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Research Article

RESEARCH ARTICLE

Transcriptomic analysis of the *GCN5* gene reveals mechanisms of the epigenetic regulation of virulence and morphogenesis in *Ustilago maydis*

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One sentence summary: *Ustilago maydis* virulence and yeast growth are epigenetically regulated by the histone acetyltransferase (Hat) Gcn5.
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ABSTRACT

Chromatin in the eukaryotic nucleus is highly organized in the form of nucleosomes where histones wrap DNA. This structure may be altered by some chemical modifications of histones, one of them, acetylation by histone acetyltransferases (HATs) that originates relaxation of the nucleosome structure, providing access to different transcription factors and other effectors. In this way, HATs regulate cellular processes including DNA replication, and gene transcription. Previously, we isolated *Ustilago maydis* mutants deficient in the *GCN5* HAT that are avirulent, and grow constitutively as mycelium. In this work, we proceeded to identify the genes differentially regulated by *GCN5*, comparing the transcriptomes of the mutant and the wild type using microarrays, to analyse the epigenetic control of virulence and morphogenesis. We identified 1203 genes, 574 positively and 629 negatively regulated in the wild type. We found that genes belonging to different categories involved in pathogenesis were downregulated in the mutant, and that genes involved in mycelial growth were negatively regulated in the wild type, offering a working hypothesis on the epigenetic control of virulence and morphogenesis of *U. maydis*. Interestingly, several differentially regulated genes appeared in clusters, suggesting a common regulation. Some of these belonged to pathogenesis or secondary metabolism.

Keywords: histone acetyltransferases; epigenetics; transcriptomes; pathogenesis; dimorphism

INTRODUCTION

In eukaryotic organisms, DNA is highly organized and compacted within the nucleus. The ultimate structural organization of DNA in the chromatin is in the form of nucleosomes, units of chromatin, consisting 147 base pairs of DNA wrapped around an octamer of the four core histone proteins (Kornberg and Lorch 1999; Lee and Workmann 2007; Battistini *et al.* 2010). This DNA compacting and organization is altered by post-translational and covalent modifications of histones that relax or compact the nucleosomes. Among such changes, histone acetylation and deacetylation play an important role. Acetylation is catalysed by histone acetyltransferases (HATs) that by addition of acetyl groups allow nucleosome relaxation and therefore accessibility of DNA to different proteins involved in transcription and its

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regulation (Braunstein *et al.* 1993; Legube and Trouche 2003). The opposing process is conducted by histone deacetylases (HDACs) which remove acetyl groups giving rise to compaction of the nucleosomes (Legube and Trouche 2003). Through their activity HATs and HDACs regulate several important cellular processes such as DNA replication, assembly of chromatin structure, gene transcription, and as an epigenetic result, cell development and function (Kuo and Allis 1998; Kuo *et al.* 1998; Legube and Trouche 2003; Lee and Workmann 2007; Liu *et al.* 2014). These proteins are evolutionarily conserved in different eukaryotic organisms, having important roles in cell fate (Kuo and Allis 1998; Marmorstein and Roth 2001; Legube and Trouche 2003; Thiagalingam *et al.* 2003; Lee and Workmann 2007; Liu *et al.* 2014). In humans, it has been demonstrated that deficient regulation of HATs activity is related to tumour development (Timmermann *et al.* 2001).

The first HAT isolated in yeast was Gcn5, which was described as a transcriptional coactivator (Georgakopoulos and Thireos 1992; Kuo *et al.* 1998). Subsequently, homologues of this gene have been characterized in other fungi such as *Cryptococcus neoformans*, where they regulate the adaptation of the fungus to its host (O'Meara *et al.* 2010), and regulating growth, conidiation and gene expression in *Trichoderma reesei* (Xin *et al.* 2013). Our working group recently isolated and characterized a *GCN5* homologue from *Ustilago maydis*, demonstrating their important role in its virulence and dimorphic transition (González-Prieto *et al.* 2014). *Ustilago maydis*, a maize pathogen, has the ability to perform a dimorphic transition of yeast to mycelium by a change in pH in the culture medium (Ruiz-Herrera *et al.* 1995). It was observed that *gcn5* mutants grow constitutively in the mycelium form, and are avirulent to maize (González-Prieto *et al.* 2014).

Ustilago Maydis is a Basidiomycota fungus that infects and completes its life and sexual cycles in maize (*Zea mays* L.) and teozintle (*Z. mays* subsp. *parviglumis*), producing the disease known as common smut. Nevertheless, under axenic conditions it is able to infect plants phylogenetically distant to maize (León-Ramírez *et al.* 2004; Méndez-Morán *et al.* 2005; Martínez-Soto *et al.* 2013; Ruiz-Herrera, Robledo-Briones and Martínez-Soto 2013), and under defined environmental *in vitro* conditions of incubation it performs a very different life and sexual cycle with the formation of basidiocarps (Cabrera-Ponce *et al.* 2012). Interestingly, *U. maydis* has been used as a model organism for the analysis of different cellular processes including gene regulation, mating and fungal virulence in plants, among others (Brefort *et al.* 2009; Ruiz-Herrera, Reynaga-Peña and Aréchiga-Carvajal 2009; Vollmeister *et al.* 2011; Ruiz-Herrera and Campos-Góngora 2012; Valdés-Santiago *et al.* 2012; Ruiz-Herrera, Robledo-Briones and Martínez-Soto 2013; León-Ramirez, Sánchez Arreguín and Ruiz-Herrera 2014; Valdés-Santiago and Ruiz-Herrera 2014).

Since there is only limited information about the genes regulated by HATs in fungi, and knowing that they are involved in epigenetic regulation of different process, we took advantage of the *U. maydis gcn5* mutants to identify all the genes regulated by the Gcn5 acetyltransferase by comparison of its transcriptome with that from the *U. maydis* wild-type strain. This way, we demonstrate that *U. maydis* pathogenesis and morphogenesis are regulated epigenetically by *GCN5*, by means of allowing accessibility to factors involved in the transcription of genes or gene clusters required for these processes.

MATERIALS AND METHODS

Ustilago maydis strains and culture conditions

The wild-type strain FB2 (a_2b_2; Banuett and Herskowitz 1989) and the constitutive avirulent monomorphic mutant GP25 (*a2b2*Δ*umgcn5*::*hyg*; González-Prieto *et al.* 2014) were used in this study. The strains were maintained in 50% glycerol at –70 °C, and recovered in liquid complete medium (MC; Holliday 1974). Growth in minimal medium (MM; Holliday 1974) took place at pH 7. Under these conditions, the wild-type strain grows in the yeast-like form, and the GP25 mutant grows constitutively as mycelium (Ruiz-Herrera *et al.* 1995; González-Prieto *et al.* 2014).

RNA isolation and microarray preparation

Ustilago maydis cells (10^6 per ml) were inoculated in MM pH 7 at 28°C under shaking conditions for 16 h. The cells were recovered by centrifugation, and RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), and purified with QIAGEN (Hilden, Germany) columns. RNA concentration was measured by absorbance at 260 nm with a Nanodrop (Thermo Scientific, Waltham, MA, USA), and its integrity was determined by electrophoresis in agarose gels. Three independent cultures (biological replicates) of the fungus were obtained, and the corresponding RNA samples were mixed together. Synthesis and labelling of cDNA, as well as microarrays hybridization, were performed by Roche NimbleGen Inc. (Reykjavík, Iceland).

