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Application of cDNA array for studying the gene expression profile of mature appressoria of *Magnaporthe grisea**

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Abstract: Appressorium is an infection structure of the phytopathogenic fungus *Magnaporthe grisea*. Analysis of gene expression profiles of appressorium development provides insight into the molecular basis of pathogenicity and control of this fungal plant disease. A cDNA array representing 2927 unique genes based on a large EST (expressed sequence tag) database of *M. grisea* strain Y34 was constructed and used to profile the gene expression patterns at mycelium and appressorium maturation stages. Compared with mycelia, 55 up-regulated and 22 down-regulated genes were identified in mature appressoria. Among 77 genes, 16 genes showed no similarity to the genome sequences of *M. grisea*. A novel homologue of peptidyl-prolyl cis-trans isomerase was found to be expressed at low-level in mature appressoria of *M. grisea*. The results indicated that the genes such as pyruvate carboxylase, phospholipid metabolism-related protein and glyceraldehyde 3-phosphate dehydrogenase involved in gluconeogenesis, lipid metabolism and glycolysis, showed differential expression in mature appressoria. Furthermore, genes such as *PTH11*, beta subunit of G protein and *SGT1* involved in cell signalling, were expressed differentially in mature appressoria. Northern blot analysis was used to confirm the cDNA array results.

Key words: *Magnaporthe grisea*, Mature appressoria, cDNA array, Gene expression profile
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INTRODUCTION

The rice blast pathosystem has emerged as a model system for studying plant-fungal interactions, because of one of attributes of the pathogen *Magnaporthe grisea* evolving an elaborate mechanism to penetrate host plant cells through a specialized infection structure known as appressorium, which can form on the hydrophobic surface of the host cells (Dean *et al*., 2005; Valent, 1990). Appressorium is also induced by hydrophobic signals in vitro 2 to 8 h after conidium germination, followed by maturation in 16 h (Lee and Dean, 1994; Talbot, 1995). Researches of individual genes on molecular mechanisms of initiation, formation and maturation of appressorium have been carried out one by one, and more than 30 related genes have been cloned (Talbot, 2003). Recently, serial analysis of gene expression (SAGE) (Irie *et al*., 2003), cDNA array (Takano *et al*., 2003), suppression subtractive hybridization (Lu *et al*., 2005), and expressed sequence tag (EST) (Ebbole *et al*., 2004; Jantasuriyarat *et al*., 2005) techniques have been adopted to study the molecular mechanism of appressorium development and pathogenesis at the systemic biology level, resulting in identification of many genes differentially expressed in appressorium development of *M. grisea*. Maturation of appressorium after its morphogenesis is a long and complex biological process. During this development stage

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turgor in appressorium generates and accumulates, which affects differentiation of penetration pegs and pathogenicity of the pathogen (Talbot, 2003; Thines *et al*., 2000). The molecular mechanism of appressorium maturation during turgor accumulation in the aspect of system biology is not well studied. In this study, we used an *M. grisea* cDNA array to detect gene expression profiles of mycelia and mature appressoria (20 h after conidium germination). Seventy-seven differentially expressed genes that are related to appressorium maturation were identified. Our data provide insight into appressorium development, as well as the molecular mechanisms mediating *M. grisea* development and pathogenesis.

MATERIALS AND METHODS

M. grisea culture and total RNA extraction

The strain of *M. grisea* Y34 used in this study was isolated from Yunnan Province of China (Zheng *et al*., 1998). The fungus was grown in oatmeal agar medium (juice after 50 g oatmeal was boiled, 15 g agar, 1 L water) for 10 d (28 °C, 12/12 h alternating day and night), and conidia suspension filtered once through Miracloth (Calbiochem, USA) was used for the following different purposes.

For vegetative mycelia, conidia suspension was cultured in liquid complete media (CM) (Talbot *et al*., 1993) for 72 h at 28 °C with a rotary shaking at 150 r/min for RNA extraction. Mycelia were collected from three independent experiments.

For appressorium formation, about 50 μl of conidia suspension (10^5 conidia/ml) was placed on transparent films (Gaoke, China) and kept for induction of appressoria at 28 °C in humid box. Twenty hours after inoculation, fluid droplets on the films were removed, the films adhering to appressoria were quickly soaked in liquid nitrogen, and appressoria were scraped with a blade off for RNA extractions from the frozen films. Appressoria was collected from three independent experiments.

