentially expressed gene population likely is insignificant. This may also be true for the pathway activities of purine metabolism and tryptophan metabolism, which rank first and seventh in the reference pathways (Tables 1 and S1), found at 37°C.

At each growth temperature, metabolically linked pathways are simultaneously influenced. For example, butanoate metabolism links to fatty acid degradation (see map at http://www.genome.jp/kegg/pathway/map/map00650.html). Propanoate metabolism links to degradation of valine, leucine, isoleucine and alanine (http://www.genome.jp/kegg-bin/show_pathway?map00640). These four pathways are actively operating at 30°C. Similarly, valine, leucine and isoleucine biosynthesis links to glycine, serine and threonine metabolism. Arginine and proline metabolism as well as histidine metabolism link to alanine, aspartate and glutamate metabolism. All of these pathways are actively operating at 37°C.

The largest class of volatile compounds produced by *A. parasiticus* grown at 30°C is derived from catabolic intermediates of leucine, isoleucine, and valine [22]. The pathway of valine, leucine and isoleucine degradation is active in *A. flavus* at 30°C, which suggests that degradation of these amino acids occurs preferably at 30°C in aspergilli. On the other hand, biosynthesis of these amino acids is active when *A. flavus* grows at 37°C (Table 1).

Aspergillus nidulans in response to hypoxia synthesizes *de novo* branched-chain amino acids of leucine, isoleucine, and valine [23]. Whether growth of *A. flavus* at suboptimal 37°C causes deprivation of adequate oxygen supply and results in hypoxic conditions is unclear. Phenylalanine metabolism (http://www.genome.jp/kegg/pathway/map/map00360.html) produces pyruvate, acetyl-CoA, fumarate, succinate, and succinyl-CoA. Tryptophan metabolism (http://www.genome.jp/kegg-bin/show_pathway?map00380+C000240) leads to acetyl-CoA production. Tyrosine metabolism (http://www.genome.jp/kegg/pathway/map/map00350.html) produces pyruvate, fumarate and succinate. These intermediates can enter the Tricarboxylic Acid (TCA) cycle (the citric acid/Krebs cycle) and may in part be involved in energy (ATP) production when *A. flavus* grows at 30°C.

Compared to those at 37°C the majority of pathways with higher activities at 30°C are involved in the production of acetyl-CoA. Acetyl-CoA has two principle fates: it either enters the TCA cycle to generate more ATP or it is used to synthesize new fatty acids. Many aspergilli contain high amounts of unsaturated fatty acids [18,24,25]. In *A. flavus* more than 70% of fatty acids in the total fatty acid content are unsaturated fatty acids, which are composed mainly of oleic acid (C18:1) and linoleic acid (C18:2) [18]. The observation that pathways of (saturated) fatty acid biosynthesis and degradation as well as biosynthesis of unsaturated fatty acids are active at 30°C may indicate the necessity of maintaining the high content of unsaturated fatty acids in *A. flavus* at the optimal

Table 1: Top ten KEGG pathways associated with differentially expressed or GO enriched genes.

KEGG metabolic pathways	Differentially expressed[a] #Seq/#Enz	GO enriched[b] #Seq/#Enz	Order of abundance[c]
30°C			
Tryptophan metabolism	25/14	25/14	1/1/7[d]
Fatty acid degradation	23/10	23/10	2/2/10
Butanoate metabolism	21/14	21/14	3/3/25
Valine, leucine and isoleucine degradation	20/14	20/14	4/4/32
Tyrosine metabolism	20/14	20/14	5/5/9
Phenylalanine metabolism	19/15	19/15	6/6/8
Fatty acid biosynthesis	18/5	18/5	7/7/35
Propanoate metabolism	18/11	18/11	8/8/33
Biosynthesis of unsaturated fatty acids	17/4	17/4	9/9/38
Metabolism of xenobiotics by cytochrome P450	17/6	17/6	10/10/19
37°C			
Starch and sucrose metabolism	9/10	--	1/--/2
Phenylalanine, tyrosine and tryptophan biosynthesis	9/15	5/12	2/7/44
Arginine and proline metabolism	8/11	6/9	3/1/5
Glycine, serine and threonine metabolism	8/4	6/3	4/2/3
Alanine, aspartate and glutamate metabolism	7/8	6/7	5/3/21
Purine metabolism	6/3	--	6/--/1
Tryptophan metabolism	6/5	--	7/--/7
Valine, leucine and isoleucine biosynthesis	5/3	5/3	8/4/61
Nitrogen metabolism	5/6	4/5	9/8/34
Pantothenate and CoA biosynthesis	5/3	5/3	10/5/43
Histidine metabolism	--	5/5	--/6/53
Butanoate metabolism	--	4/2	--/9/25
Phenylalanine metabolism	--	4/6	--/10/8

a: 849 annotated sequences at 30°C and 473 annotated sequences at 37°C were used in the KEGG pathway analysis.

b: 698 annotated sequences at 30°C and 158 annotated sequences at 37°C were used in the KEGG pathway analysis.

c: see Table S1 for ordered gene abundance for all 129 KEGG pathways of *A. flavus*.

d: order of abundance of differentially expressed genes/GO enriched genes/ whole genome gene reference.