Statistical analysis of microarrays

The type of microarrays used in this work (NimbleGen) contains five different oligonucleotides 60 nt in length designed along the full gene length by duplicate, according to a design from Scott Gold (University of Georgia). These conditions secure that data for each one of the 6883 *U. maydis* genes represents an average of 10 determinations. For scanning the arrays, GenePix 400B scan and associated software were used, and NimbleScan software based on quantile (Bolstad *et al.* 2003) and robust multi-array analysis algorithm (Irizarry *et al.* 2003) were used for data normalization. For analyses of microarrays, we used ArrayStar software from DNAStar, *P*-values less than 0.05 were considered differentially expressed and *P*-values were adjusted by the false discovery rate method (Benjamini and Hochberg 1995). A value of 2-fold change up or down was considered the cutoff to determine the genes differentially regulated. Genes whose expression values were higher in the wild-type strain were considered to be positively regulated by the HAT gene *GCN5*, and those whose expression was lower in the wild-type strain were considered to be negatively regulated by this gene. Venn diagrams were used for handling and comparison of gene groups (http://www.bioinformatics.lu/venn.php).

Quantitative real-time PCR analysis

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was employed for synthesis of the first DNA strand using 1 μg of RNA samples. RNA was quantified in a Nanodrop 2000 Spectrophotometer (Thermo Scientific), using samples of cDNA amplified by PCR using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in StepOne Real-Time PCR Systems (Applied Biosystems). Genes and primers appear in Table S1 (Supporting Information).

Functional grouping of the total differential genes

The Functional Catalogue (FunCat) online software (Ruepp *et al.* 2004) was used by the functional grouping of the total genes regulated by the HAT *GCN5* in *U. maydis*, with the support of MIPS *Ustilago maydis* Database (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/), *Ustilago maydis* Database of Broad Institute (http://www.broadinstitute.org/annotation/genome/ustilago_maydis) and *Ustilago maydis*—JGI Genome Portal (http://genome.jgi.doe.gov/Ustma1/Ustma1.home.html), and with the aid of R statistical software.

Search of consensus sequences, binding sites for transcription factors and specific genes

We used JASPAR online software (http://jaspar.binf.ku.dk/) based on the *Saccharomyces cerevisiae* genome (http://www.yeastgenome.org/) to identify consensus sequences and binding sites for transcription factors. Using BLAST of NCBI page online (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and with the support of MIPS *Ustilago maydis* Database, *Ustilago maydis* Database of Broad Institute and *Ustilago maydis*—JGI Genome Portal, we identified *U. maydis* homologous genes for the transcription factors identified. Search for genes previously described to be grouped in pathogenesis clusters, and pathogenesis genes in *U. maydis* was made based on the data previously reported by Kämper *et al.* (2006) and Martínez-Soto *et al.* (2013), and the search for genes previously reported during *U. maydis* dimorphism was made according to published data by Martínez-Soto and Ruiz-Herrera (2013).

Search for domains in unclassified proteins

The following online programs were used to search for domains in proteins not classified: SMART (Letunic, Doerks and Bork 2012), Pfam (Punta *et al.* 2012), SignalP (Bendtsen *et al.* 2004) and with the support of NCBI (http://www.ncbi.nlm.nih.gov/), MIPS *Ustilago maydis* Database, *Ustilago maydis* Database of Broad Institute and *Ustilago maydis*—JGI Genome Portal.

RESULTS AND DISCUSSION

As mentioned above, HATs regulate different cellular processes that occur in eukaryotic organisms (Kuo and Allis 1998; Kuo *et al.* 1998; Legube and Trouche 2003; Lee and Workmann 2007; Liu *et al.* 2014). Accordingly, in this work we proceeded to identify all the genes that are differentially regulated by *GCN5* in *U. maydis* by use of microarrays. To this end, we compared transcript levels of a *gcn5* mutant strain and the wild-type strain, both grown at pH 7. Since as stated, the mutant grows constitutively as mycelium independently of the pH in the culture medium (González-Prieto *et al.* 2014), comparison of the transcriptome from cells of both strains grown at pH 7, the only factors involved in the comparison were the mutation and dimorphism. This analysis revealed that 1203 genes were differentially regulated by Gcn5. Of them, 574 (47.7%) were positively regulated and 629 (52.3%) were negatively regulated in the wild-type strain (Table S2, Supporting Information); accordingly, upregulated genes are those that are under positive control by Gcn5, whereas downregulated genes are those negatively regulated by Gcn5. To validate the microarray analysis, we used a set of genes with different patterns of differential expression, whose transcription values were analysed by quantitative RT-PCR. Comparative analysis of both studies is shown in Table S3 (Supporting Information).

Functional grouping of all the differentially regulated genes showed that the highest percentage of differential genes corresponded to the following categories: unclassified with 47.5% (572 differential genes), and metabolism with 17.4% (209 differential genes) (Fig. 1). In different functional analysis, it has been found that these two categories always group the largest numbers of genes (Nantel *et al.* 2002; Doehlemann *et al.* 2008; Heimel *et al.* 2010; Morales-Vargas, Domínguez and Ruiz-Herrera 2012; Martínez-Soto *et al.* 2013; Martínez-Soto and

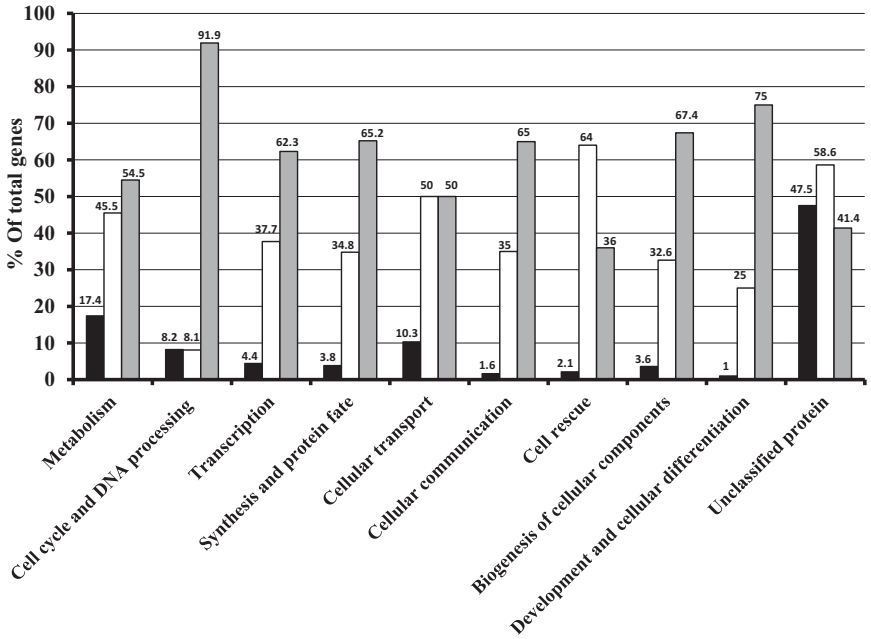

Figure 1. Functional grouping of the total genes regulated by the HAT gene *GCN5* of *U. maydis*. Black bars represent the percentage of the total genes regulated in the corresponding category; white bars represent the percentage of genes positively regulated in each category; grey bars represent the percentage of genes negatively regulated in each category. Legends under the bars are the names of the categories, and the numbers over the bars are the porcentages of differential, expressed or repressed genes in each category.

