The General Transcriptional Repressor Tup1 Is Required for Dimorphism and Virulence in a Fungal Plant Pathogen

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Abstract
A critical step in the life cycle of many fungal pathogens is the transition between yeast-like growth and the formation of filamentous structures, a process known as dimorphism. This morphological shift, typically triggered by multiple environmental signals, is tightly controlled by complex genetic pathways to ensure successful pathogenic development. In animal pathogenic fungi, one of the best known regulators of dimorphism is the general transcriptional repressor, Tup1. However, the role of Tup1 in fungal dimorphism is completely unknown in plant pathogens. Here we show that Tup1 plays a key role in orchestrating the yeast to hypha transition in the maize pathogen Ustilago maydis. Deletion of the tup1 gene causes a drastic reduction in the mating and filamentation capacity of the fungus, in turn leading to a reduced virulence phenotype. In U. maydis, these processes are controlled by the a and b mating-type loci, whose expression depends on the Prf1 transcription factor. Interestingly, Δtup1 strains show a critical reduction in the expression of prf1 and that of Prf1 target genes at both loci. Moreover, we observed that Tup1 appears to regulate Prf1 activity by controlling the expression of the prf1 transcriptional activators, rop1 and hap2. Additionally, we describe a putative novel prf1 repressor, named Pac2, which seems to be an important target of Tup1 in the control of dimorphism and virulence. Furthermore, we show that Tup1 is required for full pathogenic development since tup1 deletion mutants are unable to complete the sexual cycle. Our findings establish Tup1 as a key factor coordinating dimorphism in the phytopathogen U. maydis and support a conserved role for Tup1 in the control of hypha-specific genes among animal and plant fungal pathogens.

Introduction
Dimorphism, the capacity of certain fungi to change their morphology between yeast-like growth and a filamentous state in response to environmental signals, is frequently associated with the virulence of both animal and plant pathogenic fungi [1–6]. This morphological conversion is controlled by several conserved signaling pathways, such as the cyclic AMP-protein kinase A pathway and a mitogen-activated protein (MAP) kinase cascade [4,6–10]. Another well known transcriptional regulator controlling dimorphism is the general transcriptional repressor Tup1, which is conserved from fungi to mammals [11–16]. The mechanism of action for Tup1 has been best studied in the yeast Saccharomyces cerevisiae. In this fungus, Tup1p forms a transcriptional co-repressor complex with Ssn6p, a protein that contains tetratricopeptide repeat (TPR) motifs known to mediate protein-protein interactions [17–20]. Neither Tup1p nor Ssn6p have direct DNA binding activity and their role in transcription depends on their recruitment to promoters by specific DNA binding proteins [18,21]. Tup1p repression mechanisms include the interaction with RNA polymerase II holoenzyme components and the alteration of chromatin structure through interaction with histones H3 and H4 and histone deacetylases [22–26]. Tup1p controls S. cerevisiae dimorphism in both haploid and diploid strains. Deletions of TUP1 result in reduced haploid invasive growth and reduced diploid pseudohyphal growth, which are considered the filamentous forms of this yeast [11].

