RESEARCH PAPER

Plant Signaling & Behavior 8:8, e25059; August 2013; © 2013 Landes Bioscience

Transcriptomic analysis of *Ustilago maydis* infecting *Arabidopsis* reveals important aspects of the fungus pathogenic mechanisms

Domingo Martínez-Soto,† Angélica M. Robledo-Briones,† Andrés A. Estrada-Luna and José Ruiz-Herrera*

Departamento de Ingeniería Genética; Unidad Irapuato; Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional; Irapuato, Gto México

†These authors contributed equally to this work.

Keywords: microarrays, experimental pathosystem, *Ustilago maydis, Arabidopsis thaliana*, plant virulence, necrotrophic fungus

Abbreviations: dpi, days post-infection; DW, dry weight; FDR, false discovery rate; FunCat, functional catalogue; RMA, robust multi-array analysis; SDW, sterile distilled water

Transcriptomic and biochemical analyses of the experimental pathosystem constituted by *Ustilago maydis* and *Arabidopsis thaliana* were performed. Haploid or diploid strains of *U. maydis* inoculated in *A. thaliana* plantlets grew on the surface and within the plant tissues in the form of mycelium, inducing chlorosis, anthocyanin formation, malformations, necrosis and adventitious roots development, but not teliospores. Symptoms were more severe in plants inoculated with the haploid strain which grew more vigorously than the diploid strain. RNA extracted at different times post-infection was used for hybridization of one-channel microarrays that were analyzed focusing on the fungal genes involved in the general pathogenic process, biogenesis of the fungal cell wall and the secretome. In total, 3,537 and 3,299 genes were differentially expressed in the haploid and diploid strains, respectively. Differentially expressed genes were related to different functional categories and many of them showed a similar regulation occurring in *U. maydis* infecting maize. Our data suggest that the haploid strain behaves as a necrotrophic pathogen, whereas the diploid behaves as a biotrophic pathogen. The results obtained are evidence of the usefulness of the *U. maydis-A. thaliana* pathosystem for the analysis of the pathogenic mechanisms of *U. maydis*.

Introduction

The use of alternative hosts to understand pathogenic processes is a strategy widely used since the earliest microbiological studies on human diseases. More recently, these studies extended to fungal infections, e.g., rabbits and mice have been used as hosts to study fungal human pathogens as *Cryptococcus neoformans* and *Candida albicans*[1,2] and even *Drosophila melanogaster* has served as alternative host for studying human mycoses.[3] Additionally, model plants are used to study fungal pathogens of commercial interest. Thus *Nicotiana benthamiana* was employed as alternative host for mutants of *Colletotrichum orbiculare*, a pathogen of melon and cucumber[4] and *Brachypodium distachyon* was used as alternative host of *Magnaporthe grisea*, a rice and barley pathogen.[5] Additionally, in the pathosystems *Cryptococcus-Arabidopsis* and *Cryptococcus-Eucalyptus,* the human pathogen completed its sexual cycle.[6]

Similarly, our research group demonstrated that *Ustilago maydis* infected different plant species, unrelated to its natural host, *Zea mays* under axenic conditions,[7] establishing the *Ustilago maydis-Arabidopsis thaliana* pathosystem as model to study some virulence aspects of the fungus.[8] *A. thaliana* has the attractive characteristics of its small size, short life cycle and its complete genome sequence. Furthermore, *Arabidopsis* is a model for plant-pathogen interactions.[9,10]

U. maydis is a pathogen of maize (*Zea mays*) and teozintle (*Zea mays ssp parviglumis*), where it completes its known sexual life cycle. This starts when two haploid yeast-like cells of compatible mating types fuse giving rise to a dikaryotic hypha that infects the plant. Inside the host, the mycelium undergoes morphological changes eventually giving rise to teliospores that accumulate within tumors induced by the pathogen. Teliospores germinate to form haploid basidiospores that reinitiate the life cycle.[11,12] Interestingly, we demonstrated that *U. maydis* performs a completely different sexual life cycle with the surprising formation of basidiocarps, when incubated under defined environmental conditions.[13]

Considering the complexity of the sexual-pathogenic process of *U. maydis* in maize, the *Ustilago maydis-Arabidopsis* pathosystem appears as an attractive alternative, since no sexual cycle

*Correspondence to: José Ruiz-Herrera; Email: jruiz@ira.cinvestav.mx
Submitted: 03/27/13; Revised: 05/15/13; Accepted: 05/16/13
Citation: Martinez-Soto, D, Robledo-Briones AM, Estrada-Luna AA, Ruiz-Herrera J. Transcriptomic analysis of *Ustilago maydis* infecting *Arabidopsis* reveals important aspects of the fungus pathogenic mechanisms. Plant Signal Behav 2013:8: e25059; http://dx.doi.org/10.4161/psb.25059

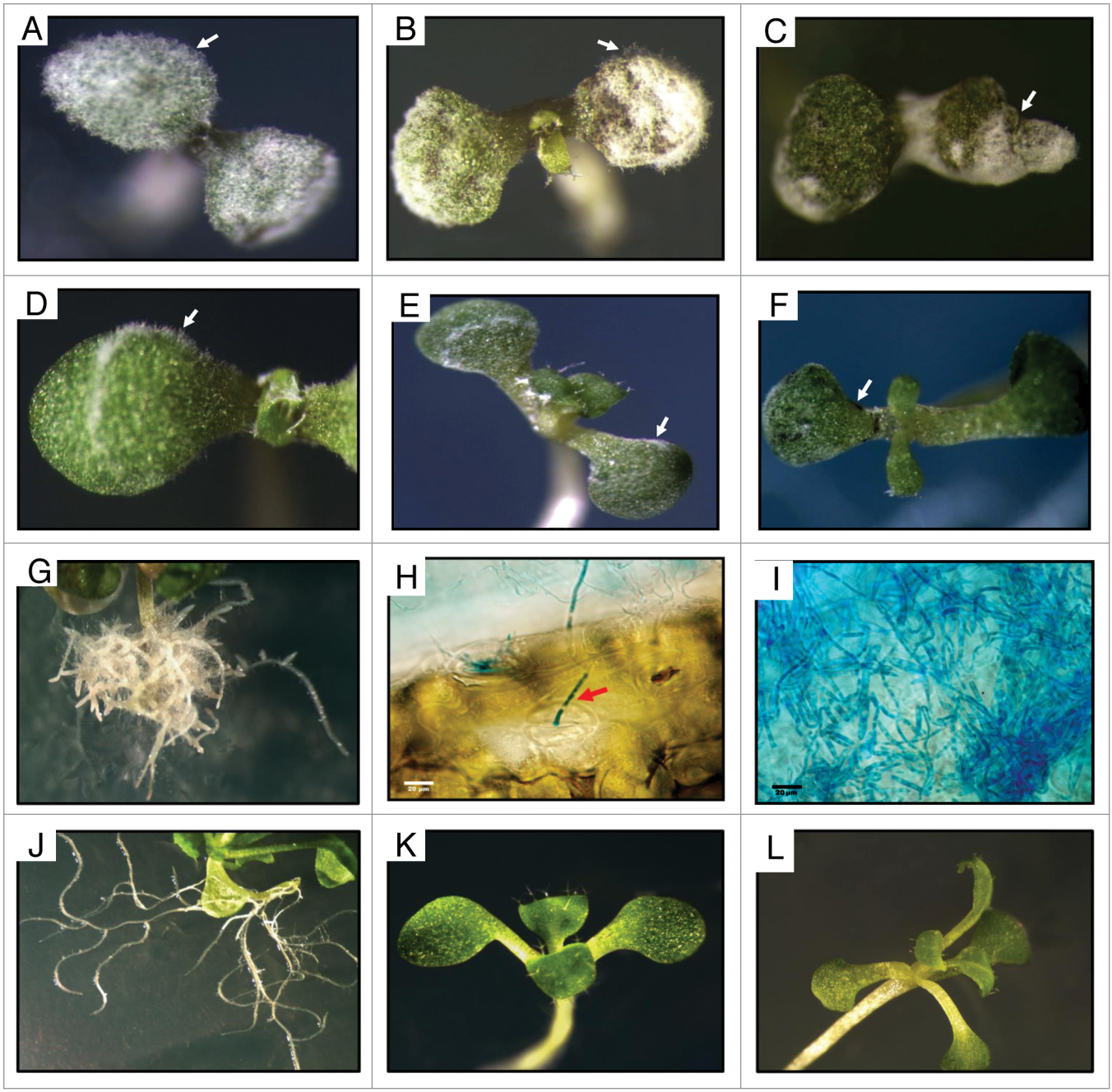

Figure 1. Appearance of *A. thaliana* plantlets infected with *U. maydis* strains. General aspect of plants inoculated with the FB2 haploid strain (**A–C**), or the Uid1 diploid strain (**D–F**) at 2, 4 or 8 d post-infection respectively. Notice in (**B**) the necrotic area (arrow), in (**C**) necrotic tissue detachment (arrow) and in (**F**) necrotic tissue points in the infected plantlets. (**G**) Severe alteration in the root system in haploid-infected plantlets 20 dpi. (**H**) Mycelium of the haploid strain entering through stomata (red arrow). (**I**) mycelium of the haploid strain growing within the infected tissue. (**J–L**) Aspect of roots and leaves of control not-inoculated plantlets. Scale bar in (**H**) and (**I**), 20 μm.

occurs, and haploid strains are pathogenic. In this study we analyzed the infection of *Arabidopsis* by haploid or diploid strains of *U. maydis* and compared the transcriptomic differences of both strains to identify factors that explained the specificity of maize infection by dikaryons or diploids and to define similarities and differences in the infection of *Arabidopsis* and maize.