Table 1. Regulation of genes of the Cell Cycle and DNA processing category by Gcn5.

Gene	Description	Fold change
um00496	Hmp1—mismatch base pair and cruciform DNA recognition protein Hmp1	2.4 up
um10852	Related to MET30-involved in regulation of sulphur assimilation genes and cell cycle progression	2.4 up
um01952	Related to UV-endonuclease UVE-1	2.4 up
um05674	nuc1-Probable NUC1-dna/rna non-specific nuclease, mitochondrial	2.3 up
um06212	Related to SSN8—DNA-directed RNA polymerase II holoenzyme and SRB subcomplex subunit, cyclin C homologue	2.0 up
um10499	Related to HOS4—subunit of the Set3 complex	2.0 up
um05186	Related to BRE1—E3 ubiquitin ligase	2.0 down
um03262	Related to Separin	2.0 down
um00290	Related to DNA topoisomerase II binding protein	2.0 down
um11010	Related to ECO1—acetyltransferase required for establishment of sister chromatid cohesion	2.0 down
um02433	Related to nuclear division protein Rft1	2.0 down
um10597	Related to UNG1—uracil-DNA glycosylase	2.0 down
um05151	Related to cyclin H	2.0 down
um03792	Related to sepB protein	2.0 down
um04416	Probable POB3—protein that binds to DNA polymerase I	2.0 down
um01458	Probable DNA polymerase delta catalytic subunit	2.0 down
um11199	Related to CDC28—cyclin-dependent protein kinase	2.0 down
um03141	Related to EXO1—exonuclease which interacts with Msh2p	2.1 down
um00366	Probable MCM6—involved in replication	2.1 down
um05568	Probable SPT16—general chromatin factor (subunit of the heterodimeric FACT complex)	2.1 down
um01679	Probable MCM3—subunit of pre-replication complex	2.1 down
um05085	Related to post-replication repair protein uvsH/nuvA	2.1 down
um10399	Related to C-type cyclin	2.1 down
um10279	Clb2 - b-type cyclin 2	2.1 down
um02718	Related to PIF1—DNA helicase involved in mitochondrial DNA repair and telomere length control	2.1 down
um04522	Related to JHD1—JmjC domain family histone demethylase	2.1 down
um04460	Related to RPB8—DNA-directed RNA polymerase I, II, III 16 KD subunit	2.2 down
um01647	Related to CDC20—cell division control protein	2.2 down
um03756	Related to DNA helicase Fdhp	2.2 down
um02823	Related to CTF18—chromosome transmission fidelity factor	2.2 down
um03095	DNA repair/recombination protein Rec2	2.2 down
um04485	Related to Cut9 interacting protein scn1	2.2 down
um03852	Related to PRI2—DNA-directed DNA polymerase alpha, 58 KD subunit (DNA primase)	2.2 down
um04529	Related to POL1—DNA-directed DNA polymerase alpha, 180 KD subunit	2.2 down
um01200	Related to histone acetyltransferase	2.2 down
um00459	Related to condensin complex subunit 3	2.2 down
um05569	Related to methylated-dna–protein-cysteine methyltransferase	2.2 down
um12335	Related to nuclear distribution protein RO11	2.2 down
um10401	Related to CHL1—protein of the DEAH box family	2.2 down
um00289	Related to inner kinetochore protein MIF2	2.2 down
um01646	Probable replication licensing factor MCM4	2.3 down
um03676	Related to RAD54—DNA-dependent ATPase of the Snf2p family	2.3 down
um02951	Related to TOF1—topoisomerase I interacting factor 1	2.3 down
um10546	Probable histone 3	2.3 down
um11521	Related to A/G-specific adenine DNA glycosylase	2.3 down
um03226	Related to component of the anaphase promoting complex	2.3 down
um11759	Related to STU1—component of the mitotic spindle	2.3 down
um10616	Probable CDC31—spindle pole body component, centrin	2.4 down
um04365	Related to SRM1 nucleotide exchange factor	2.4 down
um05141	Related to DNA polymerase kappa	2.4 down
um03501	Probable DNA topoisomerase II	2.4 down
um05156	Probable replication factor-A protein 1	2.4 down
um02059	Related to RPC31—DNA-directed RNA polymerase III	2.4 down

Table 1. *(Continued)*

Gene	Description	Fold change
um11009	Related to MSH6—DNA mismatch repair protein	2.4 down
um00950	Related to Spindle pole body component alp6	2.4 down
um05770	Protein required for initiation of DNA synthesis and for replication fork progression	2.5 down
um05207	Related to DPB3—third largest subunit of DNA polymerase II	2.5 down
um11713	Related to YCS4—subunit of condensin protein complex	2.5 down
um03987	Related to BRN1—protein required for chromosome condensation	2.5 down
um10656	Related to DNA primase 48K protein PRI1	2.5 down
um05835	Probable SMC2—chromosome segregation protein	2.5 down
um01262	DNA polymerase X-putative	2.5 down
um06131	Related to 35 kDaribonuclease H	2.6 down
um04927	Related to PSF1—subunit of the GINS complex	2.6 down
um00911	Related to MPH1—member of the DEAH family of helicases	2.7 down
um10194	Probable DNA-directed RNA polymerase II chain RPB10	2.7 down
um10910	Related to DNA repair protein rad9	2.8 down
um01445	Probable DNA replication licensing factor (nimQ)	2.8 down
um00728	Related to origin recognition complex subunit 4	2.9 down
um10865	Related to ELO1—elongase	2.9 down
um05166	Related to FUN30—protein important for chromosome integrity and segregation	2.9 down
um04905	Related to DML1—essential protein involved in mtDNA inheritance	2.9 down
um03288	Related to dna polymerase epsilon p17 subunit	3.0 down
um11355	Related to DAD4—outer kinetochore protein (part of Dam1 complex)	3.0 down
um00208	Related to SRC1—involved in sister chromatid segregation	3.1 down
um11553	Related to spindle assembly checkpoint protein	3.1 down
um02874	Related to SGS1—DNA helicase	3.2 down
um04224	Related to Myosin-like protein NUF2	3.2 down
um10752	Related to DAD1—essential subunit of the Dam1 complex	3.4 down
um11929	Related to DNA topoisomerase III alpha	3.4 down
um01691	Related to ATP-dependent DNA helicase	3.4 down
um05208	Related to MLH1—DNA mismatch repair protein	3.6 down
um02567	Related to histone acetyltransferase subunit HAT1	3.6 down
um04512	Related to DnaJ protein	3.6 down
um05028	Related to DNA2—DNA helicase	3.7 down
um05756	Related to ATP-dependent DNA helicase II, 80 kDa subunit	3.8 down
um00739	Related to DNA repair protein rad18	3.8 down
um01932	Related to PMS1—DNA mismatch repair protein	3.9 down
um05148	Related to ATP-dependent DNA helicase II, 70 kDa subunit	4.3 down
um04059	Related to anaphase control protein cut9	4.3 down
um01008	pol2—probable POL2-DNA polymerase epsilon, calytic subunit A	4.7 down
um10705	Cdk1—cyclin-dependent kinase 1	4.8 down
um04791	Related to G1/S-specific cyclin	5.9 down
um04129	Related to proliferation associated SNF2-like protein	4.8 down