Although the role of Tup1 in fungal dimorphism seems conserved, the way it controls this process frequently differs between fungi. The deletion of tup1 from the animal pathogens Candida albicans, Penicillium marneffei and Cryptococcus neoformans give clear examples of this variability. In C. albicans, the homozygous mutant for TUP1 shows a constitutive filamentation phenotype, in contrast to the situation described for S. cerevisiae, and reduced virulence [11]. In P. marneffei, however, tup1 is required for the maintenance of its filamentous form, negatively regulating yeast morphogenesis instead of filament formation [12]. In the case of C. neoformans, TUP1 is required for the formation of dikaryotic hyphae due to a mating defect of TUP1 mutant strains, and for virulence [27,28]. In addition, the molecular mechanisms and genetic pathways by which Tup1 acts in fungal dimorphism are poorly understood in most species [7,12,27–33]. This role of Tup1 in regulating the dimorphic transition is completely unknown in plant pathogenic fungi, which require different morphogenetic changes to successfully colonize their hosts and cause disease. The only data that might link Tup1 to a role in plant fungal dimorphism are...
Fungal plant pathogens cause serious damage to crops with huge social and economic consequences. To cause disease, many such fungi need to change their morphology between a yeast-like, unicellular form and a filamentous state. This change, known as dimorphism, is tightly controlled by complex genetic pathways to ensure successful pathogenic development. In animal pathogens, one of the most important genes controlling dimorphism is Tup1. In plant pathogens, however, the role for this gene is completely unknown. In this work, we describe the role of Tup1 in the dimorphism and virulence of Ustilago maydis, the plant fungal pathogen that causes maize smut disease. We show that mutant U. maydis cells lacking Tup1 are unable to properly change between yeast-like and filamentous forms, thus compromising its virulence. We look at the underlying genetic pathways, and find that Tup1 regulates key genes known to regulate dimorphism. We also show that Tup1 is essential for the production of mature fungal spores, which normally allow the fungus to disperse and infect new plants. Our results show that Tup1 is a key element in the control of both infectious and dispersable fungal forms and supports an evolutionary-conserved role for this gene in the regulation of dimorphism among animal and plant pathogenic fungi.

Author Summary

Fungal plant pathogens cause serious damage to crops with huge social and economic consequences. To cause disease, many such fungi need to change their morphology between a yeast-like, unicellular form and a filamentous state. This change, known as dimorphism, is tightly controlled by complex genetic pathways to ensure successful pathogenic development. In animal pathogens, one of the most important genes controlling dimorphism is Tup1. In plant pathogens, however, the role for this gene is completely unknown. In this work, we describe the role of Tup1 in the dimorphism and virulence of Ustilago maydis, the plant fungal pathogen that causes maize smut disease. We show that mutant U. maydis cells lacking Tup1 are unable to properly change between yeast-like and filamentous forms, thus compromising its virulence. We look at the underlying genetic pathways, and find that Tup1 regulates key genes known to regulate dimorphism. We also show that Tup1 is essential for the production of mature fungal spores, which normally allow the fungus to disperse and infect new plants. Our results show that Tup1 is a key element in the control of both infectious and dispersable fungal forms and supports an evolutionary-conserved role for this gene in the regulation of dimorphism among animal and plant pathogenic fungi.
and that it controls these processes by transcriptional activation of the Prf1 transcription factor through at least two of its direct regulators. Additionally, we show that tup1 is essential for full pathogenic development, affecting tumor formation and spore production. Our results indicate that Tup1 represents a key factor for the regulation of the pathogenic filamentous and dispersible spore forms of the corn smut fungus *U. maydis*.

**Results**

**Identification of the *U. maydis* tup1 homologue**

To identify Tup1 homologues in *U. maydis* we performed a blast search against the MIPS *U. maydis* database (MUMDB) proteome using Tup1p from the *S. cerevisiae* database (SGD) as the query sequence. A *U. maydis* protein sequence, um03280, with an e-value of 9.5e-81 and 66% similarity to *S. cerevisiae* Tup1p, was retrieved. This sequence, already annotated in MUMDB as Tup1, shows homology to Tup1 proteins from other fungi; including the animal pathogens *C. albicans* (67% similarity), *C. neoformans* (73%) and *P. marneffei* (75%) (all data in Table S1). A sequence alignment of Tup1 proteins from these organisms revealed a number of conserved domains, based on *S. cerevisiae*: (1) the tup_N domain, located in the N-terminal region, which is known to be required for Tup1p/Ssn6p complex formation; (2) seven WD40 domain repeats in the C-terminal region, that mediate protein-protein interactions and (3) a poorly conserved central region, which possesses histone binding activity in *S. cerevisiae* [24,57,58] (Figure 2, Table S2 and Figure S1).