Results

Symptoms in plantlets infected with *U. maydis* strains. Haploid or diploid strains of *U. maydis* infected *Arabidopsis* plantlets as described,[8] but the haploid induced more severe symptoms. Aerial mycelium of the haploid strain developed at the inoculation sites

after 24 h, spreading to other regions (**Fig. 1A and B**, arrows), necrotic areas developed 4 d post-infection (dpi) and tissue detachment at 8 dpi (**Fig. 1B and C**, respectively, arrows). Mycelium developed extensively within plant tissues (**Fig. 1I**), possibly penetrating through stomata (**Fig. 1H**, red arrow). The haploid strain also induced severe alterations in the root system at 20 dpi (**Fig. 1G**). On the other hand, plantlets infected with Uid1 developed scant mycelium at early periods (**Fig. 1D and E**) and necrosis points only after 8 dpi (**Fig. 1F**, arrow).

Effect of *U. maydis* inoculation on growth of *Arabidopsis* plantlets. Growth of *Arabidopsis* plantlets infected with the haploid was reduced approximately from 50 to 90 per cent after 4 dpi, whereas diploid-infected plantlets were similar to controls until 12 dpi, before slowing down. Dry weight (DW) of all plantlets was similar until 4 dpi, but thereafter DW of haploid-inoculated plantlets remained constant, while that of diploid-inoculated plantlets remained similar to controls until 8 dpi, after which they showed similar growth as the control plantlets (**Fig. 2**).

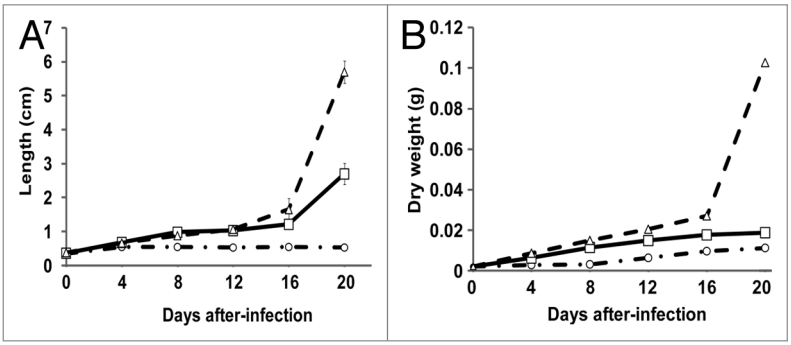

Figure 2. Growth of *A. thaliana* plantlets infected with *U. maydis* strains. (**A**) Plantlets length and (**B**) plantlets dry weight. Dotted lines with circles, plantlets infected with the haploid strain. Solid lines with squares, plantlets infected with the diploid strain. Dotted lines with triangles, control plantlets that received sterile distilled water. Results from three independent experiments with six plants in each one. Results expressed in cm or g per plantlet respectively in each graph. Bars represent standard error values.

Growth of *U. maydis* within *Arabidopsis* plantlets. Growth of *U. maydis* was measured by ergosterol and chitin, compounds that are present in fungi, but absent in plants. Amounts of chitin and ergosterol in plants infected with either strain were similar at 4 dpi, but afterwards, the levels of both compounds were higher in haploid-infected plants, whereas they decreased in diploid-infected plants, suggesting curtailment of fungal growth by the plant, something not occurring with the haploid strain. As expected, in the un-inoculated plants, no ergosterol or chitin were detected (**Fig. 3**).

Differential gene expression in *U. maydis* strains infecting *Arabidopsis*. Analysis of the genes differentially expressed in *Ustilago* strains during *Arabidopsis* infection was compared with their expression in yeast-like cells grown in liquid culture. As considered significant in most microarray analyses, a two-fold change up or down was used to consider differential expression of genes. The results obtained revealed that in the haploid 2,636 genes were differentially expressed at 1 dpi, 1,294 up-regulated and 1,342 down-regulated. In the diploid the corresponding numbers were 2,389; 1,170 and 1,219, respectively. In both cases the numbers remained almost constant during the following dpi (**Table 1**). These numbers reveal the extreme changes occurring when *U. maydis* changes its mode of life from saprophytic to pathogenic. The total number of genes differentially expressed along the infection process were 3,537; 1,703 up-regulated and 1,834 down-regulated in the haploid and 3,299; 1,621 and 1,678 respectively in the diploid; genes commonly regulated in both strains were respectively, 696, 407 and 289. **Table S1** describes the genes with the highest difference in expression (at least a twenty-fold change up or down).

Functional classification of differential genes. Functional grouping of the differentially expressed genes in either strain gave similar data (**Fig. 4**). The categories with higher gene numbers were related to metabolism, energy and metabolism regulation (19.1% in the haploid and 18.7% in the diploid of the total differential genes), transcription, protein synthesis and fate (respective values of 14.9% and 14.1%). The categories with lower gene numbers were interaction with the environment (respectively, 5.3% and 5.4%) and cell differentiation (respectively, 2.9% and 2.9%) (**Fig. 4**).

Classes of differentially regulated genes. Considering the high number of differentially regulated genes during *Arabidopsis* infection, we investigated their nature according to classes related to pathogenicity, some of which are regulated during maize infection.

Genes regulated by the bE/bW heterodimer. It has been described that *U. maydis* infection in maize by heterokarions or diploids, is regulated by a heterodimer made of the *bE* and *bW* gene products coming from the two sexually-interacting strains; this heterodimer acts as a master transcription factor for a number of genes[11,14,15] and it has been hypothesized that it is also involved in the *U. maydis* biotrophic phase.[16] Accordingly, we analyzed their regulation during *Arabidopsis* infection considering that only the diploid contains the heterodimer. *PRF1,* the gene directly regulating transcription of the heterodimer was repressed in the haploid, whereas no differential expression was observed in the diploid. Of the genes directly regulated by the heterodimer: *CLP1* (required for intracellular hyphal proliferation), *FRB52* (of unknown function) and *RBF1*,[16] only *RBF1* was up-regulated in the diploid (**Table 2**). In turn, Rbf1 regulates *BIZ1, HDP1, HDP2* and *FOX1* encoding transcription factors and two additional proteins: *KPP6* and *PCL12*. *HDP1* was up-regulated only in the haploid, *FOX1* was up-regulated in the diploid and down-regulated in the haploid, whereas no differential expression of *BIZ1* and *HDP2* was observed in any strain. *PCL12* and *KPP6* were up-regulated in both strains (**Table 2**).

Genes belonging to 12 pathogenicity clusters. Expression of genes belonging to the twelve pathogenic clusters in the diploid strain was similar to the one observed in maize.[11,15] Other genes, especially those belonging to cluster 2A whose deletion increases

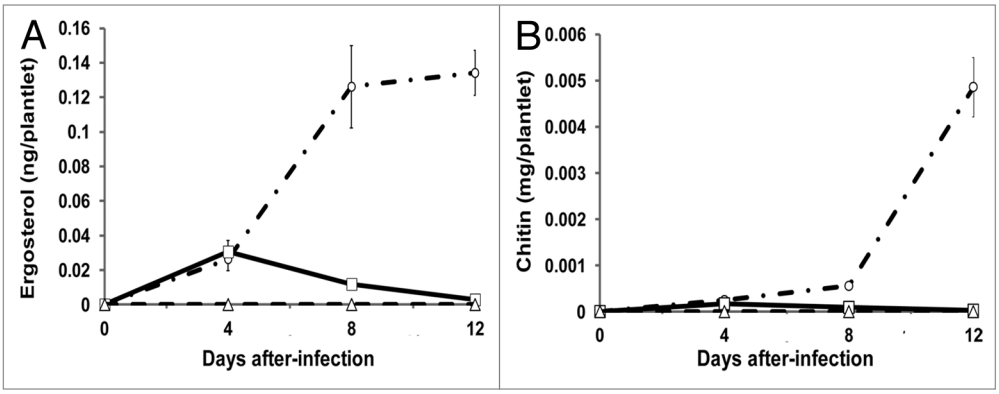

Figure 3. Development of *U. maydis* within infected *A. thaliana* plantlets. (**A**) Ergosterol determination. (**B**) Chitin determination. Empty circles with dotted lines, plantlets infected with the haploid. Empty squares with solid lines, plantlets infected with the diploid. Empty triangles with dotted lines, control plantlets that received sterile distilled water. Results from three independent experiments with 100 plant batches in each one. Results expressed as ng ergosterol or mg GlcNAC per plantlet. Bars represent standard error values.

virulence, were down-regulated in the haploid and up-regulated in the diploid as occurs in maize (**Table S2**).

Effector genes. Effectors are proteins secreted by plant pathogens that manipulate the physiology of their hosts to their benefit.[17] In biotrophic fungi, these effectors accumulate in the zone of interaction with the host inducing the suppression of plant immune responses.[18] Fox1, controlling the expression of effector-encoding genes in *U. maydis*,[19] specifically *PEP1*, the *EFF1* family and *PIT2*,[20-22] was up-regulated in the diploid and down-regulated in the haploid. *PIT2* was down-regulated in the haploid and was not differentially expressed in the diploid; *EFF1-5 to EFF1-11* were up-regulated in the diploid, whereas in the haploid *EFF1-6* and *EFF1-8* were not differentially expressed and *EFF1-7* was down-regulated. *EFF1-9, EFF1-10 and EFF1-11* were up-regulated but at most at two different dpi. In contrast, *EFF1-1* to *EFF1-4* and *PEP1* were not differentially expressed in either strain (**Table 3**).

Genes encoding hydrophobins and repellent proteins. Hydrophobins and repellent proteins are involved in *U. maydis* virulence in maize. *HUM2* has essential functions in maize infection and *REP1* is involved in aerial hyphae formation.[23,24] We found that during *Arabidopsis* infection *REP1, HUM2* and *HUM3* were up-regulated in the haploid strain, particularly *REP1* (39.3 of fold change at 1 dpi). In contrast, only *REP1* and *HUM2* were up-regulated in the diploid (not shown).

