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Analysis of nutrient-dependent transcript variations in *Neurospora crassa*

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Abstract

Nutrient-dependent variations in transcript levels of the filamentous fungus *Neurospora crassa* were studied on a microarray containing some 4700 cDNAs. Cells were grown in minimal and acetate medium. The isolated RNA was analyzed in comparison to the results obtained upon the hybridization of samples prepared from the RNA of cells grown in full medium. Altogether, 160 cDNA clones exhibited significant variations, falling into five distinct subgroups of very similar transcription profiles. This is indicative of the occurrence of a high degree of co-regulation of genes in *N. crassa*. Especially the regulation of the expression of proteins involved in metabolic pathways was found to be strongly regulated at the RNA level.

Keywords: DNA-microarray analysis; Transcriptional profiling; Correspondence analysis; Acetate metabolism; *Neurospora crassa*

1. Introduction

Ever since Beadle and Tatum (1941) formulated their one-gene–one-enzyme hypothesis on the basis of studies with *Neurospora crassa*, this filamentous fungus served as a model organism not only in genetics but also many other fields of basic research. During recent years, *N. crassa* became an important tool for studying circadian rhythms (Bell-Pedersen, 2000), cell cycle progression, recombination, and multicellular development (Davis, 2000). Many genes were detected whose expression is under developmental, photo- or circadian control, resulting in fundamental contributions to the molecular genetics of fungal photobiology (Lauter, 1996).

Despite all this successful research, only about one-tenth of the estimated 13,000 genes of *N. crassa* had been described and localized on the seven chromosomes (Radford and Parish, 1997). Therefore, genome initiatives were established with the intention to decipher the genomic sequence as a whole as a first step towards a more comprehensive understanding of the fungus' biology. Genome analysis started by ordering cosmid and BAC clones along individual chromosomes (Aign et al., 2001; Kelkar et al., 2001). Based on the physical clone maps of linkage groups II and V, sequencing of the two chromosomes was done as part of the German *Neurospora* Genome Project (Mannhaupt et al., 2003; Schulte et al., 2002) (www.mips.biochem.mpg.de/proj/neurospora). Simultaneously, a whole-genome shotgun approach was taken at the Whitehead Genome Center, Cambridge, USA (www-genome.wi.mit.edu/annotation/fungi/neurospora/index.html), recently yielding the complete genomic sequence (Galagan et al., 2003).

For an initial insight into transcriptional variations in *N. crassa*, however, we started with the creation of a microarray prior to sequence assembly and annotation. Some 4700 EST-clones of a non-normalized cDNA library (Nelson et al., 1997) were arrayed on glass slides and used to monitor nutrient-dependent functional phenomena in *N. crassa*. Total RNA was extracted from mycelia grown under different nutrient conditions and analyzed on the microarrays. Correspondence cluster analysis was performed on the resulting data in order to identify genes associated with particular functions. Five subgroups of genes with similar transcript profiles were found. Particularly the expression of proteins involved in metabolic pathways was strongly regulated at the RNA level.

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2. Materials and methods

2.1. Microarray production

Production of and hybridization to cDNA microarrays were performed as described in detail earlier (Diehl et al., 2001, 2002a) (www.dkfz.de/funct_genome). A cDNA library with about 4700 clones was kindly provided by the *Neurospora* Genome Project at the University of New Mexico (Nelson et al., 1997). Amplification of the clone inserts was carried out in 384-well microtiter dish plates with forward primer d(TA ATACGACTCACTATAGGG) and reverse primer d(AATTAACCCTCACTAAAGGG). PCR was in 25 μl of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.25 mM $MgCl_2$, 0.2 mM of each nucleotide triphosphate, 1.5 mM betaine, 0.09 mM cresol red, 0.4 μM of each primer, and 0.1 μl Taq-polymerase. Inoculation with template was done by transferring clones from growth culture by means of 384-pin replicators. After an initial denaturation at 95 °C for 5 min, 36 amplification cycles were performed with 30 s at 95 °C, 30 s at 52 °C, and 2 min at 72 °C, followed by a final elongation step at 72 °C for 10 min. After loading 1 μl on agarose gels for a quality check, the DNA-concentration in each well was increased by an evaporation process. Because of the betaine, evaporation stops at a volume of about 12 μl. This material was used for spotting without further purification (Diehl et al., 2002a).

The preparation of poly-L-lysine coated glass microscope slides (25 × 75 mm) was done as described (Eisen and Brown, 1999). Spotting of the PCR products was carried out with a SDDC-2 Microarrayer (Engineering Services, Toronto, Canada) using SMP3 pins (TeleChem International, Sunnyvale, USA), the patch size being 220 μm. Each PCR product was spotted twice. As controls, PCR products of *Arabidopsis* cDNAs and phage lambda DNA-fragments were spotted. Subsequently, the slides were left at room temperature overnight. Due to the presence of cresol red in the DNA samples, the quality of the spotting process could be assessed by scanning the slides at an excitation wavelength of 594 nm; the cresol red was subsequently washed off completely during the blocking reaction. Prior to blocking with succinic anhydride, slides were dried on a metal block at 80 °C for at least 5 s.