**Tup1 is required for full pathogenic development**

To test if Tup1 has a role during the *U. maydis* life cycle, we generated deletion mutants for *tup1* in both mating compatible strains, FB1 and FB2, replacing the *tup1* open reading frame with the carboxin resistance cassette from pMF1-cl [35]. Examination of cell growth and morphology did not reveal any statistically significant differences in either of the *tup1* mutants (Figure S2).

Since the *U. maydis* life cycle is intrinsically linked to its host, we assayed the virulence of *tup1* deletion strains. For this purpose, we infected seven day old maize seedlings with compatible mixtures of either wild-type or Δ*tup1* fungi, and scored tumor formation 14 and 21 days post-infection (dpi). We noticed a considerable reduction in the number of Δ*tup1* infected plants that developed tumors compared to wild-type infections. Moreover, the size of tumors developed by Δ*tup1* strains were also considerably reduced (Figure 3A, 3B, and Figure S3). In addition, we observed reduced plant mortality for *tup1* mutant infections, with no dead plants observed at 14 dpi and only 11% mortality versus 57% for the wild-type strain 21 dpi. (Figure 3B and Figure S3).

To ascertain whether *tup1* mutants are able to complete the sexual cycle we assayed infected plants for the presence of spores 21 dpi. Interestingly, we found no spores of spores in wild-type tumors, we could not find spores in *tup1* mutant infected plants. Microscopy analysis of the Δ*tup1* induced tumors revealed that none of the fungal hyphae observed had progressed beyond the rounded cell formation stage that occurs just before spore maturation [46] (Figure 3C).

These results indicate that *tup1* is required for full pathogenic development of *U. maydis* and support a conserved role for *tup1* in the virulence of animal and plant fungal pathogens.

Δ*tup1* cells are impaired in mating and infective filament formation

During *U. maydis* plant infection, multiple morphological changes of the fungus are required (for review see [38]). To ascertain which steps of the infectious process are responsible for the decreased amount and size of tumors generated by *tup1* mutants, we first determined the extent to which they were able to successfully undergo mating and develop dikaryon filaments. To test this, we co-spotted compatible combinations of *tup1* mutants and wild-type strains on PD-Charcoal plates, where the appearance of “fuzzy” white colonies indicates successful mating and the formation of dikaryon filaments. As shown in Figure 4A, crosses between *tup1* mutants were unable to form white fuzzy colonies, indicating a recognition or fusion defect between compatible partners, or a post-fusion filamentation defect. Similarly, crosses between *tup1* mutants and compatible wild-type strains also showed fuzzy colony formation defects. Filamentation was partially affected when FB1Δ*tup1* was crossed with wild-type FB2, showing an intermediate phenotype between wild-type and Δ*tup1* crosses. In contrast, the FB1 and FB2Δ*tup1* cross showed the same loss of fuzzy colony phenotype as the double mutant cross. In order to check whether the differences observed in FB1Δ*tup1* and FB2Δ*tup1* strains could lead to different rates of tumor formation, we performed a plant infection assay using FB1 vs FB2Δ*tup1* and FB1Δ*tup1* vs FB2 crosses. As shown in Figure S4A the infection rates of these two strains were similar and slightly different to the rates observed for the cross of both wild type strains.