Genes encoding transcription factors. We identified 111 genes encoding potential transcription factors differentially expressed during *Arabidopsis* infection. In the haploid, 45 were up-regulated and 66 down-regulated. In the diploid 47 were up-regulated and 63 down-regulated. Seventy-two genes were up or down-regulated in common in both strains (27 up and 45 down-regulated). In the haploid, gene um02301 was highly over-expressed (25.5-fold change) and um06283 was highly repressed (15.1-fold change). In the diploid, genes um00841 and um05966 were highly over-expressed (9.0 and 7.7 of fold change, respectively), whereas um06283 and um04208 were severely repressed (8.7 and 11.1 of fold change, respectively) (**Table S3**). The importance of regulation of transcription factors during maize infection is known,[16,25] in agreement with our data on *Arabidopsis* infection.

Other genes reported as involved in maize infection. Around fifty genes have been described to be involved in maize infection by *U. maydis*, as deduced from the phenotype of their corresponding mutants. In *Arabidopsis* infection we identified that 21 of them were differentially regulated. In the haploid, 12 were up-regulated and 9 were down-regulated and in the diploid 8 were up-regulated and 10 down-regulated (**Table 4**).

Genes involved in the synthesis and structure of the cell wall. Genes involved in the synthesis of structural polysaccharides. In the haploid, *CHS1* one of the genes encoding chitin synthases, was up-regulated during the whole infection period, whereas *CHS3* was down-regulated, but only at 8 dpi. In the diploid, no *CHS* gene was differentially expressed. Genes involved in the synthesis of β-1,3- or β-1,6-glucans behaved differently; *GLS* encoding β-1,3-glucan synthase was constitutively expressed in both strains, but of the genes involved in β-1,6-glucan synthesis, *CWH41* and *ROT2* homologs and three *KRE6* homologs were up-regulated (at least at one dpi), whereas three other homologs of *KRE6* were down-regulated in the haploid. In the diploid, two *KRE6* homologs were up-regulated and two down-regulated (**Table 5**). Genes encoding chitin deacetylases (*CDAs*), enzymes involved in chitosan synthesis, were up-regulated in both strains, but their fold changes were greater in the haploid. In contrast, three other *CDA* genes were down-regulated in the haploid and only two of them in the diploid (**Table 5**).

Genes involved in polysaccharide biosynthesis precursors. One gene encoding a hexokinase and one encoding a *N*-glucose acetyl transferase involved in the synthesis of uridine diphosphate GlcNAc (UDP-GlcNAc) were up-regulated in both strains, whereas a gene encoding a phosphoacetylglucosamine mutase was down-regulated in both strains. Genes involved in the synthesis of uridine diphosphoglucose (UDPGlc) and guanosine diphospho manose (GDPMan) were also differentially expressed, thus, the gene encoding UDP-glucose-hexose-1-phosphate uridylyltransferase was up-regulated in the diploid strain and the gene encoding mannose-1-phosphate guanylyltransferase showed

Table 1. Differential gene expression in *U. maydis* strains during infection of *Arabidopsis thaliana* plantlets

Strain	Genes	Days post-infection			
		1	2	4	8
Haploid (FB2)	Differential total	2,636	2,315	2,503	2,320
	Upregulated	1,294	1,132	1,216	1,152
	Downregulated	1,342	1,183	1,287	1,168
Diploid (Uid1)	Differential total	2,389	2,420	2,162	2,162
	Upregulated	1,170	1,203	1,084	1062
	Downregulated	1,219	1,217	1,078	1,100

a decrease in the level of transcription in the same strain (not shown).

Genes involved in the synthesis of glycoproteins. In the haploid, almost all the genes involved in the synthesis of the *N*-linked glycan moiety of glycoproteins were up-regulated, with the exception of *SEC59*, encoding a dolichol kinase, that was down-regulated, same as happened in the diploid, where only a few genes were up-regulated (**Table 6**). In contrast, fewer genes involved in the synthesis of the O-glycan moiety of glycoproteins were differentially regulated. Of *PMT* genes encoding enzymes that transfer the first mannose to Ser/Thre residues of proteins, only *PMT4* was up-regulated in the haploid and genes involved in further addition of mannosyl units (*KRE2* homologs) were up-regulated in both strains (**Table 6**).

Genes *GPI12*, *GPI14* and *GUP1* involved in the synthesis of the GPI anchor of GPI proteins were up-regulated in both strains. Additionally, *GPI17* and *GPI18* were up-regulated and *PER1* down-regulated in the haploid, whereas in the diploid *GPI1 and GPI8* were up-regulated and *GPI3* was down-regulated at different dpi (**Table 7**).

Genes encoding GPI proteins and proteins of the secretome. CDA genes and other genes encoding GPI-hydrolytic enzymes were up-regulated in both strains (not shown), and numerous genes encoding secreted proteins classified as unknown or with degradative, synthetic, redox or non-enzymatic functions were differentially expressed in either strain. A greater number of genes encoding enzymes that degrade polysaccharides, lipids and proteins were up-regulated, in the haploid, as compared to the diploid strain (**Table 8**).

Discussion

Ustilago maydis is a useful model for understanding phenomena of fungal pathogenesis,[11,12] and *Arabidopsis thaliana* is an excellent model for understanding plant responses to pathogens.[10] For these reasons, our working group established the experimental pathosystem *Ustilago maydis-Arabidopsis thaliana*, as model system for understanding some aspects of *Ustilago* pathogenesis.[8] In this work we analyzed the transcriptome of *U. maydis* infecting *Arabidopsis* plantlets comparing a diploid and a haploid strain of the fungus, considering that diploids (as Uid1, used in this work) and heterokaryons, but not haploids, are infective in maize, but both infect *Arabidopsis*.

We observed a drastic alteration in gene expression in *U. maydis* during *Arabidopsis* infection, in numbers similar to maize infection,[15] corresponding to more than one third of the whole genome. These numbers indicate that *U. maydis* drastically alters the levels of transcription of a great number of genes to cope with the changes taking place during its adaptation from a more or less comfortable environment where its nutritional necessities are satisfied, to the harsh conditions existing in the host, where it has to deal with the plant defenses and struggle for nutrients.

The damage caused to *Arabidopsis* plantlets by the haploid was noticeably more severe than that caused by the diploid and drastically inhibited plant growth, besides causing severe alterations in their roots. Growth of the haploid in the plant, measured by two specific parameters: ergosterol and chitin accumulation was also more abundant than growth of the diploid. These results reveal the higher virulence of the haploid, suggesting that it behaves as a necrotrophic parasite in *Arabidopsis*. These phytopathogens overturn the host defense mechanisms, destroy the plant,[26] and are resistant to host hypersensitive reactions.[27] In contrast, the diploid behaves as a biotrophic pathogen, as occurs in maize. This suggestion is supported by several pieces of evidence, e.g., the up-regulation in the diploid, contrasting with the haploid of *RBDF1* (a master regulator required for all b-dependent processes) controlled by the bE/bW heterodimer and the genes that it regulates.[16] Moreover, of the genes regulated by Rbf1, *HDP1*, involved in filamentous growth, was up-regulated only in the haploid, and *FOX1* that regulates genes encoding effector proteins, was down-regulated in the haploid and up-regulated in the diploid. These data reveal, firstly, that genes encoding both transcription factors, as well as genes encoding Pcl12 and Kpp6, can be regulated by mechanisms alternative to Rbf1, and more important, that the opposite effect on *HDP1* and *FOX1* may be related to the different pathogenic behavior of both strains, biotrophic or necrotrophic. Additionally, a larger number of effectors, important for the biotrophic stage, were up-regulated in the diploid and were even down-regulated in the haploid. Further evidence to explain the different behavior of both strains and data of gene expression revealing homologies and differences in the pathogenic behavior of *U. maydis* in maize and *Arabidopsis* are described below.

In this sense, we must indicate that genes encoding transcription factors *TUP1* and *NIT2* involved in maize infection,[28,29] were also regulated during *Arabidopsis* infection, *TUP1* being down-regulated in both strains and *NIT2* repressed only in the diploid strain. Tup1 is repressed during maize infection and *NIT2* disruption reduced virulence in maize. Behavior of homologs of genes encoding transcription factors related to virulence in different fungi was also significant: two homolog genes of *HSF1* of *C. albicans* (that is up-regulated during formation of invasive mycelium)[30,31] and are up-regulated in *U. maydis* infecting maize, were differentially expressed only in the haploid, one up and the other one down-regulated. Additionally, a homolog of *SKN7* encoding a transcription factor required for virulence in *C. albicans* and *C. neoformans*[32,33] was up-regulated only in the haploid strain. These data provide evidence of the similarity in the pathogenic mechanisms of *U. maydis* in maize and *Arabidopsis*.

