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

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**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 *Candida albicans* and *Cryptococcus neoformans* and *Drosophila melanogaster* has served as alternative host for studying human mycoses. 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 and *Brachypodium distachyon* was used as alternative host of *Magnaporthe grisea*, a rice and barley pathogen. Additionally, in the pathosystems *Cryptococcus-Arabidopsis* and *Cryptococcus-Eucalyptus*, the human pathogen completed its sexual cycle.

Similarly, our research group demonstrated that *Ustilago maydis* infected different plant species, unrelated to its natural host, *Zea mays* under axenic conditions,† establishing the *Ustilago maydis-Arabidopsis thaliana* pathosystem as model to study some virulence aspects of the fungus. *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.

*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. Interestingly, we demonstrated that *U. maydis* performs a completely different sexual life cycle with the surprising formation of basidioecarps, when incubated under defined environmental conditions.

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...
Results

Symptoms in plantlets infected with \textit{U. maydis} strains. Haploid or diploid strains of \textit{U. maydis} infected \textit{Arabidopsis} plantlets as described, 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 dpi 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).

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 and it has been hypothesized that it is also involved in the *U. maydis* biotrophic phase. 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*, 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. Other genes, especially those belonging to cluster 2A whose deletion increases
Effector genes. Effectors are proteins secreted by plant pathogens that manipulate the physiology of their hosts to their benefit. In biotrophic fungi, these effectors accumulate in the zone that manipulates the physiology of their hosts to their benefit. The importance of regulation of transcription factors during infection and defense responses is involved in aerial hyphae formation. 23,24

Hydrophobins and repellent proteins are involved in U. maydis virulence, were down-regulated in the haploid and up-regulated in the diploid as occurs in maize (Table S2).

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Hydrophobins and repellent proteins are involved in U. maydis virulence in maize. HUM2 has essential functions in maize infection and REPI is involved in aerial hyphae formation. 21,24 We found that during Arabidopsis infection REPI, HUM2 and HUM3 were up-regulated in the haploid strain, particularly REPI (39.3 fold change at 1 dpi). In contrast, only REPI and HUM2 were up-regulated in the diploid (not shown).

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Figure 3. Development of U. maydis within infected A. thaliana plantlets. (A) Ergosterol determination. (B) Chitin determination. Empty circles with solid lines, plantlets infected with the diploid. Empty circles with solid lines, plantlets infected with the haploid. 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.
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/Thr 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 *GUPI* 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 *GPI3* were up-regulated and *GPI3* was down-regulated at different dpi (Table 6).

**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 degenerative, 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 over-turn 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 Peli2 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,28,29 corresponding to more than one third of the whole genome. These data provide evidence of the similarity in the pathogenic mechanisms of *U. maydis* in maize and *Arabidopsis*.

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

| Strain       | Genes | Days post-infection |
|--------------|-------|---------------------|
| **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 1,062  |
| Downregulated  | 1,219 | 1,217 1,078 1,100  |

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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 b e/bW heterodimer during *U. maydis* infection of *A. thaliana*

| Identity | Gene | Description | Regulation |
|----------|------|-------------|------------|
| 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 |

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
explain the greater damage produced by the haploid in Arabidopsis infection. This result may help to encoding hydrolytic enzymes, some degrading the plant cell wall, were up-regulated in the haploid. This result may help to understand 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 b) and the diploid strain Uid1 (a bΔpan/a bΔodc::Hyg) were maintained and grown as described by Ruiz-Herrera et al. Arabidopsis thaliana L. Landsberg erecta (Ler) plantlets were grown on MS synthetic medium according to Mendez-Morán et al. (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 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 min and rinsed twice with sterile distilled water (SDW) by centrifugation. Finally the cells were suspended in 5 mL of SDW and cell concentration was determined as described by Reissig et al.55 The biomass of U. maydis in inoculated plantlets was determined by ergosterol and chitin 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 an RNeasy kit (Qiagen). An Agilent 2100 Bioanalyzer was used to evaluate RNA quality.