Ruiz-Herrera 2013). Within the metabolism category, genes related to metabolism of lipids were mostly overexpressed, whereas the metabolism of amino acids and iron was mostly downregulated in the wild type, i.e. repressed by Gcn5 (Fig. 1). Five categories in which genes of importance to the phenomena examined here were grouped are the following: (i) cell cycle and DNA processing, with 8.2% (99 differential genes); (ii) transcription, with 4.4% (53 differential genes); (iii) cellular transport, with 10.3% (124 differential genes); (iv) cell rescue, with 2.1% (25 differential genes); and (v) biogenesis of cellular components, with 3.6% (43 differential genes) (See Fig. 1).

In the cell cycle and DNA processing category, several genes directly related to DNA synthesis or processing were mostly downregulated in the yeast form of the wild-type strain, i.e. they are negatively regulated by Gcn5. These include genes encoding polymerases, topoisomerases, helicases, primases, histones, elongases, DNA glycosylases, other nuclear proteins, chromosome condensation proteins, chromosome segregation proteins, DNA repair proteins, kinetochore proteins, cyclins, cell division proteins and genes encoding general chromatin factors (Table 1). These results agree with the observation that during mycelial growth induced by a change in pH, genes related to this

Table 2. Regulation by Gcn5 of genes encoding transcription factors.

Gene	Description	Fold change
um10540	Related to blue light-inducible Bli-3 protein	4.5 up
um02713	Prf1—pheromone response factor Prf1	4.4 up
um00113	Related to transcriptional activator acu-15	3.4 up
um12216	Related to Mig1 protein	2.5 up
um06308	Related to RFX1 major transcriptional repressor of DNA damage-regulated genes	2.2 up
um10426	PacC—transcription factor pacC	2.2 up
um12052	bE1—b mating-type locus, bE1 allele	2.2 up
um01523	Fox1—forkhead transcription factor required for pathogenic development	2.1 up
um00114	Related to SPT3—general transcriptional adaptor or coactivator	2.1 up
um03536	Related to zinc finger protein SFP1	2.0 up
um03588	Related to transcription factor medusa	2.0 up
um00264	Related to ZAP1—metalloregulatory protein involved in zinc-responsive transcriptional regulation	2.0 down
um05338	Related to transcription factor MBP1	2.0 down
um11110	Related to HAP1—heme activator protein	2.2 down
um04293	Related to ASG1—activator of stress genes	2.8 down
um11314	Related to GIS2—putative zinc finger protein, proposed to be involved in the RAS/cAMP signaling pathway	3.4 down
um10009	Related to ARO80—positive transcription regulator of ARO9 and ARO10	3.5 down
um02301	Related to C2H2-type zinc finger protein	8.4 down

Table 3. Regulation by Gcn5 of genes of the category of cell transport.

Gene	Description	Fold change
um03411	Probable endo-1,4-beta-xylanase	183.5 up
um03034	Related to HXT5—hexose transporter with moderate affinity for glucose	23.2 up
um10211	Related to EXG1—exo-beta-1,3-glucanase (I/II), major isoform	11.3 up
um04364	exg1—probable EXG1—exo-1,3-beta-glucanase precursor	6.3 up
um05731	Cmu1—secreted chorismate mutase	5.4 up
um02139	eff1–9—effector family protein Eff1–9	3.2 up
um02137	eff1–7—effector family protein Eff1–7	3.2 up
um01898	Related to endo-1,3(4)-beta-glucanase	3.1 up
um01796	eff1–1—effector family protein Eff1–1	2.6 up
um01375	Pit2—cysteine-protease inhibitor	2.5 up
um11377	eff1–2—effector family protein Eff1–2	2.4 up
um11322	Probable beta-1,3 exoglucanase precursor	2.4 up
um03313	eff1–3—effector family protein Eff1–3	2.0 up
um03924	Rep1—repellent protein 1 precursor	33.2 down

phenomenon are upregulated (Martínez-Soto and Ruiz-Herrera 2013).

Within the transcription category, some genes encoding transcription factors were found to be mostly upregulated in the wild type (Table 2); for example um02713, um06308, um10426 and um01523 genes; coding respectively for the pheromone response factor Prf1, RFX1-a major transcriptional repressor of genes regulated by DNA damage, the transcription factor PacC and the Fox1-Forkhead transcription factor required for pathogenic development; with 4.4-, 2.2-, 2.2- and 2.1-fold changes, respectively. Prf1 transcription factor is a key regulator of gene expression, and is involved in different *U. maydis* cellular processes such as mating, cell signalling, filamentous growth and pathogenesis. It induces the expression of the *a* and *b* genes, which in turn form the bE/bW heterodimer that regulates the entire fungus pathogenic process (Hartmann, Kahmann and Bölker 1996; and revised by Brefort *et al.* 2009; Ruiz-Herrera, Reynaga-Peña and Aréchiga-Carvajal 2009; Vollmeister *et al.* 2011; Ruiz-Herrera and Campos-Góngora 2012; León-Ramirez, Sánchez Arreguín and Ruiz-Herrera 2014). On the other hand, RFX1 protein has been described as a DNA binding protein that is involved in the regulation of the cell cycle in the fission yeast (Emery *et al.* 1996); and the PacC transcription factor has been well characterized and described as involved in the response to alkaline pH through the so-called Pal/Rim pathway (Cervantes-Chávez *et al.* 2010), as well as in pathogenesis in different fungi such as *Aspergillus nidulans*, *Fusarium oxysporum*, *Coletotrichum acutatum*, *Sclerotinia sclerotiorum* and its orthologous RIM101 in *Candida albicans* (Davis 2003; Rollins 2003; Ortoneda *et al.* 2004; Mitchell, Wu and Jackson 2007; You, Choquer and Chung 2007; Hua, Yuan and Wilhelmus 2010). In *U. maydis,* this transcription factor (Aréchiga-Carvajal and Ruiz-Herrera 2005) and even the entire Pal/Rim pathway (Cervantes-Chávez *et al.* 2010) have been described to be involved in pH sensing as occurs in other fungal species, but not in dimorphism and pathogenesis. The gene encoding this transcription factor is negatively regulated in *U. maydis* during its infection of *Arabidopsis thaliana*, and also during the fungus dimorphic transition induced by a pH change to acid conditions (Martínez-Soto and Ruiz-Herrera 2013; Martínez-Soto *et al.* 2013). Previously, the interaction between

Table 4. Regulation by Gcn5 of genes of the category of cellular rescue.