In addition, we analyzed white *fuzzy* colony formation in a SG200 background, which is able to form the infective hypha without the necessity of mating with a compatible partner, because of the presence of an active *bE1/BW2* heterodimer and a constitutively expressed *mfa2* gene [59]. Significantly, SG200Δ*tup1* did not generate *fuzzy* colonies on charcoal plates, suggesting a post-fusion role for *tup1* (Figure 4B). In order to quantify the phenotype, we performed a filamentation assay by co-spotting SG200CFP [60] and SG200YFPΔ*tup1* labeled strains on PD-charcoal plates. After fuzzy colony formation, colony samples were used for the quantification of filaments formed by each strain. As shown in Figure 4C, 80% of the filaments corresponded to the wild-type strain, while only 20% belonged to the mutant. Maize infection experiments with *tup1* mutants in the SG200 background revealed similar virulence defects to what we had observed in FB1 and FB2 backgrounds (Figure S5A and S5B). Insertion of a single copy of *tup1* under the control of the constitutive *otj* promoter in the *yp* locus [34] of SG200Δ*tup1*, restored its filamentation and pathogenic capacity, indicating successful complementation (Figure 4B, Figure S5A and S5B). Moreover in the case of the FBD11 diploid strain, which also do not need to mate with a compatible partner to cause virulence, the heterozygous mutant FBD11Δ*tup1/*tup1 and the homozygous FBD11Δ*tup1*Δ*tup1* were
almost completely avirulent in leaf infection experiments (Figure S4B and S4C). Because of the reduced infection capacity of the FBD11 wild-type strain, we also performed flower infections (where we usually observe bigger tumors) with these strains to better reflect the differences between them. This experiment revealed big tumors in the wild-type strain, medium tumors in the heterozygous and small tumors in the homozygous mutant strains (Figure S4D and S4E).

These results point to a post-fusion filamentation defect as a plausible reason for the impaired pathogenicity of \(D_{tup1}\) strains. However, it has been reported that mating or filamentation defects on PD-Charcoal plates are not always conserved on the plant leaf surface [61]. To check this, we co-infected 7 day old maize seedlings with the labeled strains, SG200CFP and SG200YFP\(D_{tup1}\) and quantified filament formation on the leaf surface. As shown in Figure 4C (on plant columns), the filamentation defect seen on charcoal containing media was also apparent on the leaf surface, with only around 5% of the filaments formed corresponding to the mutant strain.

Finally, to check whether \(tup1\) could also be implicated in other morphological changes required during the \(U. maydis\) infection process, we checked for appressoria formation and the presence of clamp-like cells during mycelium expansion in \(tup1\) mutant strains. We observed that both of these structures were formed in the deletion mutants for \(tup1\) (Figure 5A and 5B), although at lower frequency than the wild type, which is very likely a consequence of the filament formation defect showed by these mutants. The frequency of appressoria formation by SG200YFP\(D_{tup1}\) was reduced to a similar extent as filament formation (Figure S5C), and mycelium expansion was reduced in \(D_{tup1}\) infected plants at 2 dpi (Figure 5C). These results, together with the capacity, albeit reduced, of \(tup1\) mutants to induce tumors in maize, suggest that those \(tup1\) mutant cells that overcome the filamentation defect are then able to undergo the morphological changes required for plant penetration and expansion. Thus, the role of \(tup1\) in the morphological changes that occur during \(U. maydis\) infection seems to be specific to the yeast-to-hypha transition.

**Induction of the \(b\) locus restores the filamentation defect of \(tup1\) mutants**

As \(tup1\) mutants are unable to form dikaryotic hyphae at wild-type levels, we wondered whether \(tup1\) regulates genes downstream...
of the \(bp\) locus, thus compromising the fungal dimorphic transition in \(tup1\) mutants. To this end, we used the AB33 strain in which expression of a compatible \(bE1/bW2\) heterodimer is under the control of the \(nar\) inducible promoter [62]. When this strain is grown in inducing conditions it forms a \(b\)-dependent filament. We found that deletion of \(tup1\) in this background did not affect its filamentation capacity (Figure 6A; see Figure S6 for quantification). This result suggests that Tup1 is affecting processes upstream of the \(bp\) locus or, alternatively, is acting on a parallel pathway regulating filamentation. To discern between these two possibilities, we extracted total RNA from SG200 and SG200 \(\Delta tup1\) fungi grown on charcoal-containing media for 48 hours and quantified the expression of \(bE\) and \(bW\) by Northern blot. We observed a strong decrease in both gene transcripts in SG200 \(\Delta tup1\) indicating that \(tup1\) is required for the normal expression of \(bp\) loci genes (Figure 6B lanes 5 and 6).