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

| Identity | Gene | Regulation in haploid (FB2) | Regulation in diploid (Uid1) |
|----------|------|-----------------------------|-----------------------------|
| 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            | -                           |
| um02138  | Eff1-8 | -                           | 4.0 up 4.4 up 3.3 up 4.2 up |
| um02139  | Eff1-9 | -                           | 2.2 up 2.2 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 |
| 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 | 4.4 down | 5.2 down | -  | -  | 3.0 down | 3.9 down | 3.1 down | -  |
| um01374     | Pit1                                      | Deletion | -  | 2.2 up | 3.9 up | 3.1 up | -  | -  | 2.2 up | 2.2 up | -  |
| um01597     | Hap2                                      | Deletion | 5.2 down | 2.8 down | 2.6 down | -  | 2.9 down | 2.2 down | -  | -  |
| um01947     | Related to cytochrome-c peroxidase precursor                                  | Deletion | 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 | 3.1 down | 2.7 down | 3.2 down | -  | 3.9 down | 3.5 down | -  | -  |
| um02377     | Probable cytochrome c peroxidase precursor                                   | Deletion | -  | -  | -  | -  | 3.7 up | 3.3 up | 2.2 up | 2.9 up | -  |
| um03280     | um10828 Tup1; probable TUP1-general transcription repressor                 | Deletion | -  | -  | -  | -  | 2.2 down | -  | -  | -  | -  |
| um03305     | Ubc3; MAP kinase                                                              | Deletion | 3.5 up | 3.1 up | 3.6 up | 3.4 up | 2.3 up | 2.3 up | -  | -  | -  | -  |
| um04258.2   | um04258 Ubc4 - MAPKK kinase                                                   | Deletion | 3.1 up | 2.4 up | 2.8 up | 2.3 up | -  | -  | -  | -  | -  | -  |
| um04744     | Gpa3; guanine nucleotide-binding protein α-3 subunit                        | Deletion | 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 | 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 | 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 | 3.3 up | 2.8 up | 3.1 up | 2.6 up | -  | -  | -  | -  | -  | -  |
| um05818     | Probable chimeric spermidine synthase/saccharopine reductase                | Deletion | -  | -  | 2.4 up | 2.7 up | 2.0 down | -  | -  | 2.0 down | -  | -  |
| um05850     | um05850.2 Polyamine oxidase (PAO)                                             | Deletion | 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 | -  | -  | -  | -  | 2.7 down | 2.7 down | 2.2 down | -  | -  | -  |
| um10672     | um10672.2 Peroxidases                                                         | Deletion | 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 | 3.0 down | 2.0 down | 2.1 down | -  | -  | -  | -  | -  | -  | -  |
| um10803     | um01516 SqI2; guanyl nucleotide exchange factor SqI2                         | Deletion | 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 | -  | 2.1 up | 2.1 up | 2.5 up | -  | -  | -  | 2.3 up | -  | -  |
| um11453     | um00648 Ubc5; probable UBC5 - E2 ubiquitin-conjugating enzyme               | Deletion | -  | -  | -  | -  | 2.5 up | 3.1 up | -  | -  | -  | -  |
| um12023     | um06025 Rop1 HMG-box transcription factor (C-terminal fragment)              | Deletion | -  | -  | 3.1 up | -  | -  | -  | -  | -  | -  | -  |
| um12024     | um05762 hdp1                                                                  | Described | 3.3 up | 3.2 up | 2.9 up | 2.5 up | -  | -  | -  | -  | -  | -  |

Table 4. Regulation of *Ustilago maydis* genes described as important for virulence in maize, during *Arabidopsis* infection.
Table 5. Differential expression of genes involved in the synthesis of structural polysaccharides of U. maydis during Arabidopsis infection