The total RNA from mycelia and appressoria of *M. grisea* was extracted immediately with Trizol™ reagent (Gibco-BRL, USA), according to the manufacturer's instructions. Contaminating genomic DNA was removed by treatment with RNase-free DNase (Promega, USA).

Preparation of cDNA arrays

We constructed a cDNA library of *M. grisea* Y34 prepared from mixed mRNA at mycelia, conidia, and different germlings of appressorium development process. From the cDNA library a total 13057 ESTs were acquired, which assembled 4756 unique ESTs. All EST and unique EST sequences can be found at our laboratory database (www.estarray.org). For the preparation of cDNA array, 4756 unique clones were amplified by PCR using universal M_{13} primers (5′-CCCAGTCACGACGTTGTAAAACG-3′ and 5′-AGCGGATAACAATTTCACACAGG-3′). The clones that did not amplify or that produced nonspecific PCR products, as determined by gel electrophoresis were discarded, while other 2927 cDNA samples were selected for array printing. The PCR products precipitated by isopropanol were resuspended in 15 μl of a denatured solution containing 0.4 mol/L NaOH and 10 mmol/L EDTA until they were printed on the arrays. The arrays were printed on Immobilon™-Ny+transfer membranes (Millipore, USA) using a GeneTAC™ G3 arrayer (Genomic Solutions, USA). Every cDNA sample was duplicated twice in each array. Additionally, several control clones from ribosomal RNA were also included.

Probe preparation and hybridization

Hybridization probes were prepared respectively from total RNA samples isolated from mycelia and mature appressoria. The RNA samples were labelled with α-^{33}P-dCTP during the first-strand reverse transcription reactions. A typical labelling reaction contained 20 mmol/L each of dATP, dTTP and dGTP, 10 mmol/L dCTP and 5 μl of α-^{33}P-dCTP (10 mCi/ml, Amersham, USA), 10 μl of 5×First-Strand Buffer, 0.5 mmol/L DTT and 400 units SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen, USA), 0.3 μg oligo(dT)$_{16}$ primer, 0.1 μg random hexamer primer and 5 μg of total RNA in a reaction volume of 50 μl. The reaction proceeded at 42 °C for 2 h, and the products were purified using a Sephadex G-50 column (Amersham, USA).

cDNA array was irradiated (60 mJ/cm^2) by UV crosslinker (UVP Inc., USA) before hybridization. The hybridizations were performed overnight at 60 °C in a hybridization oven (ThermoHybaid, UK). Subsequently, the nylon membranes were washed twice at 60 °C in 2×SSC and 0.1% (w/v) SDS for 20

min and once 60 °C in 0.1×SSC and 0.1% (w/v) SDS for 20 min. Three individual hybridization experiments were performed for each sample.

cDNA array analysis

The signals of the array were absorbed by storage phosphor screen (Kodak, Japan) for 3 d, and signal intensities were measured by scanning the storage phosphor screen with Typhoon 9200 PhosphorImager (Amersham, USA). The signal intensity of each cDNA spot on the array was quantified using software ArrayVision 6.0 (Imaging Research, Canada). With this program, the normalization between different cDNA arrays was performed by using a spot's signal intensity to divide the average signal intensity of the whole image (global normalization). Total 6 nARVOL (normalized AR volume) values were acquired for each gene per sample. The regularized *t*-test approach in the Cyber-T program (http://visitor.ics.uci.edu/genex/cybert/) was used to determine gene expression changes between two samples. All cDNA expression data are available at http://www.estarray.org/array/default.asp.

Quality control

In order to test whether PCR products of the genes on the cDNA array were right, 192 PCR products were ligated into pGEM®-T Easy vector (Promega, USA) and transformed into *Escherichia coli* DH5α cells by electroporation using a Gene Pulser Xcell™ System (Bio-Rad, USA) according to the manufacturer's instructions.

Individual recombinant colonies were inoculated into 1 ml of 2×YT (yeast extract-tryptone) liquid medium (16 g tryptone, 10 g yeast extract, 5 g NaCl in one liter) in the single wells of 96-well micro plates, and centrifuged for 15 h at 200 r/min. Plasmid DNA was extracted following the protocol of plasmid DNA purification kits (Millipore, USA). We sequenced these 192 cDNA clones from 5' and 3' end on a MegaBACE™ 1000 automated sequencer (Molecular Dynamics, USA) using standard M_{13} primers (5′-CCCAGTCACGACGTTGTAAAACG-3′ and 5′-AGCGGATAACAATTTCACACAGG-3′) and the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham, USA).