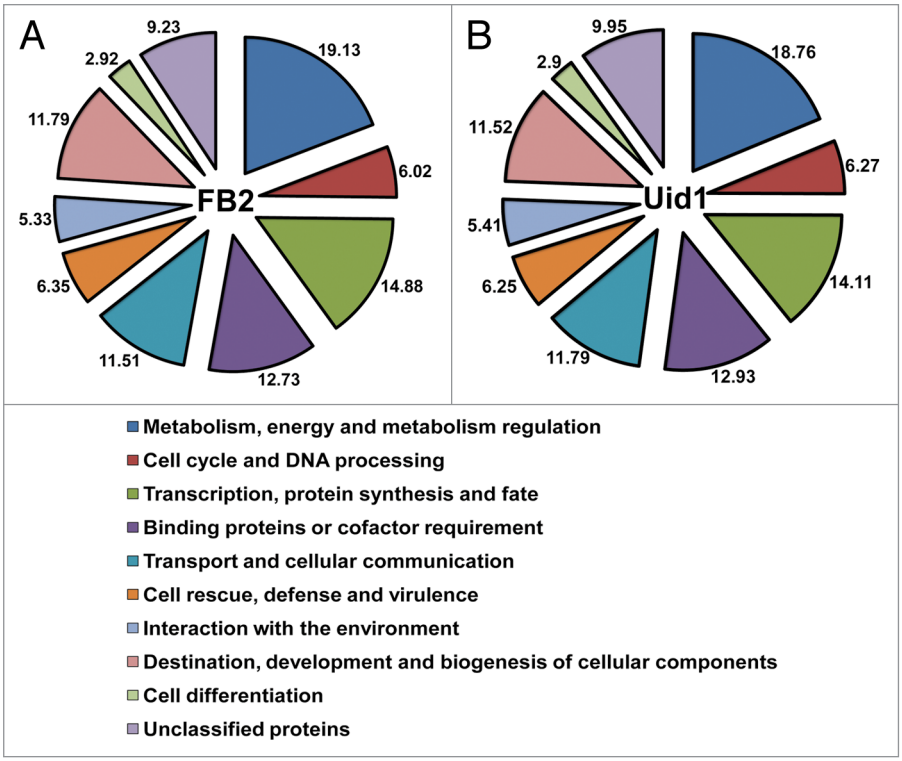

Figure 4. Functional categorization of *U. maydis* genes regulated during the infective process of *Arabidopsis*. (**A**) Differentially regulated genes in the haploid. (**B**) Differentially regulated genes in the diploid. Numbers represent the percentage of genes in a given functional category and text corresponds to the names of each category.

Functional grouping identified only a limited number of genes involved in pathogenesis, as occurs in maize.[34] This result agrees with the knowledge that *U. maydis* possesses fewer pathogenesis genes than other phytopathogenic fungi,[11] possibly in relation to its biotrophic lifestyle. Among the factors involved in pathogenesis, genes encoding G proteins and transcription factors can be cited. The number of genes involved in cell rescue and defense that may be involved in response to stress and in detoxification was small, and interestingly, were mainly up-regulated in the haploid. Worth mentioning is that genes involved in response to stress, such as oxidases were repressed in the diploid, contrasting with the haploid where significant numbers of genes responding to oxidative stress were up-regulated, possibly as a defense mechanism. Other up-regulated genes mainly in the haploid strain are involved in homeostasis, cell migration, chemotaxis, mechanical stimulus perception and perception and response to nutrients. These functions are important for the formation of intracellular hyphae involved in acquisition of nutrients, signaling, communication and avoidance.[35]

The process of *Arabidopsis* invasion, as occurs in maize, involves cell differentiation and dimorphic transition of yeast cells to invading hyphae. This process involves changes in expression of genes involved in cell wall biogenesis.[36] Accordingly, we analyzed the regulation of genes involved in this process. Some genes encoding glucanases or chitinases, probably involved in structural changes of the wall were differentially expressed during *Arabidopsis* infection. The higher number of *CDA* genes up-regulated in comparison to *CHS* genes, agrees with the observation that the hyphal surface of invasive biotrophic rust fungi, contains chitosan instead of chitin,[37] probably because chitosan, in contrast to chitin, lacks elicitor activity.

Expression of *GLS* encoding the single β-1,3-glucan synthase of *Ustilago* was constitutive during infection by both strains, agreeing with data from *in vitro* dimorphism or maize infection.[38] In contrast homologs of *ROT2* and *CWH41* from the pathway of β-1,6-glucans synthesis and *N*-glycosylation were up-regulated during *Arabidopsis* infection only in the haploid. Considering that mutants of the six homologs of *KRE6/SKN1* in *C. neoformans* were avirulent to mouse,[39] the data reveal the importance of β-1,6-glucans synthesis in fungal pathogenesis, agreeing with the more aggressive behavior of the haploid strain.

Increased protein glycosylation is required for fungal-host interaction and virulence.[40] Accordingly, genes involved in the synthesis of *N*-glycans such as *CWH41* and *ROT2* and *MSN1*

Table 2. Differential expression of genes regulated by the bE/bW heterodimer during *U. maydis* infection of *A. thaliana*

Identity		Gene	Description	Regulation	
				Haploid strain FB2	Diploid strain Uid1
um03172		Rbf1	Related to Zinc finger protein	-	up
um02438		Clp1	Related to clp1, essential for A-regulated sexual development	-	-
um01262		Frb52	DNA polymerase X-putative	up	up
um02331		Kpp6	MAP kinase	up	up
um02549		Biz1	b-induced zinc finger protein	-	-
um12024	um05762	Hdp1	Potential homeodomain transcription factor	up	-
um04928		Hdp2	Uncharacterized protein	-	-
um10529	um10529.2	Pcl12	b-dependently cyclin	up	up
um01523		Fox1	Transcriptional regulator	down	up

Table 3. Differential expression of genes encoding *U. maydis* effector proteins during *Arabidopsis* infection

Identity	Gene	Regulation in haploid (FB2)				Regulation in diploid (Uid1)			
		1 dpi	2 dpi	4 dpi	8 dpi	1 dpi	2 dpi	4 dpi	8 dpi
um01375	Pit2	2.0 down	2.2 down	-	-	-	-	-	-
um02135	Eff1-5	-	-	3.1 up	2.7 up	5.2 up	5.4 up	6.5 up	5.8 up
um02136	Eff1-6	-	-	-	-	2.6 up	2.7 up	-	2.1 up
um02137	Eff1-7	2.2 down	2.2 down	-	-	-	-	2.6 up	2.5 up
um02138	Eff1-8	-	-	-	-	4.0 up	4.4 up	3.3 up	4.2 up
um02139	Eff1-9	-	-	2.2 up	2.2 up	-	-	3.3 up	2.1 up
um02140	Eff1-10	-	-	2.1 up	3.3 up	2.6 up	-	-	-
um02141	Eff1-11	-	2.1 up	-	2.0 up	4.8 up	4.4 up	2.4 up	2.5 up

and *VRG4*, have been related to virulence.[41] In this regard, the observation that three *MSN1* homologs from *U. maydis* were up-regulated in the haploid and only two in the diploid and that the homolog of *VRG4* was up-regulated in the haploid is relevant. Regarding O-glycans, it was reported that *PMT4*, a gene involved in their synthesis, is involved in *C. albicans* and *C. neoformans* virulence,[42,43] and that its *U. maydis* mutants were avirulent to maize.[44] Agreeing with these data, we observed that this gene was up-regulated only in the haploid, correlating again with its higher virulence.

GPI proteins are important components of fungal membranes and walls and among them Yps aspartyl proteases are important in *Candida* pathogenesis,[45] agreeing with the observation that a gene encoding a Yps was up-regulated in both *Ustilago* strains. Taking into consideration that genes involved in the synthesis of the glycosylphosphatidyl inositol moiety of GPI-proteins such as *GPI12, GPI17, GPI8* are important in virulence of fungi and protozoa,[46-48] it was significant to observe that in *U. maydis* the homolog of *GPI12* was up-regulated in both strains and that *GPI17* was up-regulated only in the haploid and *GPI8* only in the diploid.

Hydrophobins and repellent proteins are important in *Ustilago* infection of maize.[23,24,49] We observed that *REP1* and *HUM2* genes were up-regulated in both strains, *REP1* to higher levels in the haploid, whereas *HUM3* was up-regulated only in this strain, suggesting their role in *Arabidopsis* infection.

Finally, it must be indicated that a higher number of genes encoding hydrolytic enzymes, some degrading the plant cell wall, were up-regulated in the haploid. This result may help to explain the greater damage produced by the haploid in *Arabidopsis* plants, agreeing with the observation that biotrophic fungi secrete a reduced number of degradative enzymes[50] and supporting our hypothesis of the different behavior of haploid and diploid strains of *U. maydis* during *Arabidopsis* infection.

In conclusion, our data that provide evidence of the importance of a number of genes in *Ustilago* virulence and that the genetic machinery used in *Arabidopsis* infection is similar to that used in maize infection. According to the evidence presented, we propose the hypothesis that the haploid strain of *U. maydis* behaves in *Arabidopsis* as a necrotrophic pathogen, in contrast to the diploid that, as occurs in maize, behaves as a biotrophic agent. We attribute this different behavior to alterations in the expression of genes encoding virulence factors, degradation enzymes, effector genes and transcription factors under the control of the bE/bW heterodimer, that obviously is absent in the haploid strain.

Materials and Methods

Fungal and plant strains, culture media and growth conditions. *Fungal strains. U. maydis* wild type strain FB2 (a_2b_2)[51] and the diploid strain Uid1 ($a_1b_1\Delta pan/a_2b_2\Delta odc::Hyg^R$)[52] were maintained and grown as described by Ruiz-Herrera et al.[36] *Arabidopsis thaliana L. Landsberg erecta* (*Ler*) plantlets were grown on MS synthetic medium according to Mendez-Morán et al.[8] (see below).

***Arabidopsis* growth conditions.** *A. thaliana Landsberg erecta* seeds were sterilized with chlorine gas. Open Eppendorf tubes containing the seeds were placed in a desiccator that contained a beaker with 100 mL of concentrated hydrochloric acid and 5 mL sodium hypochlorite. The desiccator was kept closed during 4–6 h. After of sterilization, the seeds were maintained at 4°C during 2 d. For plant growth, 80–100 seeds were placed over plates of sterile solid MS medium and incubated in a chamber at 25°C with photoperiods of 12 h.

Inoculation and measurement of plantlets growth. *U. maydis* strains were grown in shaken liquid MC at 28°C for 18 h. The cells were recovered by centrifugation at 1,000 g for 10 m and washed twice with sterile distilled water (SDW) by centrifugation. Finally the cells were suspended in 5 mL of SDW and cell concentration was determined with a Neubauer chamber. *A. thaliana* plantlets were inoculated with 1–2 μl of a cell suspension containing 10^6 cells/mL on the vegetative apex, 6 d post-planting. Control plantlets received SDW only. Plantlets were incubated under controlled conditions in a growth chamber as described above. At intervals, some plants were observed by light microscopy, directly or stained with cotton blue-lactophenol and photographed. At different periods post-inoculation plantlets were recovered and their dry weight and stem length were measured.