Gene	Description	Fold change
um06473	Related to cytochrome P450	29.7 up
um00205	Related to HSP12—heat shock protein	10.0 up
um03976	Related to PDR16—involved in lipid biosynthesis and multidrug resistance	4.0 up
um01899	Related to multidrug resistance protein	3.6 up
um11067	Probable catalase 2	3.4 up
um00154	Related to para-nitrobenzyl esterase	3.4 up
um01204	Related to PRY1—strong similarity to the plant PR-1 class of pathogen-related proteins	3.2 up
um06157	Related to Spherulin 4 precursor	2.6 up
um03888	Related to multidrug-resistant protein	2.6 up
um10368	Related to heat shock factor protein	2.4 up
um01982	Related to aminotriazole resistance protein	2.2 up
um05421	Related to multidrug resistance protein	2.2 up
um04851	Related to DOT5—nuclear thiol peroxidase	2.1 up
um10641	Related to SKT5—protoplast regeneration and killer toxin resistance protein	2.1 up
um05562	Related to stress response protein rds1p	2.1 up
um00355	Related to SGT1 protein	2.0 down
um01374	Membrane protein involved in tumor formation	2.1 down
um05393	Related to multidrug-resistant protein	2.4 down
um10170	Related to multidrug resistance protein 1	2.5 down
um03038	Related to candidate tumour suppressor dph2l1	2.5 down
um04915	Probable major allergen Mal f 1 precursor	3.2 down
um05074	Probable cytochrome P450 monooxygenase/phenylacetate hydroxylase	4.4 down

Table 5. Regulation by Gcn5 of genes involved in the biosynthesis of the cell wall.

Gene	Description	Fold change
um05811	Related to KRE6—glucan synthase subunit	8.5 up
um02758	Related to chitinase A precursor	2.9 up
um00638	Related to CDA2—sporulation-specific chitin deacetylase	2.2 up
um10120	Chs3—chitin synthase 3	2.1 up
um04357	Related to endo-1,6-beta-d-glucanase precursor	2.0 down
um05109	Related to ECM14—involved in cell wall biogenesis and architecture	2.1 down
um02840	Related to CHS5—chitin biosynthesis protein	2.1 down
um05550	Related to EXG1—exo-beta-1,3-glucanase	2.3 down
um10484	Related to ALG7—UDP-N-acetylglucosamine-1-phosphate transferase	2.5 down
um05769	Related to dolichyl-diphosphooligosaccharide–protein glycosyltransferase 67 kDa subunit precursor	2.5 down
um05036	Related to endo-1,3(4)-beta-glucanase	2.6 down
um11347	Related to GPI14—glycosylphosphatidylinositol-alpha 1,4 mannosyltransferase I	3.3 down
um05439	Related to chitin-binding protein	4.1 down
um06190	Related to chitinase	8.2 down
um03645	Related to beta-1,3-glucan-binding protein	12.8 down

Pal/Rim and MAPK pathways has been described, the latter involved in maintaining cell wall integrity (Fonseca-García, León-Ramírez and Ruiz-Herrera 2012). Finally, Fox1 is a transcription factor expressed during *U. maydis* biotrophic development, being specifically involved in virulence and tumour development in maize plants. Fox1 is regulated by Rbf1 a master transcription factor which in turn is controlled by the bE/bW heterodimer (Zahiri *et al.* 2010; revised by Vollmeister *et al.* 2011). Interestingly, the *FOX1* gene was positively regulated during infection of a *U. maydis* diploid strain in *A. thaliana*, demonstrating the similarity of the pathogenic processes developed by *U. maydis* on the experimental and natural hosts, as already pointed out (Martínez-Soto *et al.* 2013).

Within the cellular transport category, several genes involved in transport of compounds such as amino acids, sugars and lipids, as well as different genes encoding secretion proteins previously described as important in the *U. maydis* pathogenesis were differentially regulated by Gcn5 (Table 3). Of these genes, we can highlight the overexpression of genes encoding the Eff1–1, Eff1–2, Eff1–3, Eff1–7 and Eff1–9 effector proteins; polysaccharide-degrading proteins, for example endo-1,4-β-xylanase and EXG1-exo-β-1,3-glucanase; and the virulence factor Cmu1 (a secreted chorismate mutase; Djamei *et al.* 2011). The negative regulation of the repellent protein Rep1 is noticeable even more considering the high fold change observed. Repellent proteins play an important role during the formation of aerial structures (something not occurring under the culture conditions used), and virulence (Teertstra *et al.* 2009).

The observations described above that effectors, hydrolytic enzymes and a virulence factor are all positively regulated by the

GCN5 gene, most probably explain the loss of virulence by the *gcn5* mutant. In this sense, it is important to recall that effectors are secreted proteins enabling biotrophic fungi to prevent their recognition by the host immune system during the pathogenic process, and are thus involved in virulence (Hemetsberger *et al.* 2012). In addition, in the *U. maydis* genome several genes commonly regulated as a group, and described as pathogenesis clusters were previously reported (Kämper *et al.* 2006). Interestingly, some of these genes encode secreted proteins, of which some are effector proteins (Kämper *et al.* 2006; Doehlemann *et al.* 2009; Hemetsberger *et al.* 2012). When 11 of these genes were mutated, 9 of them showed a reduced virulence in maize whereas the two other mutants showed no phenotype (Khrunyk *et al.* 2010). Also, seven of these genes were also found to be upregulated during the *A. thaliana* infection by *U. maydis* (Martínez-Soto *et al.* 2013). The observation that five of these genes are positively regulated by *GCN5* is consistent with the observation that *gcn5* mutants are avirulent (Gonzalez-Prieto *et al.* 2014). The previously indicated observation that some genes encoding polysaccharide hydrolases are positively regulated by *GCN5*, e.g. the ones encoding an endo-1,4-β-xylanase (um03411) and those encoding three EXG1-exo-β-1,3-glucanases (um04364, um05550 and um10211), agrees with the concept that these enzymes are involved in the digestion of the host cell wall facilitating plant invasion by the fungus (Mueller *et al.* 2008; Geiser *et al.* 2013).

Within the cell rescue category, several genes involved in response to oxidative stress, drug resistance and heat shock were differentially regulated by *GCN5*, and most of them were upregulated (Table 4). In this sense it is important to mention that during the infection of *A. thaliana* by *U. maydis*, different stress response genes, cell rescue and defence mainly against plant resistance proteins and oxidative stress were differentially upregulated (Martínez-Soto *et al.* 2013). On the other hand, during the *U. maydis* yeast-to-mycelium dimorphic transition induced by acid pH, several genes related to cell stress were positively regulated (Martínez-Soto and Ruiz-Herrera 2013), a result that agrees with the observation that usually in dimorphic fungi under stress conditions, their dimorphic transition is induced (Banuett and Herskowitz 1994; Ruiz-Herrera *et al.* 1995; Ruiz-Herrera and Sentandreu 2002; Klose, de Sá and Kronstad 2004; Ruiz-Herrera, Reynaga-Peña and Aréchiga-Carvajal 2009; Ruiz-Herrera and Campos-Góngora 2012; Morales-Vargas, Domínguez and Ruiz-Herrera 2012; León-Ramirez, Sánchez Arreguín and Ruiz-Herrera 2014). The observation that several genes within this category were downregulated in the wild type (Table 4), and accordingly are downregulated by *GCN5*, may suggest their role in the growth of the wild type in the yeast form.