Sequence alignment was carried out with standalone BLAST against the above *M. grisea* EST sequence database. Description of preparation, analysis and quality control of cDNA array can be found in the published papers of our laboratory (Deng *et al*., 2006; Dong *et al*., 2005; Guo *et al*., 2005).

Northern blot hybridization

About 5 μg of total RNA from the above mycelia and mature appressoria was separated on a 1% (w/v) denaturing formaldehyde gel and then transferred to a nylon membrane (Millipore, USA) according to the method in (Sambrook and Russell, 2001). Candidate genes as probes came from differentially expressed genes by cDNA array analysis. Probes were labelled through PCR amplification with α-^{33}P-dCTP (Amersham, USA) using Prime-a-gene® Labelling System (Promega, USA). Hybridization methods and corresponding buffer were the same to method in (Sambrook and Russell, 2001). The hybridization signals on the membranes were absorbed by storage phosphor screen (Kodak, Japan) for 3 d, and signal intensities were measured by Typhoon 9200 PhosphorImager (Amersham, USA).

RESULTS

Appressorium induction of *M. grisea*

At 20 h after conidia inoculation on hydrophobic surfaces, more than 95% conidia germinated and formed seal brown mature appressoria, with appressorium pores being seen clearly (Fig.1). Total RNA samples from mycelia and mature appressoria were used in probe preparation for hybridization of cDNA array.

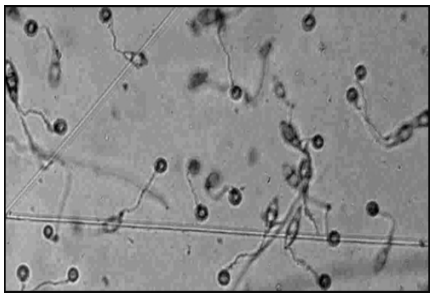

Fig.1 Appressoria of *M. grisea* Y34 on a hydrophobic surface inoculated for 20 h (16×20)

Analysis of cDNA array hybridization results

The cDNA array containing 2927 unique genes

of *M. grisea* was based on a large collection of the fungal ESTs. For cDNA array construction, PCR amplified inserts of the unique genes were spotted. In addition, a collection of control clones from ribosomal RNA were included in the cDNA array. The identity of 192 randomly chosen DNA clones from the unique genes was confirmed by resequencing. In all cases, the sequences of the clones plated on the cDNA array were identical to the original EST sequence. This confirmation increases confidence in the identity of clones spotted on the cDNA array.

In order to maximize the statistical reliability of the data, different biological and technical replicates were carried out as follows. Three biological replicates were carried out with new tissues grown under the conditions described above. In addition, for each biological replicate, two technical replicates (each PCR fragment was arrayed twice on each nylon membrane) were used for hybridization. The probe made from mature appressoria was hybridized with the cDNA array for three times and the coefficient of determination (R^2) was 0.96, 0.96 and 0.99. The same procedure was done using a probe made from mycelia, and R^2 was 0.94, 0.94 and 0.99. Above results indicated that repeat experiments on cDNA array hybridization were good and that the array data could be used for further analysis.

Fig.2 shows scanning images of the cDNA array after hybridization with isotopic nucleotide labelled probes. Images of the cDNA array were quantified using the software ArrayVision 6.0. Scatter plots of the transcript abundance of 2927 unique genes from mycelia and mature appressoria are shown in Fig.3. Signal values of most scatter dots were less than 1 [log(transformed value)<0] in mycelia and mature appressoria, which indicated the transcript abundance of most genes at both stages was low. Most of the gene dots lied close to the diagonal line, showing that the transcript abundance of these genes in mycelia and mature appressoria was similar. Some gene dots lied far from the diagonal line, indicating these genes were differentially expressed between two stages; the farther from the diagonal line the dots were, the bigger the difference in expression level.