To test if constitutive \(b\) expression could rescue the filamentation and virulence phenotypes of \(tup1\) mutants, we took advantage of the HA103 strain, which harbors a compatible \(bE1/bW2\) heterodimer under the control of constitutive promoters [52]. Deletion of \(tup1\) in HA103 did not produce the filamentation and virulence defects described for the SG200 background (Figure 6C, 6D and Figure S7), indicating that constitutive \(b\) expression partially rescues these phenotypes. To better understand the effect of \(b\) expression on the \(tup1\) mutant virulence phenotype, we used the HA103 parental strain, CL13 [59], which carries compatible \(bE1\) and \(bW2\) genes under the control of their own promoter and lacks the constitutively-expressed \(mfa2\) gene present in SG200. Deletion of \(tup1\) from CL13 led to a 90% reduction in maize tumor formation (Figure 6D and Figure S7), revealing an even clearer \(b\)-genes dependent rescue of \(tup1\) mutant phenotypes. Interestingly, the expression level of the \(b\) genes correlated with the phenotype of the wild-type and \(\Delta tup1\) strains (Figure 6B).

**Figure 4.** \(tup1\) is required for mating. (A) Mating between compatible \(U. maydis\) strains. The strains indicated (top/left) were spotted either alone or in combination and incubated on PD-charcoal plates for 24 hours at 25 °C. A white fuzzy colony appearance is indicative of successful mating and the formation of aerial dikaryotic hyphae. (B) Filament formation in SG200 and SG200\(\Delta tup1\) strains. The indicated strains were spotted alone on PD-charcoal plates. The presence of white fuzzy colonies indicates the formation of filaments. (C) Quantification of filamentation defects in the \(tup1\) deletion strain. A mixture with equal number of cells from SG200\(CFP\) and SG200\(YFP\Delta tup1\) were spotted onto charcoal plates or inoculated into maize plants. Image on the left represents the filamentation capacity of both strains on charcoal containing media. Scale bar represents 20 μm. The chart on the right indicates the number of filaments that corresponded to each strain in charcoal plates or on the plant leaf surface. Strains are indicated within the color legend. The total number of filaments counted (n) is indicated above each pair of columns. Mean values of three independent experiments and the standard deviation are shown.

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**Figure 5.** Appressorium, clamp-like cells formation and mycelium expansion of \(tup1\) mutants. (A) Appressorium formed by wild-type SG200\(CFP\) and SG200\(YFP\Delta tup1\) strains. (B) Clamp-like cells formed by wild-type and \(tup1\) mutant cells 2 dpi. (C) Visualization of mycelium expansion inside the plant tissue of the indicated strains 2 dpi. Infected leaf samples were stained with WGA-AF and propidium iodide (see Methods). Scale bars represent 20 μm.

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Moreover, when we focused on the CL13 and SG200 backgrounds, we observed that the SG200 Δtup1 strain had a b expression level, filamentation and virulence capacity comparable to the wild-type CL13 strain (Figure 6, Figure S7 and Figure S8). Thus, the effect of deleting tup1 from SG200 seems to be equivalent to removing its constitutive expression of mfa2, which would suggest a putative role for the pheromone responsive pathways in tup1 mutant phenotypes.

Tup1 is required for mfa1 gene expression, and conjugation tube formation upon pheromone stimulation

In our earlier experiment we bypassed the requirement for cell fusion by using the SG200 strain to identify a post-fusion requirement for tup1 in U. maydis filamentation. However, this experiment does not exclude a role for tup1 in mating between compatible strains as well, especially since both a and b loci genes are in the same position of the genetic pathway that controls the dimorphic transition. Moreover, as commented above, the similarity between SG200 Δtup1 and CL13 strains may reflect a role for tup1 in the transduction of the pheromone signal.