2.2. Strains and culture conditions

Mycelium of *N. crassa* wild-type strain 74-OR23-1A was kindly provided by Ulrich Schulte (Heinrich-Heine-Universität, Düsseldorf, Germany). Three different culture conditions were chosen: growth was either in minimal medium (1× Vogels's medium (Vogel, 1964) supplemented with 2% sucrose) or in acetate-medium (1× Vogel's medium supplemented with 0.5% sodium acetate); full medium (1× Vogel's medium supplemented with 2% sucrose, 0.25% yeast extract, and 0.1% casein hydrolysate) was used as control. For inoculation, conidia from a single full-medium agar plate were taken up in water and filtered. Different amounts of this material were taken for the inoculation of 2-L flasks, each filled with 500 ml growth medium. One-fourth each was used for full and minimal medium, the remaining half for the flask with acetate medium. The cultures were grown shaking at 28 °C in light for 22 h. The three mycelia were harvested at the same time by filtration, weighed for calculating the yield, immediately frozen in liquid nitrogen and stored at −70 °C.

2.3. RNA extraction and labeling

The RNA extraction from frozen mycelium was done according to the detailed protocol of Sokolovsky et al. (1990). For each medium, several separate RNA isolations were done from one single culture. For labeling, 15 μg total RNA were mixed with 2.5 μg oligo$(dT)_{12–18}$ (Invitrogen GmbH, Karlsruhe, Germany) in 14.5 μl H_2O and incubated at 70 °C for 10 min. Subsequently, first-strand cDNA synthesis was performed in a final volume of 30 μl in the presence of 15 mM each of dATP, dCTP, and dGTP, 10 mM aminoallyl-dUTP (Sigma, Deisenhofen, Germany), 15 mM dTTP, 0.1 M DTT, and 380 U SuperScriptII (Invitrogen GmbH) in 1× reaction buffer of the same manufacturer. Incubation was at 42 °C for more than 3 h. The RNA was hydrolyzed by adding 10 μl of 0.5 M EDTA and 10 μl NaOH, followed by an incubation at 65 °C for 15 min. After neutralization with 25 μl of 1 M Hepes buffer, pH 7.5, the cDNA was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer except for using water in the elution step. For conjugation to the fluorescence dyes, the eluted cDNA was dried under vacuum, dissolved in 9 μl of 0.1 M $NaHCO_3$ and added to an aliquot of the monofunctional NHS-esters of Cy3 or Cy5 (Amersham Biosciences, Freiburg, Germany). Coupling was in the dark at room temperature for 1 h. Unbound dye was quenched by adding 4.5 μl of 4 M hydroxylamine followed by a 15 min incubation in the dark. The labeled sample was purified again with the QIAquick Purification Kit.

2.4. Hybridization and signal detection

Each hybridization sample was dried in a Speedvac centrifuge and dissolved in 25 μl hybridization buffer made of 5% dextran sulfate, 3× SSC (450 mM NaCl and 45 mM sodium citrate, pH 7.5), 1% SDS, 5× Denhardts solution, and 50% formamide. The sample was denatured at 80 °C for 10 min. Hybridization was done under a coverslip of 24 × 40 mm in a humidified hybridization chamber (TeleChem International, Sunnyvale, USA) at

42 °C for 16 h. Slides were then slightly agitated in 2× SSC and 0.1% SDS until the coverslip was swept away. Subsequently, they were washed in 1× SSC for 3 min and in 0.2× SSC for 30 s and dried immediately by centrifugation at 1800 rpm for 2 min. The fluorescence signals were detected using a ScanArray5000 confocal laser scanner (Packard BioChip Technologies, Billerica, USA). Quantification of the signal intensities was done with the GenePix Pro 3.0 software package (Axon Instruments, Union City, USA).

2.5. Data evaluation

Data quality assessment, normalization and correspondence cluster analysis were performed with the MIAME-compatible analysis and data warehouse software package M-CHiPS (Multi-Conditional Hybridization Intensity Processing System), in which currently more than 4000 hybridization experiments are stored (www.dkfz-heidelberg.de/tbi/services/mchips; Beissbarth et al., 2000; Fellenberg et al., 2001, 2002). Eight data points per gene and individual experimental condition (duplicate spots on each array, four hybridizations per sample) were accumulated. Signal intensities of repeated hybridizations were normalized and significance levels assessed by two stringency criteria. The highly stringent "min–max separation" is calculated by taking the minimum distance between all data points of two conditions. The less stringent criteria, called "standard deviation separation," is defined as the difference of the means of the two data sets diminished by 1 SD. In the tables, a color code indicates the two stringency measures. According to these criteria, data were classified as being of high, medium or no significance.