Differentially expressed genes in mature appressoria

The Cyber-T program calculates the fold change and significance of the difference in expression. In order to maximize the screening reliability of differentially expressed genes, genes that met the following three criteria were identified as differentially expressed genes: (1) confidence level of $P<0.01$, (2) fold change cutoff of the signal value being more than 3, (3) larger signal value of gene with at least one of any two stages being larger than 0.2 (nARVOL). According to these criteria, 77 differentially expressed genes were

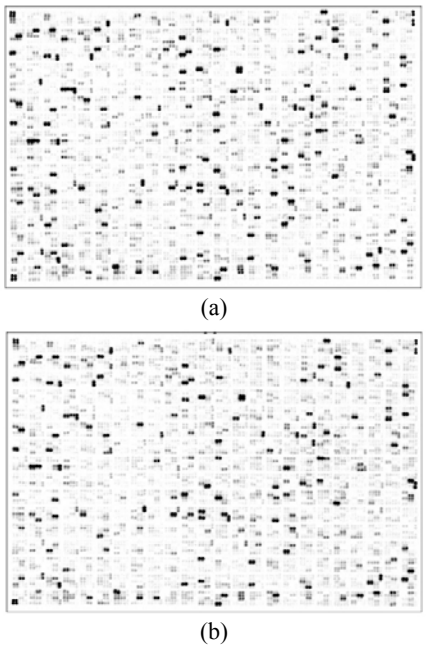

Fig.2 Scanning images of the cDNA array after hybridization with isotopic nucleotide [α-^{33}P]-labelled probes. (a) Mycelia; (b) Appressoria

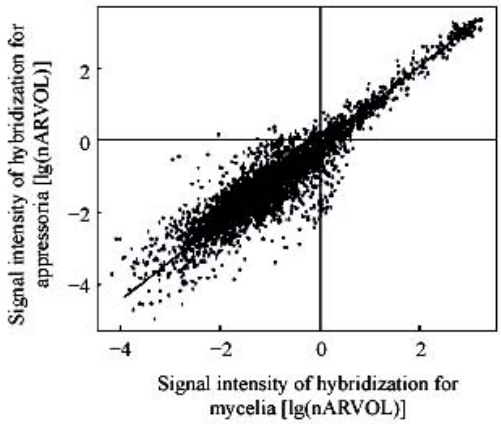

Fig.3 Scatter plot comparing the cDNA array hybridization signal values of mycelia and mature appressoria

identified in mature appressoria compared with mycelia. Of these, 55 genes were up-regulated and 22 genes were down-regulated (Table 1).

The sequences of 77 differentially expressed genes from mature appressoria of *M. grisea* Y34 were performed BLASTn searches against the draft sequences of the *M. grisea* 70-15 genome (http://www.broad.mit.edu/annotation/fungi/magnaporthe/). A total of 61 genes could be matched with the *M. grisea* genome database ($E<10^{-3}$), the remaining 16 genes showed no similarity to the genome sequences of *M. grisea* ($E\geq10^{-3}$) (Table 1). We know that only about 4% genome sequence of *M. grisea* was not obtained (Dean *et al*., 2005). So the 16 unique transcripts could not match the genome sequences of *M. grisea* 70-15, probably because they fall within gaps of genome sequences although they may also represent genes unique to *M. grisea* Y34.

In order to annotate the 77 differentially expressed genes, we performed BLASTx searches against the NCBI (the National Center for Biotechnology Information) protein database (Table 1). A total 32 genes showed no homology with identified proteins deposited in the NCBI database ($E\geq10^{-3}$). These genes were considered unknown novel genes differentially expressed in the mature appressoria of *M. grisea*. Other 45 genes had significant matches ($E<10^{-3}$) and were given functional annotation. Among the 45 annotated genes, only 12 genes were annotated known or hypothetical protein of *M. grisea*, the remaining 33 genes had hits with proteins of other origins.

Novel homologue of peptidyl-prolyl cis-trans isomerase

A gene *CYP1* encoding peptidyl-prolyl cis-trans isomerase (PPIase) (GenBank accession No. AF293848) can serve as a virulence determinant during plant infection (Viaud *et al*., 2002), another homologue of PPIase (GenBank accession No. AI068531) was found up-regulated in conidia of *M. grisea* (Takano *et al*., 2003). We found a homologue of PPIase (GenBank accession No. CK923388) down-regulated in mature appressoria. BLASTn searches were performed against *M. grisea* genome database with the results indicating three clones CK923388, AF293848 and AI068531 could match respectively three predicted genes MG08104.4 (E value=10^{-48}), MG10447.4 (E value=0) and MG07389.4 (E value=10^{-174}). We further compared conserved domains of three predicted genes encoding proteins using NCBI Conserved Domain Search Database (http://ncbi.nih.gov/Structure/cdd/cdd.shtml) (Fig.4). The clone AI068531 had PPIC (peptidyl-prolyl isomerase C)-type PPIase domain (rotamase). Clones CK923388 and AF293848 had a cyclophilin_ABH domain, but the domain laid in different location of open reading frame of two genes, N-terminal extension of the former gene and C-terminal extension of the later gene. So the homologue of PPIase identified from this study was a novel gene expressing weakly in mature appressria of *M. grisea*.