| Identity | Gene     | Subcellular 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     |                                     | 3.2 up 3.4 up 2.1 up 2.3 up | - - - - |
| um10120  | CHS3     |                                     | - - - 2.3 down | - - - - |
| um01143  | Cell wall|                                     | 2.6 down 2.2 down | - - 2.5 down 2.8 down | - |
| um01788  |          |                                     | - - 2.7 down | - - - - |
| um02019  |          |                                     | - 2.7 up 5.4 up | 8.1 up 2.6 up | - 2.4 up |
| 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  |          |                                     | - 2.7 up 5.4 up | 8.1 up 2.6 up | - 2.4 up |
| um11723  |          |                                     | 3.4 up 3.1 up | 2.7 up 2.7 up | - - - - |
| um04405  | ROT2*    |                                     | 6.0 down 4.3 down | 7.9 down 5.6 down | - - - - |
| um00857  |          |                                     | 2.1 up 2.0 up | - - - - |
| um03569  |          |                                     | 2.0 up 2.5 up | - - - - |
| 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 down | - - - - |
| um05809  |          |                                     | 3.3 up 5.2 down | - - - - |
| 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 |

*Homologs to Saccharomyces cerevisiae genes.

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

| Sequence identity | Homolog gene | 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 | - - - - |
| um10262          | VRG4         |          | 2.1 up - - - - | - - - - |
| um04198          | OST3         | Oligosaccharide transference | 3.8 up 2.8 up 3.6 up 2.8 up | 2.9 up 2.2 up 2.0 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/ | 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          | HTM1         |          | 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/Thr | 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 |

*Homologs to S. cerevisiae genes.
NimbleScan software was used to import the scanned images and data extraction. Normalization was made with NimbleScan software using quantile and the algorithm Robust Multi-array Analysis (RMA). 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. 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) was used for functional annotation of differentially expressed genes.

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

| Identity  | Homolog gene | Function | Process | FB2 1 dpi | FB2 2 dpi | FB2 4 dpi | FB2 8 dpi | Uid1 1 dpi | Uid1 2 dpi | Uid1 4 dpi | Uid1 8 dpi |
|-----------|--------------|----------|---------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| um00213   | GPII         | Modified of PI with GlcNAc (GPI-GnT complex) |         | -         | -         | -         | -         | 3.4 up    | 3.2 up    | 2.6 up    | 2.5 up    |
| um00302   | GPII2        | Deacetylation |         | 2.9 up    | 4.5 up    | 3.3 up    | 4.1 up    | -         | -         | 2.0 up    | 2.4 up    |
| um1347    | GPII4        | Transfer of mannose |         | 2.4 up    | 2.9 up    | 2.6 up    | 2.3 up    | -         | -         | -         | -         |
| um05554   | GPII8        | Transfer of mannose |         | -         | -         | -         | -         | 2.3 up    | 2.0 up    | -         | -         |
| um05709   | GPI8         |          |         | 2.3 up    | 2.5 up    | 2.9 up    | 2.2 up    | -         | -         | -         | -         |
| um01565   | GPII7        | Removal of acyl group of GPI-anchor |         | 4.5 down  | 3.9 down  | 4.3 down  | 3.9 down  | -         | -         | -         | -         |
| um04859   | PER1         | Lipid Remodeling |         | 5.3 up    | 4.1 up    | 4.9 up    | 4.5 up    | 2.8 up    | 2.9 up    | 3.0 up    | 3.1 up    |
| um03003   | GUP1         | Lipid remodeling with longer fatty acids |         | -         | -         | -         | -         | -         | -         | -         | -         |

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 | 1 dpi | 2 dpi | 4 dpi | 8 dpi |
| 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     |
| Degradation             | Up       | 6     | 5     | 5     | 5     | 6     | 5     | 5     | 5     |
| Nucleases               | 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     | 3     | 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     | 8     | 8     | 8     | 7     | 7     |
| Non-enzymatic           | Up       | 4     | 4     | 2     | 2     | 4     | 4     | 4     | 4     |
|                         | Down     | 3     | 3     | 5     | 5     | 5     | 5     | 5     | 6     |
Bioinformatic searches. Besides a classification of all regulated genes, in silico searches of specific genes previously reported in the following groupings, were made: belonging to pathogenesis clusters, encoding proteins from the secretome, encoding hydrophobins or repellent proteins, regulated by the bE/bW heterodimer, involved in the synthesis and organization of the cell wall, 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, KEGG, Pfam SUPERFAMILY 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.

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

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