Correspondence Analysis is an explorative computational method for the study of associations between variables. Much like principle component analysis, it displays a low-dimensional projection of the data into a plane. It does it simultaneously for two variables, thus revealing associations between them. In the analysis of array-based transcript analysis (Fellenberg et al., 2001), the display of genes and experiments proved very valuable for biological data interpretation.

3. Results

To construct a microarray for initial transcriptional profiling analyses in *N. crassa*, some 4700 random cDNA clones were PCR-amplified using universal primers. According to the TIGR *N. crassa* Gene Index, about 70% of the cDNA clones contain unique ESTs (http://www.tigr.org/tdb/tgi/ncrgi). The PCR products were checked by agarose gel electrophoresis for amount and quality (Fig. 1). The average insert size was 1.5 kb. Amplification was successful with 96% of the cDNA clones yielding at least 1.5 μg DNA per reaction. After reducing the volume by evaporation, each PCR product had a concentration of at least 120 ng/μl, with the average concentration being 250 ng/μl. Spotting was performed by contact printing with split pins, depositing about 1 nl DNA per spotting event.

Mycelium of *N. crassa* was grown at three different conditions. While the growth rate was nearly the same for the cultures in full medium and minimal medium, resulting in 12.8 and 11.1 g filtered mycelia, respectively, the growth rate in acetate medium was quite low, resulting in only 5 g filtered mycelia, although it had been inoculated with twice as much conidia. Therefore, it cannot be excluded that some changes in the expression profiles might reflect not only different nutrient conditions but also different developmental states of the mycelia. Replicated RNA preparations were made and pooled prior to hybridization. RNA from mycelium that had been grown on full medium was defined as control. The samples were labeled with either Cy3 or Cy5 and analyzed in competitive hybridizations of condition versus control. Each hybridization was repeated four times. To prevent bias caused by preferential label incorporation into particular sequences, the dyes were swapped after two hybridizations. Because of the presence of duplicate spots on the microarrays, eight data points per gene and individual experimental condition were generated. Unspecific hybridization to non-homologous sequences did not occur, as could be seen on the spots generated with the control DNA-fragments. After scanning and quantification of the spot intensities, data were normalized and filtered. The complete data set can be downloaded from our webpage at www.dkfz.de/funct_genome. In total, 160 PCR fragments contained sequence that was significantly differentially transcribed.

The results were subjected to a correspondence cluster analysis (Fellenberg et al., 2001). One major advantage of this process is its ability to present the results of cluster analyses on different but corresponding factors in one plot. In Fig. 2, not only the gene clusters (black dots) but also the results of clustering the individual hybridizations (colored squares) are shown, which simultaneously represent the respective experimental conditions. Co-localization of genes and experiments/conditions is indicative for a strong association between them. In addition,

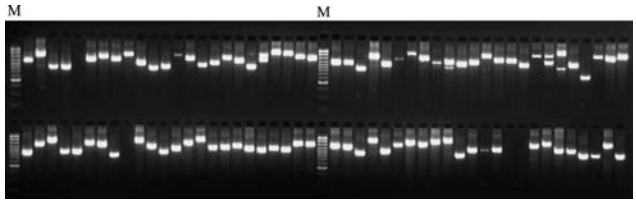

Fig. 1. Quality check by agarose gel electrophoresis of PCR-amplification. Typical results for 96 randomly selected cDNA clone inserts are shown. "M" designates the lanes with size markers.