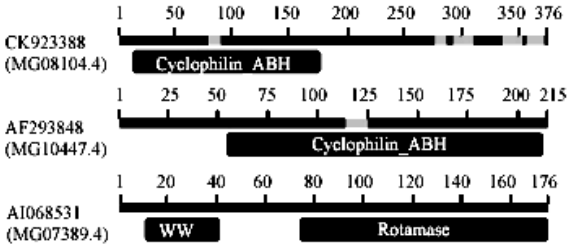

Fig.4 Comparison of conserved domains of open reading frames of peptidyl-prolyl cis-trans isomerase homologues

CK923388, cyclophilin_ABH [cyclophilin A, B and H-like cyclophilin-type peptidyl-prolyl cis-trans isomerase (PPIase) domain], E value=10^{-70}; AF293848, cyclophilin_ABH, E value=10^{-61}; AI068531, rotamase (PPIC-type PPIase domain), E value=10^{-16}, WW [domain with 2 conserved Trp (W) residues], E value=10^{-4}. The number above bold lines indicates the number of amino acids in open reading frames of peptidyl-prolyl cis-trans isomerase homologues

Northern blot analysis

Northern blot analysis was done to confirm the cDNA array results. Eleven genes for analysis were randomly picked from 77 differentially expressed genes (Fig.5). Seven genes (CK918905, CK917797, CK926361, CK919831, CK926422, CK922573 and CK925364) were up-regulated in mature appressoria compared with mycelia. The remaining four genes (CK926411, CK914039, CK923388 and CK921137) were down-regulated in mature appressoria. These results are consistent with the cDNA array results and confirm the cDNA array experiment is reliable.