Within the category of biogenesis of cellular components, most repressed genes involved in the biosynthesis of the cell wall and membrane were grouped. Of the few overexpressed genes involved in the cell wall biosynthesis, we may cite um05811, um00638 and um10120, respectively, encoding a KRE6-glucan synthase subunit, a CDA2-sporulation-specific chitin deacetylase and chitin synthase 3 with 8.5-, 2.2- and 2.1-fold change, respectively (Table 5). Our working group previously reported the isolation and mutation of the *Chs3* gene, but no significant phenotypic abnormalities were observed (Xoconostle-Cázares, León-Ramírez and Ruiz-Herrera 1996), most probably because the function of the disrupted gene is substituted by other Chs protein (s) (*U. maydis* contains eight *CHS* genes; Ruiz-Herrera *et al.* 2008). In this sense, a complete transcriptomic analysis of the *U. maydis* enzymes involved in the synthesis of the cell wall during its dimorphic transition revealed the differential expression of several homologous genes of *KRE* and *CDA* (chitin deacetylase) (Robledo-Briones and Ruiz-Herrera 2013). These results suggest that only some aspects of cell wall synthesis and hydrolysis are under epigenetic control either positive or negative by Gcn5.

Table 6. Regulation by Gcn5 of genes involved in the biosynthesis of cell membrane.

Gene	Description	Fold change
um04742	Related to stomatin	11.0 up
um02222	Related to membrane protein Dik6	2.2 up
um00350	erg5—probable ERG5—C-22 sterol desaturase	2.0 up
um11875	Probable ERG20—farnesyl-pyrophosphate synthetase	2.1 down
um04127	Probable ERG26—C-3 sterol dehydrogenase (C-4 decarboxylase)	2.2 down
um11655	Related to BRL1—essential nuclear envelope integral membrane protein	2.2 down
um04374	Erg9—farnesyl-diphosphate farnesyltransferase	2.5 down
um05293	Probable oligosaccharyltransferase	3.0 down
um11962	Probable ERG24—C-14 sterol reductase	3.2 down
um01498	erg4—probable ERG4—sterol C-24 reductase	3.3 down
um03662	Erg11—sterol 14 alpha-demethylase	3.6 down
um01934	Erg2—C-8 sterol isomerase	9.8 down
um05068	Probable arylsulfatase	28.5 down

In relation to the category of cell membrane biosynthesis, it was interesting to observe the negative control of several genes involved in the biosynthesis of sterols (Table 6). An explanation for this result is difficult to find, we can only suggest that this change is probably related to cell shape, and that the mycelium form might be richer in ergosterol than the yeast form. Only genes encoding stomatin, peroxisomal membrane protein 20 and membrane protein Dik6 were positively regulated by Gcn5, with 11.0, 4.0 and 2.2 values of fold change, respectively. Stomatin was originally described as an integral protein of the erithrocyte membrane where its absence causes stomatocytosis, a rare haemolytic anaemia in humans (Stewart 1997), and further stomatin proteins have been shown to play a role in ion channel regulation and membrane trafficking. Peroxisomal protein 20 is involved in peroxisome biogenesis (Ma and Subramani 2009). Membrane protein Dik6 has been reported to be a virulence factor of *U. maydis*, and a target of the bE/bW heterodimer (Heimel *et al.* 2010). These results suggest the active role of Gcn5 in membrane synthesis and in the formation of peroxisomes involved in the metabolism of C_2 compounds.

As described above, analysis of the *U. maydis* genome revealed the existence of gene clusters in what were named 'pathogenic clusters' (Kämper *et al.* 2006). Interestingly, we also found that 131 genes regulated by Gcn5 were arranged into 41 clusters ranging from two to seven genes (Fig. 2). Some of these genes encode unclassified proteins, for which *in silico* search of domains was performed (see Table S4, Supporting Information). A total of 21 of these gene clusters were part of the pathogenic

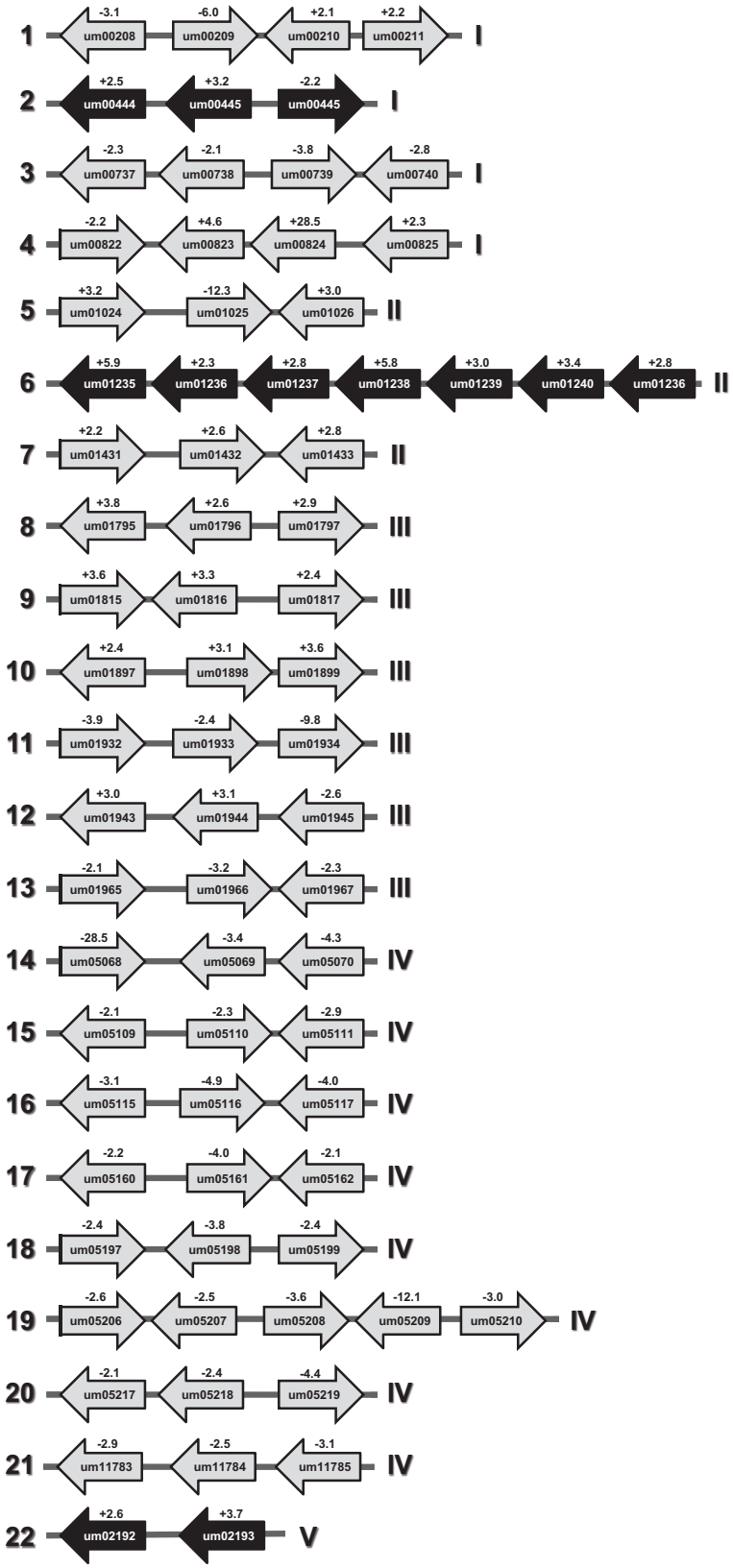

Figure 2. Schematic representation of the 41 gene clusters differentially regulated by *GCN5*. The arrows to right or left indicate the direction of gene transcription, but not genes sizes or the space between them. Text within each arrow is the gene ID. Black arrows represent genes previously described as clusters of pathogenesis by Kämper *et al.* (2006). White arrows represent genes previously described by Hewald *et al.* (2006) and Teichmann *et al.* (2007), as clusters involved in the biosynthesis of mannosylerythritol lipids and biosynthetic gene cluster for a secreted cellobiose lipid respectively. Numbers above arrows indicate positive or negative regulation by *GCN5*. Arabic numerals indicate cluster number as described in the text. Roman numerals indicate the number of chromosome in which the gene clusters are localized.