Table 1
Identity of cDNAs that exhibited differential transcript levels

Clone #	Putative identity	Accession #	Fold change of transcript level relative to control (full medium)	
			Minimal medium	Acetate medium
Group I				
sc10c2	No homology		-4.2	-4.8
w6h2	No homology	AI398949	-2.8	-5.0
Group II				
nm3e10	Ncrassa grg1/ ccg1	X14801	-3.2	+1.1
nm4e11	No homology	AA898270	-4.9	+1.0
nm4h11	Halorhodopsin (light-induced chloride pump)	AI392478	-3.6	+1.0
nm6b7	No homology	AA901660	-4.5	-1.2
nm8b5	Subtilisin-like serine protease (Podospora anserina)	AACO3564	-4.3	+1.1
sc7d8	Extracellular matrix protein precursor (Fusarium oxysporum f. sp. lycopersici)	AAL47843	-3.8	-1.4
sm2c6	C. elegans C25D7.i gene product, chromosome III	AI392184	-4.1	-1.3
sm6e6	Ncrassa grg1/ ccg1	X14801	-3.8	+1.3
sm7a4	Ncrassa grg1/ ccg1	X14801	-4.1	+1.3
Group III				
nc2d2	No homology	AI397728	-1.3	-6.9
sc5b12	No homology	AI399009	-1.3	-5.2
sc6b4	No homology	BF072412	-1.1	-4.6
sc7b4	Hypothetical protein SPAC10F6.16, S. pombe	BF739665	-1.1	-4.6
sc7c6	No homology		-1.3	-8.3
sc8b3	Related to tropomyosin TPM1, N. crassa	CAD21417	-1.2	-3.9
Group IV				
nc1b7	No homology	AA901911	+9.3	+2.7
nc3g6	No homology	AA901910	+6.6	+1.6
nm7b10	No homology	AA901915	+4.3	+1.3
nm7h12	No homology	AA898678	+10.9	+2.3
sc1c6	nmt1 protein homolog - thiamine biosynthesis enzyme	AI392375	+14.2	+2.3
sc1h3	nmt1 protein homolog - thiamine biosynthesis enzyme	AI392532	+10.7	+2.1
sc2h11	nmt1 protein homolog - thiamine biosynthesis enzyme	AI392549	+12.3	+3.5
sc5f6	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399633	+3.8	+1.7
sc5g1	Stress-inducible protein ST135	AI397603	+9.8	+1.2
sc5h8	Translation elongation factor 2	AI416427	+5.8	-1.2
sc7f9	nmt1 protein homolog - thiamine biosynthesis enzyme	BF072529	+13.3	+3.0
sm3b10	N. crassa CyPBP37 /peptidylprolyl isomerase	AJ297565	+5.7	-1.3
sm3c9	nmt1 protein homolog - thiamine biosynthesis enzyme	BF072566	+18.7	+3.6
sm3g7	nmt1 protein homolog - thiamine biosynthesis enzyme	BF072643	+14.0	+2.6
sm4f8	nmt1 protein homolog - thiamine biosynthesis enzyme	BF072767	+12.0	+2.9
sm6g12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI392549	+14.3	+4.1
sp1f9	nmt1 protein homolog - thiamine biosynthesis enzyme	AI392287	+7.2	+1.6
sp3h4	Ncrassa CyPBP37 /peptidylprolyl isomerase	AJ297565	+8.5	+1.0
sp8h3	N. crassa CyPBP37 /peptidylprolyl isomerase	AJ297565	+7.9	-1.1
sp9a7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+8.6	+1.2
w10a3	60S ribosomal proteins (assorted)	AI399017	+11.1	+2.1
w10b8	Stress-inducible protein ST135	AI398503	+10.4	+1.2
w10b9	Transaldolase	AI398504	+7.3	+1.6
w10c9	60S ribosomal proteins (assorted)	AI398507	+5.2	+1.7
w10d3	No homology	AI399034	+4.8	-1.2
w10e11	Glyceraldehyde-3-phosphate dehydrogenase	AI399527	+7.1	+1.9
w10e2	ADP/ATP carrier protein, N. crassa	AI398885	+3.1	+1.4
w10g4	No homology	AI398902	+5.8	+2.1
w10h1	40.3 kD protein C17C9.12 in chromosome I, S. pombe	AI399263	+7.0	+1.2
w10h7	Ubiquinol-cytochrome C reductase complex subunit VIII	AI399120	+9.9	+1.3
w10h9	Glucokinase	AI399289	+12.7	+1.8
w13a4	No homology	AI398990	+9.1	+1.9
w13b6	60S ribosomal proteins (assorted)	AI398442	+7.7	+2.3
w13b8	No homology	AI399378	+6.0	+1.2
w13c6	Peroxisomal-like protein, Aspergillus fumigatus	AI398824	+8.6	+2.7
w13e1	Minor allergen Alt A VII, Alternaria alternata	AI398455	+10.0	+3.4
w13e12	No homology	AI398998	+5.4	+1.0
w13e4	Acyl carrier protein, mitochondrial precursor (ACP), N. crassa (NADH-ubiquinone oxidoreductase 9.6 KD subunit)	AI398454	+3.9	+2.1
w13e9	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398578	+8.3	+2.5
w13f8	ATP synthase, subunit 9	AI399001	+6.6	+1.5
w13h4	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398578	+9.4	+2.5
w13h9	nmt1 protein homolog - thiamine biosynthesis enzyme	AI397535	+3.4	+1.3

Table 1 (*continued*)