Table 1 Differentially expressed genes in mature appressoria of *M. grisea*

Clone	GenBank No.	Annotation	*E* value	Signal intensity		Fold change [b]
				Mycelia	Appressoria	
Up-regulated						
p3fmgcf_003968	CK916894	Pyruvate carboxylase 2 (*Candida robusta*)	0	3.5	13.8	4.0
p3fmgc_016531	CK925431	Related to coclaurine N-methyltransferase (*Neurospora crassa*)	2E−110	5.0	15.4	3.1
p3fmgc_014963	CK924418	Similar to dTDP-D-glucose 4,6-dehydratase (*Arabidopsis thaliana*)	1E−77	1.6	4.8	3.1
p3fmgc_017013	CK925780	Heterochromatin protein 1 (*Drosophila melangaster*)	7E−10	0.6	4.0	6.3
p3fmgc_015770	CK924917	Cutinase transcription factor 1 beta (*Fusarium solani*)	0	3.1	9.5	3.1
p3fmgcf_004759	CK917547	60S ribosomal protein L27 (*Cryptococcus neoformans*)	2E−14	0.5	1.9	3.8
p3fmgc_014039	CK923805	60S ribosomal protein L20 (*Candida robusta*)	0	2.3	6.9	3.0
p3fmgc_009740	CK920976	60S acidic ribosomal protein P2 (*Fusarium culmorum*)	1E−20	0.4	1.2	3.1
p3fmgcf_003681	CK916638	60S acidic ribosomal protein P0 (*Podospora anserina*)	0	0.9	2.9	3.1
p3fmgcf_003730	CK916679	Protein transport protein sec73 (*Schizosaccharomyces pombe*)	0	0.2	0.9	3.8
p3fmgc_006682	CK918982	H^+/nucleoside cotransporter (*Aspergillus fumigatus*)	7E−167	1.4	4.6	3.2
p3fmgc_010096	CK921272	Nucleoporin nup184 (*Schizosaccharomyces pombe*)	8E−44	0.5	2.4	4.5
p3fmgc_015331	CK924608	Tubulin alpha-1 chain (*Aspergillus nidulans*)	0	4.0	13.1	3.3
p3fmgc_007888	CK919831	Hydrophobin-like protein Mpg1 precursor (*Magnaportha grisea*)	0	0.4	1.5	4.0
p3fmgc_007879	CK919824	Putative zinc knuckle domain protein (Byr3) (*Aspergillus fumigatus*)	1E−25	0.4	1.3	3.1
p3fmgc_015536	CK924732	Putative NADH-ubiquinone oxidoreductase B18 subunit (*Aspergillus fumigatus*)	6E−29	3.4	10.8	3.2
p3fmgcf_005058	CK917797	Phospholipid metabolism-related protein (*Cryptococcus neoformans*)	1E−24	0.9	3.3	3.5
p3fmgc_015911	CK924986	Putative transcriptional regulator (*Magnaporthe grisea*)	2E−40	1.9	8.6	4.4
p3fmgc_006559	CK918905	Putative Sgt1 protein (*Aspergillus fumigatus*)	9E−101	6.4	21.7	3.4
p3fmgc_018263	CK926361	G-protein beta like WD repeat protein (*Fusarium oxysporum*)	0	0.9	2.8	3.0
p3fmgc_016443	CK925364	Integral membrane protein (*Aspergillus fumigatus*)	3E−95	0.4	1.3	3.1
p3fmgc_018350	CK926422	*UVI-1* gene induced by near-UV light (*Bipolaris oryzae*)	4E−72	0.3	1.7	5.0
p3fmgc_012062	CK922573	Signal recognition particle 14 kD protein (*Aspergillus fumigatus*)	3E−11	0.4	1.6	4.0
p3fmgc_019369	CK927054	Copper metallothionein (*Neurospora crassa*)	7E−4	0.3	0.9	3.2
p3fmgc_010478	CK921529	Hypothetical protein MG03625.4 (*Magnaporthe grisea*)	0	0.2	0.8	3.4
p3fmgc_006276	CK918694	Predicted protein MG04367.4 (*Magnaporthe grisea*)	0	0.5	1.7	3.4
p3fmgc_013687	CK923540	Hypothetical protein MG04654.4 (*Magnaporthe grisea*)	0	1.4	4.8	3.5
p3fmgc_006351	CK918755	Hypothetical protein MG04836.4 (*Magnaporthe grisea*)	0	1.8	5.8	3.2
p3fmgc_016377	CK925316	Hypothetical protein MG09618.4 (*Magnaporthe grisea*)	0	3.1	9.8	3.1
p3fmgc_012651 [a]	CK923046	Unknown	−	3.1	12.9	4.1
p3fmgcf_004538	CK917359	Unknown	−	1.8	6.2	3.4
p3fmgc_010655 [a]	CK921636	Unknown	−	2.7	9.2	3.4
p3fmgc_007568	CK919581	Unknown	−	1.1	3.3	3.0
p3fmgc_017202	CK925886	Unknown	−	1.0	3.2	3.2
p3fmgc_006167 [a]	CK918615	Unknown	−	1.1	3.8	3.5
p3fmgc_005954	CK918534	Unknown	−	3.7	13.1	3.6
p3fmgc_016946	CK925761	Unknown	−	6.8	25.2	3.7
p3fmgc_018594	CK926628	Unknown	−	1.1	5.3	4.9
p3fmgc_009430 [a]	CK920812	Unknown	−	8.2	25.4	3.1
p3fmgc_009675 [a]	CK920940	Unknown	−	1.5	5.0	3.4
p3fmgcf_004668	CK917466	Unknown	−	2.3	8.4	3.7