***Ustilago maydis* growth in *A. thaliana* seedlings.** The biomass of *U. maydis* in inoculated plantlets was determined by ergosterol[53] and chitin[54] measurements. N-acetylglucosamine (GlcNAC) was determined as described by Reissig et al.[55]

Isolation of RNA and microarrays hybridization of microarrays. Total RNA from *Ustilago* cells (three experiments in triplicates) or *Arabidopsis* plantlets (four experiments with 150 plants each) was isolated using Trizol (Invitrogen) and purified with

Table 4. Regulation of *Ustilago maydis* genes described as important for virulence in maize, during *Arabidopsis* infection

Identity		Description	Reference	Fold change during *Arabidopsis thaliana* infection							
				Days post-infection with the haploid strain FB2				Days post-infection with the diploid strain Uid1			
				1	2	4	8	1	2	4	8
um00641	um11450	Hgl1; Hgl1p, required for dimorphism and teliospore formation	Deletion[67]	4.4 down	5.2 down	-	-	3.0 down	3.9 down	3.1 down	-
um01374		Pit1	Deletion[21]	-	2.2 up	3.9 up	3.1 up	-	-	2.2 up	2.2 up
um01597		Hap2	Deletion[68]	5.2 down	2.8 down	2.6 down	-	2.9 down	2.2 down	-	-
um01947		Related to cytochrome-c peroxidase precursor	Deletion[69]	2.6 down	16.5 down	14.8 down	48.2 down	-	-	3.0 down	3.6 down
um02374		Srt1; related to monosaccharide transporter	Deletion[70]	3.1 down	2.7 down	3.2 down	-	3.9 down	3.5 down	-	-
um02377		Probable cytochrome c peroxidase precursor	Deletion[69]	-	-	-	-	3.7 up	3.3 up	2.2 up	2.9 up
um03280	um10828	Tup1; probable TUP1-general transcription repressor	Deletion[28]	-	-	-	-	2.2 down	-	-	-
um03305		Ubc3; MAP kinase	Deletion[71]	3.5 up	3.1 up	3.6 up	3.4 up	2.3 up	2.3 up	-	-
um03315		Ukb1; serine/threonine protein kinase b-related Ukb1	Deletion[72]	3.1 up	2.4 up	2.8 up	2.3 up	-	-	-	-
um04258.2	um04258	Ubc4 - MAPKK kinase	Deletion[73]	-	2.0 down	-	-	-	-	-	-
um04474		Gpa3; guanine nucleotide-binding protein α-3 subunit	Deletion[74]	2.3 up	2.8 up	2.8 up	4.3 up	3.2 up	3.3 up	3.9 up	3.8 up
um04580		O-mannosylation	Deletion[75]	3.3 up	-	2.3 up	2.0 up	2.5 up	2.0 up	-	2.0 up
um05261		Ubc2; MAP kinase pathway-interacting protein	Deletion[76]	2.9 down	2.7 down	2.8 down	2.0 down	4.2 down	4.2 down	3.6 down	2.5 down
um05433		Pmt4; probable PMT4; dolichyl-phosphate-mannose–protein *O*-mannosyltransferase	Deletion[44]	3.3 up	2.8 up	3.1 up	2.6 up	-	-	-	-
um05818		Probable chimeric spermidine synthase/saccharopine reductase	Deletion[77]	-	-	2.4 up	2.7 up	2.0 down	-	-	2.0 down
um05850	um05850.2	Polyamine oxidase (PAO)	Deletion[78]	3.5 up	2.4 up	2.4 up	2.2 up	2.3 up	-	2.2 up	2.0 up
um10417	um04252	Nit2; related to transcription factor ScGATA-6	Deletion[29]	-	-	-	-	2.7 down	2.7 down	2.2 down	-
um10672	um10672.2	Peroxidases	Deletion[69]	2.7 down	-	2.3 down	2.3 down	4.5 down	5.2 down	4.1 down	5.7 down
um10792	um00999	Related to S-adenosylmethionine decarboxylase (spe-2)	Deletion[79]	3.0 down	2.0 down	2.1 down	-	-	-	-	-
um10803	um01516	Sql2; guanyl nucleotide exchange factor Sql2	Deletion[80]	2.7 down	2.6 down	2.5 down	2.2 down	2.1 down	2.5 down	-	-
um11410	um02513	Crk1; Cdk-related kinase 1	Deletion[81]	-	2.1 up	2.1 up	2.5 up	-	-	-	2.3 up
um11453	um00648	Ubc5; probable UBC5 - E2 ubiquitin-conjugating enzyme	Deletion[73]	-	-	-	-	2.5 up	3.1 up	-	-
um12033	um06025	Rop1 HMG-box transcription factor (C-terminal fragment)	Deletion[82]	-	-	3.1 up	-	-	-	-	-
um04456	um10537	Adr1 - protein kinase A, catalytic subunit	Described[83]	3.3 up	3.2 up	2.9 up	2.5 up	-	-	-	-
um12024	um05762	hdp1	Described[16]	-	-	5.6 up	2.0 up	-	-	-	-

Table 5. Differential expression of genes involved in the synthesis of structural polysaccharides of *U. maydis* during *Arabidopsis* infection

Identity	Gene	Subcelular localization of protein	Haploid (FB2)				Diploid (Uid1)			
			1 dpi	2 dpi	4 dpi	8 dpi	1 dpi	2 dpi	4 dpi	8 dpi
um10718	*CHS1*	Cell wall	3.2 up	3.4 up	2.1 up	2.3 up	-	-	-	-
um10120	*CHS3*		-	-	-	2.3 down	-	-	-	-
um01143	*CDA*		-	2.5 down	-	3.8 down	3.1 down	3.0 down	2.1	2.0 down
um01788			2.6 down	2.2 down	-	-	2.5 down	2.8 down	-	-
um02019			-	-	2.7 down	-	-	-	-	-
um05792			-	2.7 up	5.4 up	8.1 up	2.6 up	-	2.4 up	-
um11922			24.2 up	31.0 up	29.0 up	27.1 up	10.9 up	9.9 up	15.1 up	12.0 up
um01639	*GLS*	Endoplasmic Reticulum	-	-	-	-	-	-	-	-
um11723	*CWH41*[a]		3.4 up	3.1 up	2.7 up	2.7 up	-	-	-	-
um04405	*ROT2*[a]		2.1 up	2.1 up	2.0 up	-	-	-	-	-
um00857	*KRE6/ SKN1*[a]	Golgi Apparatus	2.0 up	-	2.5 up	-	-	-	-	-
um03569			6.0 down	4.3 down	7.9 down	5.6 down	-	-	-	-
um05718			3.4 down	3.7 down	3.0 down	3.2 down	2.2 down	2.1 down	-	-
um05807			3.3 up	5.2 up	-	-	-	-	-	-
um05809			18.9 up	27.0 up	8.1 up	10.1 up	3.7 up	3.9 up	6.8 up	4.9 up
um05811			4.0 down	10.3 down	7.9 down	2.5 down	5.4 down	4.8 down	3.4 down	3.2 down

[a]Homologs to *Saccharomyces cerevisie* genes.

Table 6. Differential expression of *U. maydis* genes involved in *N*- and *O* glycosylation during *Arabidopsis* infection

Sequence identity	Homolog gene[a]	Function	Haploid (FB2)				Diploid (Uid1)			
			1 dpi	2 dpi	4 dpi	8 dpi	1 dpi	2 dpi	4 dpi	8 dpi
um10484	*ALG7*	Oligosaccharide synthesis	5.4 up	4.1 up	2.7 up	2.5 up	8.1 up	4.6 up	4.0 up	4.3 up
um11771	*ALG14*		3.1 up	-	-	-	2.5 up	2.1 up	-	-
um11547	*ALG1*		2.3 up	-	2.2 up	-	-	-	-	-
um05209	*ALG11*		2.1 up	2.2 up	2.3 up	-	-	-	-	-
um02433	*RFT1*		2.5 up	2.6 up	2.1 up	-	-	-	-	-
um11341	*ALG12*		4.2 up	3.8 up	3.7 up	2.8 up	6.5 up	5.3 up	4.7 up	3.6 up
um04779	*SEC59*		2.7 down	2.4 down	2.2 down	2.1 down	2.4 down	2.5 down	2.1 down	-
um01231	*ALG5*		2.3 up	2.2 up	2.3 up	2.0 up	2.0 up	2.0 up	2.1 up	2.3 up
um05547	*ALG10/DIE2*		-	-	-	-	2.1 up	-	-	-
um01062	*VRG4*		2.1 up	-	-	-	-	-	-	-
um04198	*OST3*	Oligosaccharide transference	3.8 up	2.8 up	3.6 up	2.8 up	2.8 up	2.9 up	2.2 up	2.0 up
um05293	*STT3*		3.5 up	3.8 up	3.6 up	3.6 up	2.4 up	2.2 up	2.5 up	2.3 up
um11723	*CWH41/GLS1*		3.4 up	3.1 up	2.7 up	2.7 up	-	-	-	-
um04405	*ROT2*		2.1 up	2.1 up	2.0 up	-	-	-	-	-
um01957	*MNS1, MNL1/ HTM1*	Modification of oligosaccharide	4.0 up	1.6 up	3.0 up	2.1 up	7.3 up	4.6 up	8.1 up	5.7 up
um02227			2.8 up	2.5 up	2.6 up	2.7 up	4.9 up	5.6 up	6.8 up	5.5 up
um10494			3.7 up	2.3 up	2.5 up	2.1 up	-	-	-	-
um05433	*PTM4*	Transfer of first mannose to Ser/Thre	3.1 up	2.8 up	3.0 up	2.6 up	-	-	-	-
um01154	*KRE2*	Modification with additional mannose	3.8up	3.6 up	3.2 up	3.2 up	2.9 up	2.8 up	3.4 up	3.2 up
um01821			2.0 up	-	-	-	2.1 up	2.4 up	2.2 up	2.5 up

[a]Homologs to *S. cerevisiae* genes.