Clone #	Putative identity	Accession #	Fold change of transcript level relative to control (full medium)	
			Minimal medium	Acetate medium
w1b4	nmt1 protein homolog – thiamine biosynthesis enzyme	AI398512	+8.5	+1.9
w1d12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398520	+4.1	-1.3
w1e12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399073	+7.7	+2.9
w1e2	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398525	+10.1	+2.7
w1e6	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399241	+9.9	+3.5
w1f12	Stress-inducible protein ST135	AI398574	+4.9	+1.1
w1g2	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398576	+9.2	+2.1
w1g5	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398578	+8.2	+2.1
w1h7	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398589	+3.8	+1.1
w6b10	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399314	+8.0	+2.8
w6d3	nmt1 protein homolog – thiamine biosynthesis enzyme	AI398910	+8.5	+1.6
w6e7	No homology	AI399322	+6.7	+3.3
w6e9	Stress-inducible protein ST135	AI398924	+2.6	+1.2
w6f12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398938	+5.9	+1.4
w6f7	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399212	+7.4	+2.0
w6f8	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399213	+5.4	+1.5
w6h10	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399220	+9.0	+2.4
w7a6	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398533	+4.6	+1.6
w7b8	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398540	+5.4	+1.8
w7d1	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398810	+7.5	+2.0
w7d12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398815	+7.0	+1.1
w7d5	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398548	+3.9	+1.7
w7d9	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398642	+3.4	+1.7
w7g3	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398645	+7.0	+1.9
w7h1	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398720	+6.9	+2.2
w8a10	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398808	+6.1	+2.4
w8a2	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398549	+7.4	+2.5
w8a5	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398550	+7.2	+1.8
w8b11	Glyceraldehyde-3-phosphate dehydrogenase	AI398835	+4.9	+1.3
w8b3	Stress-inducible protein ST135	AI398663	+6.1	+1.1
w8c10	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398808	+4.5	+2.1
w8d9	Probable ATP-dependent RNA-Helicase DBP5 (Helicase CA5/6)	AI398873	+8.3	+1.8
w8f11	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398692	+11.5	+2.7
w8h1	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398703	+12.2	+2.7
w8h2	Histidine-3 protein, N. crassa	AI398704	+9.6	+2.8
w8h4	Stress-inducible protein ST135	AI398472	+4.5	+1.4
w8h8	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398708	+8.7	+2.2
w9a10	No homology		+12.3	+3.2
w9b1	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398766	+7.8	+1.1
w9b12	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399334	+9.2	+3.1
w9c3	No homology	AI399353	+3.5	+1.5
w9c5	nmt1 protein homolog - thiamine biosynthesis enzyme	AY007661	+3.8	+1.5
w9e12	No homology	AI397526	+8.4	+2.0
w9e5	Glyceraldehyde-3-phosphate dehydrogenase	U56397	+3.1	+1.1
w9e7	nmt1 protein homolog - thiamine biosynthesis enzyme	AI397522	+8.2	+2.2
w9f4	nmt1 protein homolog - thiamine biosynthesis enzyme	AI399337	+7.0	+2.2
w9g11	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398975	+3.6	+1.3

Table 1 (*continued*)

w9h9	nmt1 protein homolog - thiamine biosynthesis enzyme	AI398988	+7.8	+1.8
Group V				
nc1e6	No homology	AA901617	+8.4	+35.2
nc2c2	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392004	+8.5	+26.8
nc3a11	No homology	AA898660	+1.2	+5.8
nc3e4	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392088	+12.3	+42.6
nc3e6	No homology	AI397804	+3.5	+17.8
nc3g3	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+14.7	+31.5
nc5d7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392042	+13.8	+40.7
nc5e3	No homology	AI397785	+3.1	+7.1
nc5g2	Acetolactate synthase, small subunit homolog precursor	AI392061	+2.1	+9.2
nc5g7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392064	+11.3	+36.8
nc5h1	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392068	+7.2	+23.2
nc5h3	ccg-6, putative polypeptide, N. crassa	AI392069	+1.1	+4.8
np1h1	No homology		+8.1	+49.3
np2b10	No homology	AA898959	+2.0	+6.4
sc10c5	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+18.3	+41.1
sc10d10	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+19.9	+39.4
sc10d12	No homology		+6.0	+24.4
sc10e2	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+15.7	+35.0
sc10e4	No homology		+4.5	+18.6
sc1a2	Cyclophilin A, N. crassa, translation elongation factor 2	AI392522	+5.3	+14.2
sc1a6	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392524	+22.1	+73.5
sc1a7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392485	+28.3	+86.6
sc1a8	No homology	AI399383	+2.2	+3.5
sc1b10	No homology	AI398298	+2.2	+8.1
sc1c11	No homology	AI398326	+14.3	+96.8
sc1e11	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392487	+16.9	+41.0
sc2a2	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392361	+12.6	+31.7
sc2c11	No homology		+4.6	+25.7
sc2h3	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392426	+10.3	+29.7
sc2h8	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI392426	+13.9	+29.2
sc3c3	No homology	AI398340	+20.2	+68.9
sc3d1	ORF YGL141w, hypothetical 105.6 kD protein in MRF1-SEC27 intergenic region	AI392495	+2.6	+16.2
sc4a7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+20.7	+52.9
sc4c7	No homology		+18.9	+46.2
sc4d1	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+10.7	+38.8
sc4d3	No homology		+2.6	+8.2
sc4f2	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+12.8	+31.1
sc4f4	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+2.6	+3.5
sc4f7	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+13.3	+37.9
sc4f9	Trichoderma harzianum 14.3.3 protein / Hypocrea jecorina fft1 gene for 14.3.3 prot	U24158	+3.7	+9.7
sc4h10	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+17.3	+34.7
sc5f2	Pyruvate decarboxylase (cfp gene product)	AI397597	+8.5	+23.7
sc5h1	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	AI416425	+19.8	+57.1
sc5h3	Pyruvate decarboxylase (cfp gene product)	AI397668	+3.6	+6.8
sc7e8	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	BF072518	+9.3	+18.8
sc7f2	No homology	BF072523	+1.9	+9.2
sc8d10	60S ribosomal proteins (assorted)	AI399017	+3.4	+11.3
sc8d3	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+13.9	+32.1
sc8d5	No homology		+2.5	+8.1
sc8d8	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	BF072518	+7.3	+11.1
sc8h11	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+18.3	+53.6
sc9a6	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	BF072518	+12.7	+21.3
sm6b10	ccg-2 (hydrophobin precursor; rodlet protein; blue light induced protein 7)	X67339	+21.2	+40.1