30°C growth temperature. The finding that the main cellular component involved at 30°C growth is the fatty acid synthase complex also supports this notion. An increase in fatty acid unsaturation has been well known for many fungi at lower growth temperatures [25-27].

Of the annotated *A. flavus* NRRL3357 genes in the current genome database at NCBI, JCVI, and Broad Institute 5,613 encode either conserved hypothetical proteins or hypothetical proteins. Among these genes 1,115 (20%) were further mapped and annotated with GO terms in this study, which gives a final total of 8,987 annotated genes. Therefore, 67% (8,987/13,485) of the total *A. flavus* genes can be used in functional genomics studies. Although this number is far from complete, it is an intrinsic drawback of all current GO databases. However, the percentage of annotated genes in the *A. flavus* GO database is in the range of genes annotated with GO terms including those of rice, sweet potato, hazelnut and salamander [28-31]. Besides the genes encoding conserved and/or hypothetical proteins, an additional 1,025 novel *A. flavus* genes have been identified from assembled RNA-Seq transcript data [16]. For *A. oryzae*, which is closely related to *A. flavus*, 1,116 novel transcripts are also reported [32]. The actual gene number of *A. flavus* likely is greater than originally predicted. Future inclusion of these genes if annotated will substantially increase the fidelity of the results for *A. flavus* functional genomics studies.

Online construction of a GO database is time-consuming. The blast, mapping and annotation steps in this work took well over 550 CPU hours. When necessary the current database can be updated periodically as more information about those unannotated genes is obtained. This can be achieved by individual or collaborating research groups to divide the genes to be annotated into manageable portions for generating GO sub-databases. These sub-databases then can be merged into the current database by using the "import annotation file" function of Blast2GO as an updated annotation file. This would greatly reduce the processing time in comparison to constructing an entirely new GO database. Despite nucleotide sequence variations in orthologs of related *Aspergillus* species, such as *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. minisclerotigenes*, *A. arachidicola*, *A. pseudonomius*, *A. sojae*, and *A. oryzae* [33,34], the encoded proteins should yield the same GO terms. GO databases for these aspergilli can now be constructed based on the current *A. flavus* GO database. The *A. flavus* orthologs can first be filtered out by using the NCBI BLAST+ tool [35]; only genes unique to each species need to be further annotated as indicated above. In conclusion, the *A. flavus* GO database constructed in this study should help to expedite the progress of functional genomics of *A. flavus* and related aspergilli.

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Supplemental Material

Table S1: *Aspergillus flavus* KEGG Pathways.

***Aspergillus flavus* KEGG Pathways**	**#Seqs**	**#Enzs**
Purine metabolism	222	59
Starch and sucrose metabolism	138	37
Glycine, serine and threonine metabolism	116	30
Pyrimidine metabolism	98	31
Arginine and proline metabolism	85	39
Amino sugar and nucleotide sugar metabolism	83	28
Tryptophan metabolism	80	22
Phenylalanine metabolism	78	21
Tyrosine metabolism	77	17
Fatty acid degradation	75	11
Glycolysis / Gluconeogenesis	75	27
Aminobenzoate degradation	73	13
Glycerolipid metabolism	72	15
Pentose and glucuronate interconversions	70	20
Drug metabolism - cytochrome P450	68	5
Oxidative phosphorylation	67	10
Galactose metabolism	67	21
Pyruvate metabolism	65	30
Metabolism of xenobiotics by cytochrome P450	64	6
Cysteine and methionine metabolism	62	32
Alanine, aspartate and glutamate metabolism	61	25
Fructose and mannose metabolism	61	23
Thiamine metabolism	60	8
Glycerophospholipid metabolism	58	30
Butanoate metabolism	55	20
Methane metabolism	54	23
Pentose phosphate pathway	53	21
Glyoxylate and dicarboxylate metabolism	53	21
Drug metabolism - other enzymes	53	11
Aminoacyl-tRNA biosynthesis	53	23
Glutathione metabolism	51	17
Valine, leucine and isoleucine degradation	50	19
Propanoate metabolism	49	17
Nitrogen metabolism	49	18
Fatty acid biosynthesis	48	9
Carbon fixation pathways in prokaryotes	48	19
sphingolipid metabolism	46	13
Biosynthesis of unsaturated fatty acids	46	7
Phenylpropanoid biosynthesis	46	6
Lysine degradation	45	12
Riboflavin metabolism	44	12
Citrate cycle (TCA cycle)	42	20
Pantothenate and CoA biosynthesis	42	11
Phenylalanine, tyrosine and tryptophan biosynthesis	39	23
Carbon fixation in photosynthetic organisms	37	15
Retinol metabolism	37	2
Benzoate degradation	36	16
beta-Alanine metabolism	35	10
Arachidonic acid metabolism	35	7
Biotin metabolism	33	8
Cyanoamino acid metabolism	33	5
Steroid hormone biosynthesis	33	6
Histidine metabolism	32	11
Lysine biosynthesis	32	10
Naphthalene degradation	31	2
Chloroalkane and chloroalkene degradation	31	6
Inositol phosphate metabolism	28	17