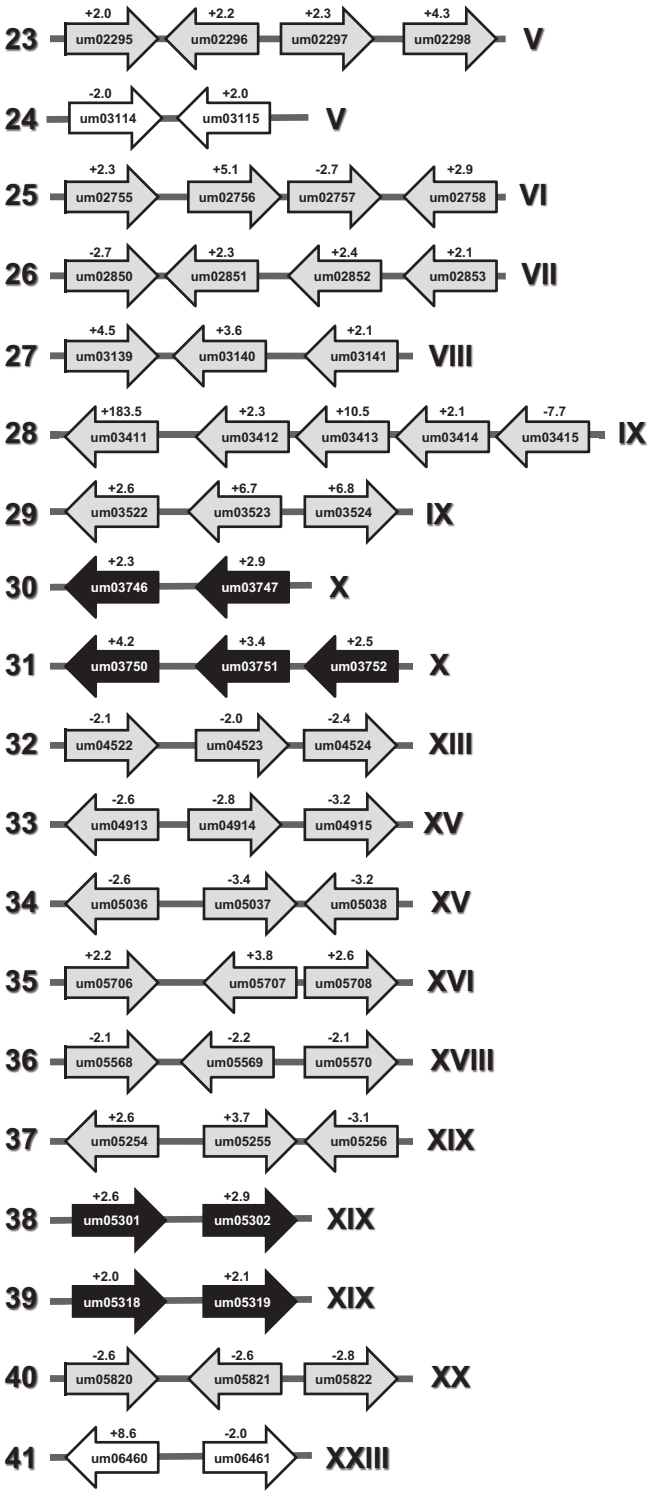

Figure 2. *(Continued)*

clusters described previously by Kämper *et al.* (2006) (Fig. 3). Accordingly, cluster 2 containing um00444, um00445 and um00446 genes is part of the cluster described as 1A; cluster 6, containing genes um01235, um01236, um01237, um01238, um01239, um01240 and um01241, is part of the cluster described before as 2A; cluster 22, containing genes um02192 and um02193, is part of the cluster described previously as 5A; clusters 30, containing genes um03746 and um03747 and cluster 31 including genes um03750, um03751 and um03752 are part of the cluster described before as 10A; and finally clusters 38 with genes um05301 and um05302, and cluster 39 containing genes um05318 and um05319, are part of the cluster 19A. Most of these genes were upregulated during the *U. maydis* pathogenic process on maize (Kämper *et al.* 2006) and *A. thaliana* (Martínez-Soto

et al. 2013), and some of these genes are expressed organ specifically, since they are essentials for tumour formation (Skibbe *et al.* 2010). Interestingly, in this work we also found that almost all of the identified genes were also upregulated, with the exception only of um00446 gene, which was downregulated by Gcn5. Mutation of some clusters served to demonstrate their role in maize pathogenesis, whereas in other cases, mutation did not affect virulence. For example, deletion of clusters 2 and 22 did not affect virulence, deletion of cluster 6 increased virulence, deletion of clusters 29 and 30 reduced virulence; and a more drastic reduction in virulence occurred by deletion of clusters 37 and 38 (Kämper *et al.* 2006). Overexpression of these genes grouped in clusters by Gcn5 is additional evidence demonstrating how *GCN5* gene is involved in *U. maydis* virulence, not only in maize, but also in *A. thaliana* infection, where some of them were found differentially regulated (Martínez-Soto *et al.* 2013). Interestingly, four of the genes differentially regulated in clusters by *GCN5* are part of previously described clusters (Hewald *et al.* 2006; Teichmann *et al.* 2007) as to be involved in the biosynthesis of mannosylerythritol lipids [um03114 (*mat1*) and um03115 (*mmf1*)] and a secreted cellobiose lipid [um06460 (*fas2*) and um06461 (*atr1*)], respectively (Fig. 2). We consider that possibly *GCN5* regulates the formation of biosurfactants and some other secondary metabolites.

In order to obtain information on the mechanism of regulation of the genes organized in clusters, and based on the *S. cerevisiae* genome, we searched for consensus sequences and binding sites for transcription factors in their promoter regions. In these analyses, we identified different consensus sequences and the transcription factors that bind to these sequences, the most represented were Gln3 (GATAA), Msn4 (AGGGG), Msn2 (AGGGG), Hap2 (TTGGT), Rmg1 (AGGGG) and Arg80 (AGACGC). Gln3 regulates the transcription of genes under nitrogen catabolite repression (Minehart and Magasanik 1991; Magasanik and Kaiser 2002); Mns4 and Mns2 are transcriptional regulators of stress-responsive genes (Martínez-Pastor *et al.* 1996; Görner *et al.* 1998; Sunnaker *et al.* 2013); Hap2 is part of a transcriptional regulatory complex of the expression of respiratory genes (Pinkham and Guarente 1985; Olesen and Guarente 1990); Rgm1 is a possible transcription factor involved in the expression of genes related with the catabolism of monosaccharides, aldehydes metabolism and telomeric and subtelomeric elements (Estruch 1991); and Arg80 is a transcription factor involved in gene regulation in response to arginine (Dubois, Bercy and Messenguy 1987).