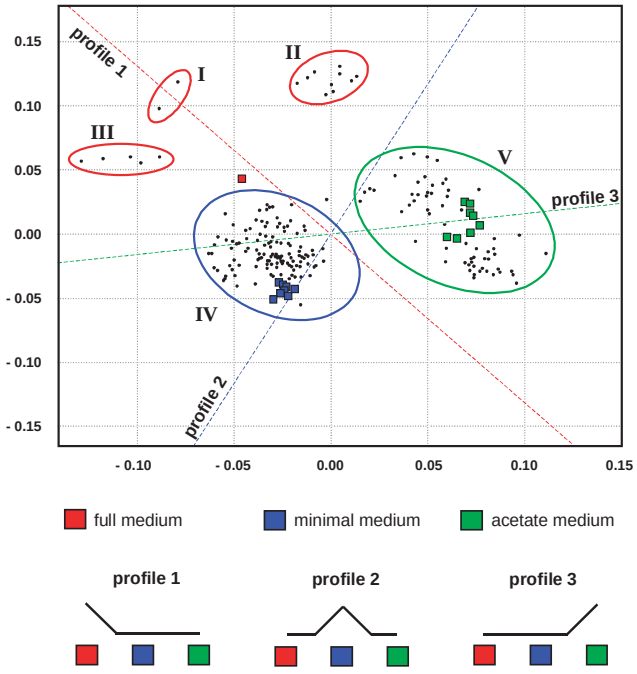

Fig. 2. Correspondence cluster analysis. In the resulting biplot, each hybridization of an individual condition is depicted as a colored square, cDNAs as a black dot. Only such cDNAs are shown, which exhibited significant differential transcription levels. As a consequence of the normalization process, only the median of all control hybridizations is shown in the diagram as a single red square instead of the individual hybridization events. Three guiding lines are displayed in the diagram. They correspond to the transcription profiles of virtual genes, which exhibit a signal in one condition only. Their transcription profiles are represented below the biplot. The closer a gene lies to one of these guidelines, the better its expression is described by the related ideal profile.

virtual transcription profiles were calculated on the basis of the results and projected into the plot.

In the experiment, hybridizations with material from minimal medium (blue squares) and acetate medium (green squares) are clearly separated and also distinct from the full medium controls. In contrast, the individual hybridization events within each condition series are positioned close to each other, indicating a high degree of experimental reproducibility.

The differentially transcribed genes are clustered in five distinct groups (I–V), highlighted by encirclements. The red-bordered groups I, II, and III contain 2, 9, and 6 cDNAs, respectively, which exhibited a higher signal intensity—thus RNA level—when mycelium was cultured in full medium. Apart from genes with no homology to known genes, these groups include genes that take part in the building of the cell structure, like tropomyosin or an

The color-code indicates the significance of the variation. A red or blue label marks an up-regulation or down-regulation, respectively, that is significant according to the highly stringent "min–max separation" criterion. Data shown in yellow and light blue represent a significant up- or down-regulation, respectively, according to the less stringent "standard deviation separation."

extracellular matrix protein precursor, or genes that participate in the cell signaling, such as halorhodopsin. The cDNAs in group I are located relatively close to the line of virtual profile 1, indicating that their respective profiles are of a very similar nature. In contrast, the cDNAs in group II are located right between the lines of profiles 1 and 2. Compared to the cluster of the minimal medium experiments (blue squares), the group II cDNAs are also positioned much more opposite to the centroid, than group I cDNAs. This indicates that in addition to up-regulated RNA levels in full medium cells, the respective genes are strongly down-regulated in minimal medium. The same is true for the genes of group III with respect to acetate medium.