Table 7. Expression of *U. maydis* genes involved in GPI-anchor synthesis during *Arabidopsis* infection

Identity	Homolog gene	Function	Process	FB2				Uid1			
				1 dpi	2 dpi	4 dpi	8 dpi	1 dpi	2 dpi	4 dpi	8 dpi
um00213	*GPI1*	Modified of PI with GlcNAc (GPI-GnT complex)	Synthesis of GPI anchor from PI	-	-	-	-	3.4 up	3.2 up	2.6 up	2.5 up
um00302	*GPI12*	Deacetylation		-	2.0 up	-	-	2.5 up	2.0 up	2.0 up	-
um11347	*GPI14*	Transfer of mannose		2.9 up	4.5 up	3.3 up	4.1 up	-	-	2.0 up	2.4 up
um05554	*GPI18*	Transfer of mannose		2.4 up	2.9 up	2.6 up	2.3 up	-	-	-	-
um05709	*GPI8*		Catalytic site	-	-	-	-	2.3 up	2.0 up	-	-
um01565	*GPI17*			2.3 up	2.5 up	2.9 up	2.2 up	-	-	-	-
um04859	*PER1*	Removal of acyl group of GPI-anchor	Lipid Remodeling	4.5 down	3.9 down	4.3 down	3.9 down	-	-	-	-
um03003	*GUP1*	Lipid remodeling with longer fatty acids		5.3 up	4.1 up	4.9 up	4.5 up	2.8 up	2.9 up	3.0 up	3.1 up

Table 8. Numbers of *U. maydis* genes encoding secreted proteins regulated during *Arabidopsis* infection

Function		Regulate	Haploid (FB2)				Diploid (Uid1)			
			1 dpi	2 dpi	4 dpi	8 dpi	1 dpi	2 dpi	4 dpi	8 dpi
Degradation	Unknown	Up	54	50	62	57	61	72	62	76
		Down	29	37	27	32	17	20	30	22
	Polysaccharidases	Up	17	16	19	15	10	9	11	9
		Down	0	0	0	0	1	1	1	1
	Peptidases	Up	9	7	8	6	4	4	4	5
		Down	0	0	2	2	2	0	0	2
	Nucleases	Up	6	5	5	5	5	6	5	5
		Down	0	0	0	0	0	0	0	0
	Lipases and esterases	Up	4	5	6	8	4	5	3	5
		Down	0	1	1	0	1	1	1	1
	Phytases	Up	3	2	3	2	2	3	3	1
		Down	0	0	0	0	0	0	0	0
	Synthesis	Up	3	4	5	4	4	2	4	2
		Down	1	2	3	3	1	4	2	3
	Redox	Up	12	11	12	9	7	5	7	8
		Down	3	3	3	3	8	8	8	7
	Non-enzymatic	Up	4	4	2	2	4	4	4	4
		Down	3	3	5	5	5	5	5	6

QIAGEN columns (Cat. 28104). RNA concentration was measured by its absorbance at 260 nm and its integrity observed by electrophoresis in denaturing agarose gels. cDNA synthesis and labeling and hybridization of the microarrays were performed by Roche NimbleGen Inc.

Design, image analysis, normalization and analysis of microarrays. The microarrays used were high density (one channel) arrays from NimbleGene, according to a design from Scott Gold (University of Georgia). Five different oligonucleotide probes per gene (60 nt in length) per duplicate represented each one of the 6,883 genes of the *U. maydis* genome. GenePix 4000B scan and associated software were used to scan the arrays. Roche NimbleScan software was used to import the scanned images and data extraction. Normalization was made with NimbleScan software using quantile[56] and the algorithm Robust Multi-array Analysis (RMA).[57] Microarray analyses were made with DNAStar ArrayStar software, comparing data from *Arabidopsis* infected plants against *U. maydis* grown in culture medium. Data obtained from plants that received SDW were used as controls. *P*-values obtained were adjusted by the false discovery rate (FDR) method.[58] p-values inferior to 0.05 were considered significant. A two-fold change up or down was used to consider differential expression of genes. The Functional Catalogue (FunCat)[59] was used for functional annotation of differentially expressed genes.

Bioinformatic searches. Besides a classification of all regulated genes, in silico searches of specific genes previously reported in the following groups, were made: belonging to pathogenesis clusters,[11] encoding proteins from the secretome,[60,61] encoding hydrophobins or repellent proteins,[24] regulated by the bE/bW heterodimer,[16,25] involved in the synthesis and organization of the cell wall,[62] and those previously reported as important during maize infection (specific references cited in "Results"). Additionally, we performed in silico searches of genes encoding transcription factors using online programs UniProt,[63] KEGG,[64] Pfam,[65] SUPERFAMILY[66] and MIPS *Ustilago maydis* DataBase (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was partially supported by Consejo Nacional de Ciencia y Tecnología (CONACYT), México. Thanks are given to Dr. Lucila Ortiz and Dr. Doralinda Guzmán for assistance in some analyses and Dr. Scott Gold for permission to use his microarray design. D.M.S. and A.M.R.B. are doctoral students with fellowships from CONACYT.

Supplemental Material

Supplemental material may be found here:
http://www.landesbioscience.com/journals/psb/article/25059/

References

1. Perfect JR, Savani DV, Durack DT. Comparison of itraconazole and fluconazole in treatment of cryptococcal meningitis and candida pyelonephritis in rabbits. Antimicrob Agents Chemother 1986; 29:579-83; PMID:3010846; http://dx.doi.org/10.1128/AAC.29.4.579
2. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot Cell 2003; 2:1053-60; PMID:14555488; http://dx.doi.org/10.1128/EC.2.5.1053-1060.2003
3. Kontoyiannis DP, Lewis RE. Studying fungal pathogenesis in *Drosophila.* Microbe 2010; 5:291-5
4. Chen N, Hsiang T, Goodwin PH. Use of green fluorescent protein to quantify the growth of Colletotrichum during infection of tobacco. J Microbiol Methods 2003; 53:113-22; PMID:12609730; http://dx.doi.org/10.1016/S0167-7012(02)00234-8
5. Parker D, Beckmann M, Enot DP, Overy DP, Rios ZC, Gilbert M, et al. Rice blast infection of *Brachypodium distachyon* as a model system to study dynamic host/pathogen interactions. Nat Protoc 2008; 3:435-45; PMID:18323815; http://dx.doi.org/10.1038/nprot.2007.499
6. Xue C, Tada Y, Dong X, Heitman J. The human fungal pathogen *Cryptococcus* can complete its sexual cycle during a pathogenic association with plants. Cell Host Microbe 2007; 1:263-73; PMID:18005707; http://dx.doi.org/10.1016/j.chom.2007.05.005
7. León-Ramírez C, Cabrera-Ponce JL, Martínez-Espinoza A, Herrera-Estrella L, Méndez-Morán L, Reynaga-Peña G, et al. Infection of alternative host plant species by *Ustilago maydis.* New Phytol 2004; 164:337-46; http://dx.doi.org/10.1111/j.1469-8137.2004.01171.x
8. Méndez-Morán L, Reynaga-Peña CG, Springer PS, Ruiz-Herrera J. *Ustilago maydis* infection of the non-natural host *Arabidopsis thaliana.* Phytopathology 2005; 95:480-8; PMID:18943312; http://dx.doi.org/10.1094/PHYTO-95-0480
9. Davis KR, Hammershmidt R. *Arabidopsis thaliana* as a model for plant-pathogen interactions. In: The American Phytopathological Society, ed. St. Paul, USA, 1993:134.
10. Innes R. Arabidopsis as a model host in molecular plant pathology. In: Singh RP, Singh US, ed(s). Molecular methods in plant pathology. FL, USA: Boca Raton CRC Press, 1995:203-19.
11. Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ, et al. Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis.* Nature 2006; 444:97-101; PMID:17080091; http://dx.doi.org/10.1038/nature05248
12. Ruiz-Herrera J, León-Ramírez CG. Development and dimorphism of the phytopathogenic basidiomycota *Ustilago maydis.* In: Ruiz-Herrera J, ed. Dimorphic fungi. Their importance as models for differentiation and fungal pathogenesis. Ed. Bentham eBooks elSBN: 978-1-60805-364-3, 2012:105-16.
13. Cabrera-Ponce JL, León-Ramírez CG, Verver-Vargas A, Palma-Tirado L, Ruiz-Herrera J. Metamorphosis of the Basidiomycota *Ustilago maydis*: transformation of yeast-like cells into basidiocarps. Fungal Genet Biol 2012; 49:765-71; PMID:22921263; http://dx.doi.org/10.1016/j.fgb.2012.07.005
14. Brefort T, Doehlemann G, Mendoza-Mendoza A, Reissmann S, Djamei A, Kahmann R. *Ustilago maydis* as a Pathogen. Annu Rev Phytopathol 2009; 47:423-45; PMID:19400641; http://dx.doi.org/10.1146/annurev-phyto-080508-081923
15. Skibbe DS, Doehlemann G, Fernandes J, Walbot V. Maize tumors caused by *Ustilago maydis* require organ-specific genes in host and pathogen. Science 2010; 328:89-92; PMID:20360107; http://dx.doi.org/10.1126/science.1185775
16. Heimel K, Scherer M, Vranes M, Wahl R, Pothiratana C, Schuler D, et al. The transcription factor Rbf1 is the master regulator for *b*-mating type controlled pathogenic development in *Ustilago maydis.* PLoS Pathog 2010; 6:e1001035; PMID:20700446; http://dx.doi.org/10.1371/journal.ppat.1001035
17. Torto TA, Li S, Styer A, Huitema E, Testa A, Gow NA, et al. EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen *Phytophthora.* Genome Res 2003; 13:1675-85; PMID:12840044; http://dx.doi.org/10.1101/gr.910003
18. Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G. The *Ustilago maydis* effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. PLoS Pathog 2012; 8:e1002684; PMID:22589719; http://dx.doi.org/10.1371/journal.ppat.1002684
19. Zahiri A, Heimel K, Wahl R, Rath M, Kämper J. The *Ustilago maydis* forkhead transcription factor Fox1 is involved in the regulation of genes required for the attenuation of plant defenses during pathogenic development. Mol Plant Microbe Interact 2010; 23:1118-29; PMID:20687802; http://dx.doi.org/10.1094/MPMI-23-9-1118
20. Doehlemann G, van der Linde K, Assmann D, Schwammbach D, Hof A, Mohanty A, et al. Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLoS Pathog 2009; 5:e1000290; PMID:19197359; http://dx.doi.org/10.1371/journal.ppat.1000290
21. Doehlemann G, Reissmann S, Assmann D, Fleckenstein M, Kahmann R. Two linked genes encoding a secreted effector and a membrane protein are essential for *Ustilago maydis*-induced tumour formation. Mol Microbiol 2011; 81:751-66; PMID:21692877; http://dx.doi.org/10.1111/j.1365-2958.2011.07728.x
22. Khrunyk Y, Münch K, Schipper K, Lupas AN, Kahmann R. The use of FLP-mediated recombination for the functional analysis of an effector gene family in the biotrophic smut fungus *Ustilago maydis.* New Phytol 2010; 187:957-68; PMID:20673282; http://dx.doi.org/10.1111/j.1469-8137.2010.03413.x
23. Teertstra WR, Deelstra HJ, Vranes M, Bohlmann R, Kahmann R, Kämper J, et al. Repellents have functionally replaced hydrophobins in mediating attachment to a hydrophobic surface and in formation of hydrophobic aerial hyphae in *Ustilago maydis.* Microbiology 2006; 152:3607-12; PMID:17159213; http://dx.doi.org/10.1099/mic.0.29034-0
24. Müller O, Schreier PH, Uhrig JF. Identification and characterization of secreted and pathogenesis-related proteins in *Ustilago maydis.* Mol Genet Genomics 2008; 279:27-39; PMID:17917743; http://dx.doi.org/10.1007/s00438-007-0291-4
25. Vollmeister E, Schipper K, Baumann S, Haag C, Pohlmann T, Stock J, et al. Fungal development of the plant pathogen *Ustilago maydis.* FEMS Microbiol Rev 2012; 36:59-77; PMID:21729109; http://dx.doi.org/10.1111/j.1574-6976.2011.00296.x
26. Govrin EM, Levine A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea.* Curr Biol 2000; 10:751-7; PMID:10898976; http://dx.doi.org/10.1016/S0960-9822(00)00560-1
27. Mayer AM, Staples RC, Gil-ad NL. Mechanisms of survival of necrotrophic fungal plant pathogens in hosts expressing the hypersensitive response. Phytochemistry 2001; 58:33-41; PMID:11524110; http://dx.doi.org/10.1016/S0031-9422(01)00187-X
28. Elías-Villalobos A, Fernández-Álvarez A, Ibeas JI. The general transcriptional repressor Tup1 is required for dimorphism and virulence in a fungal plant pathogen. PLoS Pathog 2011; 7:e1002235; PMID:21909277; http://dx.doi.org/10.1371/journal.ppat.1002235
29. Horst RJ, Zeh C, Saur A, Sonnewald S, Sonnewald U, Voll LM. The *Ustilago maydis* Nit2 homolog regulates nitrogen utilization and is required for efficient induction of filamentous growth. Eukaryot Cell 2012; 11:368-80; PMID:22247264; http://dx.doi.org/10.1128/EC.05191-11
30. Nicholls S, MacCallum DM, Kaffarnik FAR, Selway L, Peck SC, Brown AJP. Activation of the heat shock transcription factor Hsf1 is essential for the full virulence of the fungal pathogen *Candida albicans.* Fungal Genet Biol 2011; 48:297-305; PMID:20817114; http://dx.doi.org/10.1016/j.fgb.2010.08.010