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Clone	GenBank No.	Annotation	*E* value	Signal intensity		Fold change [b]
				Mycelia	Appressoria	
p3fmgc_006635 [a]	CK918945	Unknown	−	1.4	4.3	3.1
p3fmgc_007193 [a]	CK919313	Unknown	−	0.4	2.2	5.2
p3fmgc_015653 [a]	CK924820	Unknown	−	0.4	1.9	4.6
p3fmgc_015571 [a]	CK924761	Unknown	−	0.6	2.1	3.3
p3fmgc_010647 [a]	CK921629	Unknown	−	0.4	1.6	3.9
p3fmgc_016358	CK925300	Unknown	−	0.5	1.6	3.2
p3fmgc_012659 [a]	CK923053	Unknown	−	0.3	1.2	3.6
p3fmgcf_004487	CK917315	Unknown	−	1.1	3.7	3.3
p3fmgc_012479 [a]	CK922902	Unknown	−	0.4	1.8	4.0
p3fmgc_016180	CK925181	Unknown	−	0.3	1.4	4.9
p3fmgcf_004752	CK917541	Unknown	−	8.4	25.4	3.0
p3fmgc_008171 [a]	CK920009	Unknown	−	0.4	1.3	3.4
p3fmgc_012910	CK923215	Unknown	−	2.4	7.7	3.2
p3fmgc_014484 [a]	CK924087	Unknown	−	0.3	0.8	3.1
Down-regulated						
p3fmgc_018329	CK926411	Glyceraldehyde 3-phosphate dehydrogenase (*Podospora anserina*)	0	3.9	0.6	6.0
p3fmgcf_000490	CK913975	RNA 3'-terminal phosphate cyclase-like protein (*Candida robusta*)	0	3.2	1.0	3.2
p3fmgcf_004048	CK916959	Heat shock protein Sti1 (*Candida robusta*)	7E−16	1.0	0.3	3.7
p3fmgc_013455	CK923388	Peptidyl-prolyl cis-trans isomerase (*Neurospora crassa*)	9E−115	1.6	0.5	3.3
p3fmgc_016518	CK925421	Possible copper transport protein Ctr2 (*Candida robusta*)	1E−19	1.2	0.3	4.4
p3fmgc_017343	CK925929	Related to GTPase activating protein (*Neurospora crassa*)	0	2.0	0.6	3.3
p3fmgc_009930	CK921137	b-ZIP transcription factor IDI-4 (*Podospora anserina*)	3E−15	1.0	0.2	4.9
p3fmgc_010939	CK921810	Reverse transcriptase (*Magnaporthe grisea*)	1E−179	3.5	1.1	3.3
p3fmgcf_000222	CK913775	Elongation factor 1-alpha (*Aspergillus oryzae*)	0	1.7	0.6	3.0
p3fmgc_007834	CK919784	Putative TBC domain protein (*Aspergillus fumigatus*)	7E−100	1.2	0.3	3.6
p3fmgcf_000566	CK914039	Integral membrane protein, Pth11 (*Magnaporthe grisea*)	5E−17	2.8	0.6	4.4
p3fmgc_005845	CK918469	Putative transposase (*Magnaporthe grisea*)	0	1.3	0.3	4.0
p3fmgc_017874	CK926198	Related to spore coat protein sp96 precursor (*Neurospora crassa*)	4E−12	3.5	0.5	7.3
p3fmgc_007636	CK919625	Male sterility protein 2 (*Arabidopsis thaliana*)	4E−15	1.3	0.4	3.3
p3fmgc_006724	CK919007	Hypothetical protein MG00432.4 (*Magnaporthe grisea*)	0	1.9	0.6	3.0
p3fmgc_013422	CK923366	Hypothetical protein MG10198.4 (*Magnaporthe grisea*)	0	3.0	0.9	3.4
p3fmgc_015509 [a]	CK924710	Unknown	−	155.5	48.7	3.2
p3fmgc_017596	CK926044	Unknown	−	1.2	0.2	4.9
p3fmgcf_003124 [a]	CK916195	Unknown	−	0.8	0.2	3.9
p3fmgc_007826	CK919778	Unknown	−	2.8	0.7	3.8
p3fmgc_005375	CK918157	Unknown	−	2.5	0.5	4.7
p3fmgcf_000736	CK914184	Unknown	−	1.2	0.4	3.0

[a] indicates that clone showed no similarity to the genome sequences of *M. grisea* 70-15 ($E \geq 10^{-3}$); [b] indicates that fold changes reached statistical significance of the difference in expression level between mature appressoria and mycelia (confidence level of $P<0.01$)

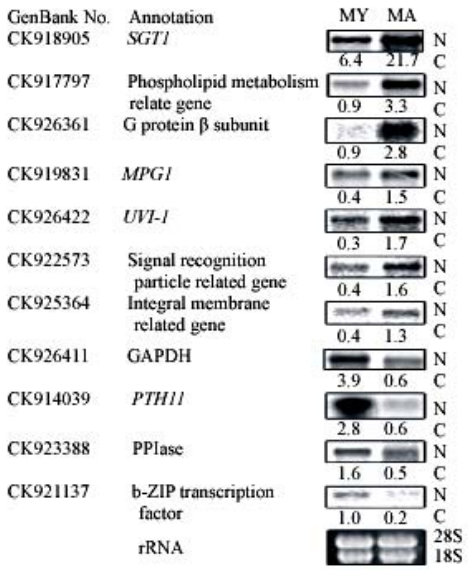

Fig.5 Confirmation of cDNA array data by Northern blot analysis of differentially expressed genes in *M. grisea*

MY: Mycelia; MA: Mature appressoria; N: Northern blot; C: cDNA array nARVOL value

DISCUSSION

Metabolism changes related to appressorium turgor

Strong turgor in appressorium allows a penetration peg to invade the host tissue. Accumulation of high concentration glycerol in appressorium was considered to be the main cause of turgor (de Jong *et al*., 1997). Glycerol is a degradation product of triacyglycerol, and can also come from gluconeogenesis demonstrated by a mutant research of gene *ICL1* encoding isocitrate lyase in *M. grisea* (Thines *et al*., 2000; Wang *et al*., 2003). We also found a homologue of pyruvate carboxylase involved in gluconeogenesis up-regulated in mature appressoria. The gene might be related to glycerol production involved in turgor accumulation and still remained to verify its function in appressoria.