The 90 cDNAs represented in group IV are mainly up-regulated in minimal medium, in part to a rather low degree, while down-regulated in acetate medium. In this group, the majority of the genes encode metabolic enzymes, such as glucokinase, glyceraldehyde-3-phosphate dehydrogenase or transaldolase, for example. Also included are the genes of the 60S ribosomal protein and translation elongation, both participating in protein biosynthesis. Group V, encircled by a green line, finally, consists of 53 cDNAs, which are up-regulated in acetate medium but not in minimal or full medium. In this group are the transcripts of *ccg-2* and *ccg-6*, two genes that are involved in the circadian rhythm, and also the acetolactate synthase gene, which is part of the amino acid biosynthesis pathway. The expression of these genes is very roughly described by the virtual transcription profile 3, although they could be divided into two subgroups at either side of the profile line.

All 160 cDNAs, on which significantly differential transcription of the related genes could be observed, were sequenced. Thirty-eight clones did not show in BLAST analyses any apparent homology to other sequences. The remaining 122 cDNAs represent genes of known function. Because of the redundancy in the cDNA library, they actually represent only 31 different genes. All these data are listed in Table 1. A color-code indicates the signifi-

Table 2
List of known genes with varying transcript levels

Gene	Biochemical process	Transcript level FM	MM	AM
Hypothetical protein SPAC10F6.16, *S. pombe*	Unclassified			
Subtilisin-like serine protease (*Podospora anserina*)	Protein synthesis			
Extracellular matrix protein precursor (*Fusarium oxysporum* f. sp. lycopersici)	Cell structure: extracellular matrix			
Related to tropomyosin TPM1, *N. crassa*	Cell structure: cytosceleton			
Halorhodopsin (light-induced chloride pump)	Cell signaling: transport/channel protein			
N. crassa grg1/ccg1	Circadian rhythm			
C. elegans C25D7.i gene product, chromosome III	Unclassified			
Hypocrea jecorina fft1 gene for 14.3.3 prot	Intracellular signaling cascade			
Acetolactate synthase, small subunit homolog precursor	Amino acid biosynthesis			
ccg-2 (hydrophobin precursor; rodlet protein; blue light-inducedprotein 7)	Circadian rhythm			
ccg-6, putative polypeptide, *N. crassa*	Circadian rhythm			
Cyclophilin A, N. crassa, translation elongation factor 2	Stress response			
ORF YGL141w, hypothetical 105.6-kDa protein in MRF1-SEC27 intergenic region	Unclassified			
Pyruvate decarboxylase (cfp gene product)	Carbohydrate metabolism			
nmt1 protein homolog—thiamine biosynthesis enzyme	Thiamine biosynthesis			
Acyl carrier protein, mitochondrial precursor (ACP)	Oxidative phosphorylation			
ADP/ATP carrier protein, *N. crassa*	Oxidative phosphorylation			
ATP synthase, subunit9	Oxidative phosphorylation			
Ubiquinol–cytochrome *c* reductase complex subunit VIII	Oxidative phosphorylation			
Glucokinase	Glycolysis			
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis			
Transaldolase	Pentose-phosphate shunt			
Histidine-3 protein, *N. crassa*	Histidine biosynthesis			
Probable ATP-dependent RNA-helicase DBP5 (Helicase CA5/6)	Nucleic acid metabolism			
Translation elongation factor 2	Protein biosynthesis			
Ncrassa CyPBP37/peptidylprolyl isomerase	Regulation of mitosis			
Stress-inducible protein ST135	Stress response			
60S ribosomal proteins (assorted)	Protein biosynthesis			
40.3 kDa protein C17C9.12 in chromosome I, *S. pombe*	Unclassified			
Minor allergen Alt A VII, *Alternaria alternata*	Unclassified			
Peroxisomal-like protein, *Aspergillus fumigatus*	Unclassified			

cance of the variation in transcript levels: red and dark-blue indicate data that is significant according to the highly stringent "min–max separation" criterion, while yellow and light-blue represent significance according to the less stringent "standard deviation separation" parameter (see Section 2). A summary of the differentially expressed genes is given in Table 2.

4. Discussion

As an initial step for the comprehensive analyses on the genome of *N. crassa*, we used some 4700 EST clones from a cDNA library to construct a microarray. For our studies, RNA from mycelium grown under three different nutrient conditions was analyzed. *N. crassa* has a rich history in biochemical genetics of carbon utilization and is known to adapt to many carbon sources (Davis, 2000). The minimal medium contained only sucrose as carbon source, while sodium acetate is acting as such in the acetate medium. In contrast, the full medium contained not only sucrose but also casein hydrolysate and yeast extract. It was used as control, to which the cells grown in minimal medium and acetate medium were compared. In the analysis, there was one major observation. Quite a few genes were up-regulated in minimal or acetate medium but not in full medium, while only few genes exhibited the opposite behavior. This observation is in accordance to results seen in yeast. ter Linde et al. (1999) described for glucose-limited cultures of *Saccharomyces cerevisiae* a higher number of transcripts at a detectable level than in glucose-rich cultures. This may be due to the decline of glucose catabolite repression as a result of the low glucose concentration in the minimal and acetate medium (de Jong-Gubbels et al., 1999). Out of the characterized genes (Table 2), one-third of the genes that were up-regulated in minimal or acetate medium are concerned with metabolism. Apparently, *N. crassa* activates more resources to metabolism when cultured in a poor carbon source. From studies in *Escherichia coli* (Oh et al., 2002), it is known that growing on acetate leads to an induction of the transcription of metabolic genes. Because acetate acts as a carbon source to *N. crassa*, although of poor quality, the up-regulation of the metabolic genes occurs at a lower degree when the cells were growing on acetate rather than minimal medium.