Isoquinoline alkaloid biosynthesis	27	8
Steroid biosynthesis	27	11
Porphyrin and chlorophyll metabolism	26	16
Valine, leucine and isoleucine biosynthesis	25	8
Sulfur metabolism	25	14
Other glycan degradation	24	6
Terpenoid backbone biosynthesis	24	15
Phosphatidylinositol signaling system	23	12
Toluene degradation	23	6
alpha-Linolenic acid metabolism	23	6
Capriolactam degradation	22	5
Styrene degradation	22	7
Fatty acid elongation	21	7
N-Glycan biosynthesis	20	11
One carbon pool by folate	20	15
Linoleic acid metabolism	20	4
Nicotinate and nicotinamide metabolism	19	12
Geraniol degradation	19	4
Tropane, piperidine and pyridine alkaloid biosynthesis	19	5
Glycosaminoglycan degradation	17	3
Taurine and hypotaurine metabolism	17	6
Caffeine metabolism	17	3
Ubiquinone and other terpenoid-quinone biosynthesis	16	5
Various types of N-glycan biosynthesis	15	7
Glycosphingolipid biosynthesis - ganglio series	15	2
T cell receptor signaling pathway	14	1
Vitamin B6 metabolism	14	4
Streptomycin biosynthesis	14	8
Selenocopound metabolism	13	8
Ether lipid metabolism	13	8
Ascorbate and aldarate metabolism	13	7
Limonene and pinene degradation	13	2
Chlorocyclohexane and Chlorobenzene degradation	13	6
Folate biosynthesis	12	8
Novaobiocin biosynthesis	12	5
Synthesis and degradation of ketone bodies	11	5

Primary bile acid biosynthesis	11	3
C5-Branched dibasic acid metabolism	11	5
Dioxin degradation	11	2
Betalain biosynthesis	11	3
Aflatoxin biosynthesis	9	2
Polycyclic aromatic hydrocarbon degradation	9	1
Glycosphingolipid biosynthesis-globo series	8	2
Penicillin and cephalosporin biosynthesis	8	4
mTOR signaling pathway	8	2
Flouroenzoate degradation	8	3
Peptidoglycan biosynthesis	8	4
Steroid degradation	7	3
Tetracycline biosynthesis	6	1
Atrazine degradation	5	2
D-Alanine metabolism	5	2
D-Arginine and D-ornithine metabolism	5	1
Butirosin and neomycin biosynthesis	5	2
Glucosinolate biosynthesis	5	1
Phosphonate and phosphinate metabolism	4	5
D-Glutamine and D-glutamate metabolism	4	2
Flavonoid biosynthesis	4	2
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	4	1
Biosynthesis of ansamycins	4	1
Other types of O-glycan biosynthesis	3	1
Xylene degradation	3	2
Biosynthesis of terpenoids and steroids	3	1
Sesquiterpenoid and triterpenoid biosynthesis	3	3
Carotenoid biosynthesis	3	1
Polyketide sugar unit biosynthesis	3	2
Flavone and flavonol biosynthesis	2	1
Lipoic acid metabolism	2	2
Indole alkaloid biosynthesis	2	1
Biosynthesis of vancomycin group antibiotics	2	1
Biosynthesis of siderophore nonribosomal peptides	2	2
Bisphenol degradation	1	1
Ethylbenzene degradation	1	1