Some carbohydrates such as glycogen also participate in turgor generation in appressoria (Talbot, 2003). We found up-regulation of phospholipid metabolism-related gene involved in lipid catabolism and down-regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) homolog involved in glycolysis in mature appressoria. Takano *et al*.(2003) also showed the down-regulation of GAPDH in developing appressoria using cDNA array analysis. All the above results might be cues of induced lipid catabolism and reduced glycolysis in appressorium development. Reduction in glycolysis in appressoria is unlikely to ensure macromolecules or carbohydrates accumulation (Talbot, 2003). It more likely reflected that the carbohydrates such as glycogen are not degraded in appressorium development and that lipid catabolism is the main source of energy and glycerol.

Genes related to cell signaling of appressorium

Cell signaling pathways regulate appressorium formation and development (Dean, 1997). *PTH11* gene encoding a plasma membrane protein mediates appressorium formation of *M. grisea* (DeZwaan *et al*., 1999). Our data showed that a homologue of *PTH11* had lower expression in mature appressoria. It might imply that *PTH11* had no significant relation to appressorium maturation.

The heterotrimetric G proteins, which are composed of α, β and γ subunits, are essential in signal transduction and regulation of fungal growth and development. We found a homologue of *FGB1* encoding β subunit of G protein highly expressed in mature appressoria. Gene *FGB1* regulates Fusarium development and virulence through multiple signaling pathways (Delgado-Jarana *et al*., 2005). In this study the homologue of *FGB1* showed no significant homology to *MGB1* gene of *M. grisea*, which also encoded β subunit involved in appressorium formation of *M. grisea* (Nishimura *et al*., 2003). We believed that the homologue was a novel member of G protein β subunit of *M. grisea* and might be related to cell signaling in appressorium maturation.

Mitosis is regulated by signals inside cells. There is a single mitosis before appressoium formation and mitosis starts again only in infectious hyphae of *M. grisea* (Dean, 1997). Sgt1 protein is involved in kinetochore assembly of mitosis and regulation of adenylate cyclase activity in cAMP signal transduction pathways in eukaryotic cell (Dubacq *et al*., 2002; Steensgaard *et al*., 2004). In *M. grisea*, we identified a homologue of *SGT1* gene, the transcript abundance of which is highest in mature appressoria. Because there is no mitosis in appressorium maturation, up-regulation of *SGT1* might reflect activation of the cAMP pathway responsible for appressorium maturation.

Comparison of differentially expressed genes in appressoria with results of previous studies

Mature appressoria is dispensable for penetration into host plant cell. We found 45 annotated genes among 77 genes differentially expressed in mature appressoria of *M. grisea*. We compared our annotated genes with results from previous analyses of subtracted cDNA libraries, SAGE, promotor-*egfp* (a red-shifted green fluorescent gene) fusion system, microarray in appressorium initiation, formation and maturation of *M. grisea* (Banno *et al*., 2003; Irie *et al*., 2003; Kamakura *et al*., 1999; Lu *et al*., 2005; Takano *et al*., 2003). Thirty-nine annotated genes were isolated in mature appressoria for the first time in this study, besides 8 homologues of hydrophobin, metallothionein, dTDP-D-glucose 4,6-dehydratase, UVI-1, PPIase, GAPDH, integral membrane protein Pth11, elongation factor 1-alpha and hypothetical protein MG09618.4 identified in previous studies on appressorium development of *M. grisea*. Among the 8 genes, some genes such as PPIase might be novel homologues different from previous studies. We found four genes encoding different ribosomal proteins up-regulated in mature appressoria, but a gene encoding elongation factor 1-alpha was up-regulated in mycelia and down-regulated in mature appressoria. A similar phenomenon was also found in previous study. It was possible that the translational machinery was not identical between appressoria and mycelia (Takano *et al*., 2003).

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