Among the genes that are mainly induced by growth on minimal medium (group IV), not only the genes participating in metabolism are very eye-catching, but also the gene for NMT1, a protein that takes part in the biosynthesis of thiamine. As shown in detail earlier (Hansen et al., 1998; Maundrell, 1990), the expression of *nmt1* (= no message in thiamine) is completely suppressed by thiamine (vitamin B_1). As the full medium contains yeast-extract and is therefore rich in vitamin B_1, *nmt1* is not transcribed at this growth condition. Neither minimal medium nor acetate medium contained vitamin B_1, leading to an induced expression of *nmt1*. The high copy number (see Table 1) of *nmt1* in the cDNA library can also be attributed to this induction, because minimal medium was used for the production of the library (Nelson et al., 1997). In addition, the induction of *his-3*, a gene coding for the HIS-3-protein complex was observed. It was even higher in minimal medium than in acetate medium, again an indication for the overall lower cell activity in the poor carbon source acetate. The gene *his-3* takes part in the histidine biosynthesis, and earlier studies with Northern blots (Legerton and Yanofsky, 1985) have shown that the transcript level of *his-3* is strongly induced, when the mycelium was cultured in a medium completely lacking histidine. Studies on the phytopathogenic fungus *Fusarium* spp. have shown an up-regulation of the stress-inducible gene *sti35* after treatment with alcohol, different chemicals or heat (Choi et al., 1990). This up-regulation is also seen in mycelia of *N. crassa*, when changing growth conditions from full to minimal medium. Growing on minimal medium is a stressful situation for the fungus, because of the starvation of amino acids and other nutrients. This leads to the elevation of the transcript level of *sti35*. A similar effect was also observed in yeast (Wodicka et al., 1997). We did not see an induction of *sti35*, when growth took place on acetate medium, however. The detailed mechanism of the stress-response with *sti35* is yet unknown.

The transcript level of cyclophilin A is 14-fold elevated in acetate medium compared to full medium, and increased by a factor of five in minimal medium. Cyclophilin A is known to be induced as a stress-response (Joseph et al., 1999). Again, this induction indicates the stress put upon *N. crassa* when growing in minimal or acetate medium. Interestingly, the glucose-repressible gene *grg-1* was down-regulated in minimal medium while identical RNA levels were found in full and acetate medium.

One of the genes from group V that are mainly induced by growing in acetate medium is *ccg-2*, which encodes for a rodlet protein (Lauter et al., 1992). This protein is part of the surface of the spores of *N. crassa*. It is also known as *bli-7* (blue light induced), and is not only inducible by blue light but also by glucose and nitrogen starvation (Eberle and Russo, 1992). According to the studies of Turian and Bianchi (1972), conidiation is induced upon growth in liquid culture with a poor carbon source, hence explaining the induction of the conidiation genes *ccg-2* and *ccg-6*. The 14.3.3 protein, whose expression was also up-regulated in acetate medium, participates in a nutrient sensing pathway, shown in *A. thaliana* (Cotelle et al., 2000). Growing on media having acetate as a sole carbon source leads to an induction of the synthesis of the glyoxylate shunt

enzymes and others. Unfortunately, some of these were missing on our microarray. Few others showed variations that were not considered significant after passing through our filtering measures. A global analysis clearly awaits the arrival of a comprehensive microarray with all relevant genes and possibly more refined means of analysis (Townsend and Hartl, 2002). The existence of many acetate-requiring mutants (*ace*) (Kuwana and Okumura, 1979; Santosa and Kuwana, 1992; Strauss, 1957) as well as mutants unable to use acetate (*acu*) (Flavell and Fincham, 1968a,b, Flavell and Woodward, 1971) will facilitate further studies. Overall, the present analysis provides an initial view at the degree of variation of RNA levels in *N. crassa*. Since genomic sequencing and the initial sequence annotation are complete by now, the production of a DNA-microarray containing all genes will be pursued. Preferably, this microarray should be made of a minimal tiling path of the genomic shotgun clones used for sequencing, then containing by definition all open reading frames, irrespective of the quality of the sequence annotation. Similar to work on other organisms (Diehl et al., 2002b; Stjepandic et al., 2002), such an array will not only provide a tool for transcriptional studies on all genes of *N. crassa* but additionally permit analyses on the non-coding portions of the genome.

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