Also we identified *U. maydis* homologues for transcription factors predicted on the *S. cerevisiae* genome, and some of them appeared differentially regulated by *GCN5*. For example, um04293 gene described as *ASG1* activator of stress genes is 2.8 times negatively regulated and is homologous to *S. cerevisiae ASG1*, a zinc cluster protein and regulator trascriptional involved in stress respose (Akache, Wu and Turcotte 2001; MacPherson, Lacochelle and Turcotte 2006); um00113 gene described as transcriptional activator Acu-15 is 3.4 times positively regulated is homologous to yeast *CAT8*, a zinc cluster transcriptional activator for derepression of several of gluconeogenic genes (Hedges, Proft and Entian 1995); um05338 gene described as transcription factor *MBP1* is 2.0 times repressed is homologous to yeast *MBP1*, a trascription factor involved in the cell cycle regulation (Koch *et al.* 1993); and um10426 gene described as transcription factor PacC (Aréchiga-Carvajal and Ruiz-Herrera 2005) is 2.2 times positively regulated and homologous to yeast *RIM101*, a CysHis2 zinc finger transcriptional repressor involved in the adaptation to alkaline growth conditions, cell wall assembly and sporulation (Lamb and Mitchell 2003; Castrejon *et al.*

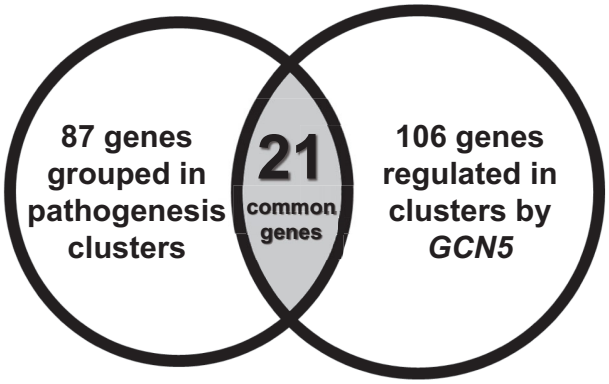

Figure 3. Venn diagram showing that 21 of the genes grouped in clusters differentially regulated by *GCN5* also belong to the clusters of pathogenesis described by Kämper *et al.* (2006).

2006). Some of the transcription factors described here have been previously described for *U. maydis* by genetic, transcriptomic or bioinformatics analysis. For example, transcription factor Hap2 is activated by the MAPK pathway, regulates master transcription factor Prf1, and its mutation affects mating and pathogenesis of the fungus (Mendoza-Mendoza *et al.* 2009; Brefort *et al.* 2009; Vollmeister *et al.* 2011; Martínez-Soto *et al.* 2013).

Some further results provide a possible explanation of the role of *GCN5* on *U. maydis* morphogenesis. Thus, we found that the 154 genes differentially regulated during the *U. maydis* dimorphism induced by pH change (Martínez-Soto and Ruiz-Herrera 2013) were also differentially regulated by Gcn5. Interestingly, most of the overexpressed genes during the mycelial growth were negatively regulated by Gcn5, and most of the overexpressed genes during yeast growth were also overexpressed by Gcn5 (see Fig. 4 and Table S5, Supporting Information). According to these data, *GCN5* is required for the expression of genes involved in yeast growth of the fungus, and represses the ones involved in mycelial growth. Therefore, when *GCN5* is mutated, the genes involved in yeast growth are repressed, and those necessary for mycelial growth are constitutively expressed. As a result, the mutant grows constitutively in the form of mycelium (González-Prieto *et al.* 2014). Previously, we identified by a proteomic analysis two proteins whose expression was regulated by Gcn5, and that were specifically involved in the *U. maydis* dimorphism (Martínez-Salgado *et al.* 2013). The first one, an Hmp1 (,ismatch base pair and cruciform DNA recognition protein) encoded by the gene um00496, and the second one, an uncharacterized protein encoded by the gene um03284. These proteins were up- and downaccumulated respectively in the fungus mycelial form. Interestingly, and according to the data reported here, they were respectively negatively and positively regulated by Gcn5, indicating that um00496 is involved in yeast growth, and um03284 in mycelial growth in *U. maydis*.

In summary, our data are evidence that histone acetylation by Gcn5 is involved in the transcription of genes related to pathogenesis, virulence and yeast-like fungal growth in *U. maydis*. These data described here provide evidence to explain the epigenetic mechanism through wich the *GCN5* gene encoding a HAT regulates these processes. They also demonstrate the benefits of massive transcription analysis comparing wild type and specific mutants, by means of the use microarrays.

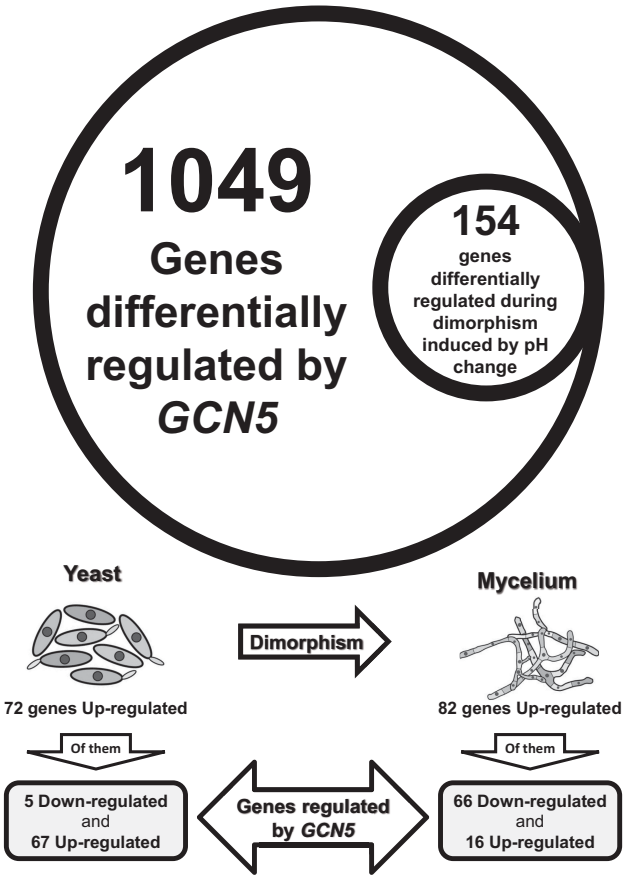

Figure 4. Venn diagram showing that all genes differentially regulated during *U. maydis* dimorphism induced by pH change are also differentially regulated by GCN5. Most overexpressed genes in the yeast growth are positively regulated by GCN5, and most overexpressed genes in the mycelial growth are negatively regulated by *GCN5*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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