31. Du H, Guan G, Xie J, Sun Y, Tong Y, Zhang L, et al. Roles of *Candida albicans* Gat2, a GATA-type zinc finger transcription factor, in biofilm formation, filamentous growth and virulence. PLoS ONE 2012; 7:e29707; PMID:22276126; http://dx.doi.org/10.1371/journal.pone.0029707
32. Singh P, Chauhan N, Ghosh A, Dixon F, Calderone RS. SKN7 of Candida albicans: mutant construction and phenotype analysis. Infect Immun 2004; 72:2390-4; PMID:15039366; http://dx.doi.org/10.1128/IAI.72.4.2390-2394.2004
33. Wormley FL Jr., Heinrich G, Miller JL, Perfect JR, Cox GM. Identification and characterization of an *SKN7* homolog in *Cryptococcus neoformans.* Infect Immun 2005; 73:5022-30; PMID:16041017; http://dx.doi.org/10.1128/IAI.73.8.5022-5030.2005
34. Brachmann A, Weinzierl G, Kämper J, Kahmann R. Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis.* Mol Microbiol 2001; 42:1047-63; PMID:11737646; http://dx.doi.org/10.1046/j.1365-2958.2001.02699.x
35. Smith SE, Smith FA. Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol 1990; 114:1-38; http://dx.doi.org/10.1111/j.1469-8137.1990.tb00370.x
36. Ruiz-Herrera J, León-Ramirez C, Guevara-Olvera L, Cárabez-Trejo A. Yeast-mycelial dimorphism of haploid and diploid strains of *Ustilago maydis.* Microbiology 1995; 141:695-703; http://dx.doi.org/10.1099/13500872-141-3-695
37. El Gueddari NE, Rauchhaus U, Moerschbacher BM, Deising HB. Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. New Phytol 2002; 156:103-12; http://dx.doi.org/10.1046/j.1469-8137.2002.00487.x
38. Robledo-Briones M, Ruiz-Herrera J. Transcriptional regulation of the genes encoding chitin and β-1,3-glucan synthases from *Ustilago maydis.* Curr Microbiol 2012; 65:85-90; PMID:22538468; http://dx.doi.org/10.1007/s00284-012-0129-0
39. Gilbert NM, Donlin MJ, Gerik KJ, Specht CA, Djordjevic JT, Wilson CF, et al. *KRE* genes are required for β-1,6-glucan synthesis, maintenance of capsule architecture and cell wall protein anchoring in *Cryptococcus neoformans.* Mol Microbiol 2010; 76:517-34; PMID:20384682; http://dx.doi.org/10.1111/j.1365-2958.2010.07119.x
40. Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, et al. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans.* J Biol Chem 2005; 281:90-8; PMID:16263704; http://dx.doi.org/10.1074/jbc.M510360200
41. Nishikawa A, Poster JB, Jigami Y, Dean N. Molecular and phenotypic analysis of CaVRG4, encoding an essential Golgi apparatus GDP-mannose transporter. J Bacteriol 2002; 184:29-42; PMID:11741841; http://dx.doi.org/10.1128/JB.184.1.29-42.2002
42. Rouabhia M, Schaller M, Corbucci C, Vecchiarelli A, Prill SK, Giasson L, et al. Virulence of the fungal pathogen *Candida albicans* requires the five isoforms of protein mannosyltransferases. Infect Immun 2005; 73:4571-80; PMID:16040968; http://dx.doi.org/10.1128/IAI.73.8.4571-4580.2005
43. Olson GM, Fox DS, Wang P, Alspaugh JA, Buchanan KL. Role of protein O-mannosyltransferase Pmt4 in the morphogenesis and virulence of *Cryptococcus neoformans.* Eukaryot Cell 2007; 6:222-34; PMID:17142566; http://dx.doi.org/10.1128/EC.00182-06
44. Fernández-Alvarez A, Elías-Villalobos A, Ibeas JI. The O-mannosyltransferase PMT4 is essential for normal appressorium formation and penetration in *Ustilago maydis.* Plant Cell 2009; 21:3397-412; PMID:19880800; http://dx.doi.org/10.1105/tpc.109.065839
45. Kaur R, Ma B, Cormack BP. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata.* Proc Natl Acad Sci USA 2007; 104:7628-33; PMID:17456602; http://dx.doi.org/10.1073/pnas.0611195104
46. Richard M, Ibata-Ombetta S, Dromer F, Bordon-Pallier F, Jouault T, Gaillardin C. Complete glycosylphosphatidylinositol anchors are required in *Candida albicans* for full morphogenesis, virulence and resistance to macrophages. Mol Microbiol 2002; 44:841-53; PMID:11994163; http://dx.doi.org/10.1046/j.1365-2958.2002.02926.x
47. Lillico S, Field MC, Blundell P, Coombs GH, Mottram JC. Essential roles for GPI-anchored proteins in African trypanosomes revealed using mutants deficient in GPI8. Mol Biol Cell 2003; 14:1182-94; PMID:12631733; http://dx.doi.org/10.1091/mbc.E02-03-0167
48. Chang T, Milne KG, Güther ML, Smith TK, Ferguson MA. Cloning of *Trypanosoma brucei* and *Leishmania major* genes encoding the GlcNAc-phosphatidylinositol de-N-acetylase of glycosylphosphatidylinositol biosynthesis that is essential to the African sleeping sickness parasite. J Biol Chem 2002; 277:50176-82; PMID:12364327; http://dx.doi.org/10.1074/jbc.M208374200
49. Wösten HA, Bohlmann R, Eckerskorn C, Lottspeich F, Bölker M, Kahmann R. A novel class of small amphipathic peptides affect aerial hyphal growth and surface hydrophobicity in *Ustilago maydis.* EMBO J 1996; 15:4274-81; PMID:8861956
50. Mendgen K, Hahn M. Plant infection and the establishment of fungal biotrophy. Trends Plant Sci 2002; 7:352-6; PMID:12167330; http://dx.doi.org/10.1016/S1360-1385(02)02297-5
51. Banuett F, Herskowitz I. Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. Proc Natl Acad Sci USA 1989; 86:5878-82; PMID:16594058; http://dx.doi.org/10.1073/pnas.86.15.5878
52. Ruiz-Herrera J, León-Ramírez C, Cabrera-Ponce JL, Martínez-Espinoza AD, Herrera-Estrella L. Completion of the sexual cycle and demonstration of genetic recombination in *Ustilago maydis in vitro.* Mol Gen Genet 1999; 262:468-72; PMID:10589834; http://dx.doi.org/10.1007/s004380051107
53. Martin F, Delareulle C, Hilbert JL. An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycol Res 1990; 94:1059-64; http://dx.doi.org/10.1016/S0953-7562(09)81333-6
54. Specht CA, Liu Y, Robbins PW, Bulawa CE, Iartchouk N, Winter KR, et al. The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. Fungal Genet Biol 1996; 20:153-67; PMID:8810520; http://dx.doi.org/10.1006/fgbi.1996.0030
55. Reissig JL, Storminger JL, Leloir LF. A modified colorimetric method for the estimation of N-acetylamino sugars. J Biol Chem 1955; 217:959-66; PMID:13271455
56. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003; 19:185-93; PMID:12538238; http://dx.doi.org/10.1093/bioinformatics/19.2.185
57. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4:249-64; PMID:12925520; http://dx.doi.org/10.1093/biostatistics/4.2.249
58. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc, B 1995; 57:289-300
59. Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, et al. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res 2004; 32:5539-45; PMID:15486203; http://dx.doi.org/10.1093/nar/gkh894
60. Mueller O, Kahmann R, Aguilar G, Trejo-Aguilar B, Wu A, de Vries RP. The secretome of the maize pathogen *Ustilago maydis.* Fungal Genet Biol 2008; 45:S63-70; PMID:18456523; http://dx.doi.org/10.1016/j.fgb.2008.03.012
61. Ruiz-Herrera J, Ortiz-Castellanos L, Martínez AI, León-Ramírez C, Sentandreu R. Analysis of the proteins involved in the structure and synthesis of the cell wall of *Ustilago maydis.* Fungal Genet Biol 2008; 45:S71-6; PMID:18508396; http://dx.doi.org/10.1016/j.fgb.2008.04.010
62. Robledo-Briones M, Ruiz-Herrera J. Regulation of genes involved in cell wall synthesis and structure during *Ustilago maydis* dimorphism. FEMS Yeast Res 2013; 13:74-84; PMID:23167842; http://dx.doi.org/10.1111/1567-1364.12011
63. UniProt Consortium. Update on activities at the Universal Protein Resource (UniProt) in 2013. Nucleic Acids Res 2013; 41:D43-7; PMID:23161681; http://dx.doi.org/10.1093/nar/gks1068
64. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEEG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 1999; 27:29-34; PMID:9847135; http://dx.doi.org/10.1093/nar/27.1.29
65. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, et al. The Pfam protein families database. Nucleic Acids Res 2012; 40:290-301; PMID:21896617; http://dx.doi.org/10.1093/nar/gkr1065
66. Gough J, Karplus K, Hughey R, Chothia C. Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol 2001; 313:903-19; PMID:11697912; http://dx.doi.org/10.1006/jmbi.2001.5080
67. Dürrenberger F, Laidlaw RD, Kronstad JW. The hgl1 gene is required for dimorphism and teliospore formation in the fungal pathogen *Ustilago maydis.* Mol Microbiol 2001; 41:337-48; PMID:11489122; http://dx.doi.org/10.1046/j.1365-2958.2001.02528.x
68. Mendoza-Mendoza A, Eskova A, Weise C, Czajkowski R, Kahmann R. Hap2 regulates the pheromone response transcription factor prf1 in *Ustilago maydis.* Mol Microbiol 2009; 72:683-98; PMID:19400774; http://dx.doi.org/10.1111/j.1365-2958.2009.06676.x
69. Molina L, Kahmann R. An *Ustilago maydis* gene involved in H_2O_2 detoxification is required for virulence. Plant Cell 2007; 19:2293-309; PMID:17616735; http://dx.doi.org/10.1105/tpc.107.052332
70. Wahl R, Wippel K, Goos S, Kämper J, Sauer N. A novel high-affinity sucrose transporter is required for virulence of the plant pathogen *Ustilago maydis.* PLoS Biol 2010; 8:e1000303; PMID:20161717; http://dx.doi.org/10.1371/journal.pbio.1000303
71. Mayorga ME, Gold SE. Characterization and molecular genetic complementation of mutants affecting dimorphism in the fungus *ustilago maydis.* Fungal Genet Biol 1998; 24:364-76; PMID:9756717; http://dx.doi.org/10.1006/fgbi.1998.1078
72. Abramovitch RB, Yang G, Kronstad JW. The ukb1 gene encodes a putative protein kinase required for bud site selection and pathogenicity in *Ustilago maydis.* Fungal Genet Biol 2002; 37:98-108; PMID:12223194; http://dx.doi.org/10.1016/S1087-1845(02)00030-0
73. Andrews DL, Egan JD, Mayorga ME, Gold SE. The *Ustilago maydis* ubc4 and ubc5 genes encode members of a MAP kinase cascade required for filamentous growth. Mol Plant Microbe Interact 2000; 13:781-6; PMID:10875339; http://dx.doi.org/10.1094/MPMI.2000.13.7.781
74. Regenfelder E, Spellig T, Hartmann A, Lauenstein S, Bölker M, Kahmann R. G proteins in *Ustilago maydis*: transmission of multiple signals? EMBO J 1997; 16:1934-42; PMID:9155019; http://dx.doi.org/10.1093/emboj/16.8.1934

75. Fernández-Álvarez A, Marín-Menguiano M, Lanver D, Jiménez-Martín A, Elías-Villalobos A, Pérez-Pulido AJ, et al. Identification of O-mannosylated virulence factors in *Ustilago maydis.* PLoS Pathog 2012; 8:e1002563; PMID:22416226; http://dx.doi.org/10.1371/journal.ppat.1002563

76. Mayorga ME, Gold SE. The ubc2 gene of *Ustilago maydis* encodes a putative novel adaptor protein required for filamentous growth, pheromone response and virulence. Mol Microbiol 2001; 41:1365-79; PMID:11580841; http://dx.doi.org/10.1046/j.1365-2958.2001.02606.x

77. Valdés-Santiago L, Cervantes-Chávez JA, Ruiz-Herrera J. *Ustilago maydis* spermidine synthase is encoded by a chimeric gene, required for morphogenesis, and indispensable for survival in the host. FEMS Yeast Res 2009; 9:923-35; PMID:19624748; http://dx.doi.org/10.1111/j.1567-1364.2009.00539.x

78. Valdés-Santiago L, Guzmán-de-Peña D, Ruiz-Herrera J. Life without putrescine: disruption of the gene-encoding polyamine oxidase in *Ustilago maydis* odc mutants. FEMS Yeast Res 2010; 10:928-40; PMID:20840600; http://dx.doi.org/10.1111/j.1567-1364.2010.00675.x

79. Valdés-Santiago L, Cervantes-Chávez JA, Winkler R, León-Ramírez CG, Ruiz-Herrera J. Phenotypic comparison of samdc and spe mutants reveals complex relationships of polyamine metabolism in *Ustilago maydis.* Microbiology 2012; 158:674-84; PMID:22222500; http://dx.doi.org/10.1099/mic.0.055954-0

80. Müller P, Katzenberger JD, Loubradou G, Kahmann R. Guanyl nucleotide exchange factor Sql2 and Ras2 regulate filamentous growth in *Ustilago maydis.* Eukaryot Cell 2003; 2:609-17; PMID:12796306; http://dx.doi.org/10.1128/EC.2.3.609-617.2003

81. Garrido E, Voss U, Müller P, Castillo-Lluva S, Kahmann R, Pérez-Martín J. The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. Genes Dev 2004; 18:3117-30; PMID:15601825; http://dx.doi.org/10.1101/gad.314904

82. Orth AB, Rzhetskaya M, Pell EJ, Tien M. A serine (threonine) protein kinase confers fungicide resistance in the phytopathogenic fungus *Ustilago maydis.* Appl Environ Microbiol 1995; 61:2341-5; PMID:7793954

83. Zavrel M, Majer O, Kuchler K, Rupp S. Transcription factor Efg1 shows a haploinsufficiency phenotype in modulating the cell wall architecture and immunogenicity of *Candida albicans.* Eukaryot Cell 2012; 11:129-40; PMID:22140230; http://dx.doi.org/10.1128/EC.05206-11

84. Shah JC, Clancy MJ. IME4, a gene that mediates MAT and nutritional control of meiosis in *Saccharomyces cerevisiae.* Mol Cell Biol 1992; 12:1078-86; PMID:1545790

85. Aréchiga-Carvajal ET, Ruiz-Herrera J. The RIM101/pacC homolog from the basidiomycete *Ustilago maydis* is functional in multiple pH-sensitive phenomena. Eukaryot Cell 2005; 4:999-1008; PMID:15947192; http://dx.doi.org/10.1128/EC.4.6.999-1008.2005

86. Brefort T, Müller P, Kahmann R. The high-mobility-group domain transcription factor Rop1 is a direct regulator of prf1 in *Ustilago maydis.* Eukaryot Cell 2005; 4:379-91; PMID:15701800; http://dx.doi.org/10.1128/EC.4.2.379-391.2005