To test this possibility, we extracted total RNA from a FB1 Δtup1 vs FB2 Δtup1 cross grown on charcoal-containing media for 24 hours and compared mfa1 and be1 expression with a wild-type strains cross by Northern blot. In the wild-type cross, as a result of the recognition of pheromones by receptors of opposite mating types, activation of pheromone responsive pathways takes places, which is reflected in the expression of genes at both a and b loci. In the case of the tup1 mutant cross, however, we observed reduced mfa1 and be1 expression (Figure 7A), indicating that tup1 is necessary for wild-type expression of these genes. Accordingly, FB1 Δtup1 and FB2 Δtup1 strains drastically reduced conjugation hyphae formation upon stimulation with synthetic pheromones of the opposite mating type (Figure 7B and 7C).

Thus, tup1 is required for signal transduction upon stimulation with pheromone and expression of genes at both a and b loci, which is reflected in the observed pre and post-fusion defects of Δtup1 cells.

Tup1 controls a and b loci genes downstream of the MAP kinase cascade

The expression of a and b loci genes is controlled by the cAMP and MAP kinase pathways through their common effector Prf1. To situate tup1 within this genetic context, we used the FB1P<sub>agr</sub> fus7DD strain, which harbors a constitutively active allele of fus7 MAPKK under the control of the arabinose inducible promoter csg1 [51] (see Figure 1 for components of the MAP

![](Figure 6. Genetic interaction between tup1 and the b mating-type locus. (A) Induction of b-compatible heterodimer in the AB33 background. Expression of be and bw genes was induced by a shift from ammonium (OFF) to nitrate (ON) containing minimal media. b-dependent filament formation could be observed both in wild-type and tup1 mutant strains. Pictures were taken 5 hours post-induction. Scale bars represent 20 µm. (B) b-gene expression level in wild-type and tup1 deletion strains of CL13 (a1 be1/bw2), SG200 (a1 mfa2 be1/bw2) and HA103 (a1 (be1/bw2)con). 10 µg of total RNA extracted from each strain grown on charcoal minimal media for 48 hours at 25 °C was loaded per lane. Methylene blue stained rRNA was used as loading control. Numbers indicate the relative signal of be gene in regard to rRNA. (C) Filamentation capacity of the indicated strains growing on PD-charcoal plates during 24 hours at 25 °C. White fuzzy colonies indicates b-dependent filament formation. (D) Representative images showing the most prevalent disease category for wild-type and tup1 mutant infected plants. Strains are indicated below. The FB1 (a1 be1/bw1) background, which harbors an incompatible b-heterodimer, was used as control. Scale bar represent 1 cm. doi:10.1371/journal.ppat.1002235.g006
Tup1 is required for expression of prf1 transcription factor

We have shown that tup1 seems to regulate the expression level of a and b loci genes acting downstream of the MAP kinase cascade. Since the Prf1 transcription factor is the genetic element connecting the MAP kinase cascade and the mating-type genes, we measured prf1 expression level in a FB1 Prf1-fuz7DD background under inducing conditions. The removal of tup1 prevented the increase in prf1 expression (Figure 8A), indicating that tup1 is required for prf1 expression upon MAP kinase cascade induction. Moreover, the filamentation defects on charcoal-containing media as well as on the plant surface were rescued with the constitutive expression of prf1 (Figure 8C and 8D). These results strongly suggest that tup1 affects mating and b-dependent filament formation through control of prf1 transcription factor expression level rather than by controlling the expression of a and b loci genes directly.

Tup1 deletion affects the expression of several b and pheromone/fuz7DD regulated genes as well as the rop1 transcription factor

As constitutive bE/bW expression did not fully complement Δtup1 phenotypes, we were interested in identifying other Tup1 regulated genes, that might also have roles in the dimorphic transition and virulence in U. maydis. For this purpose we performed a microarray analysis with custom Affimetrix array (MPIUstilagoA), covering 3823 of the 6787 predicted genes, and compared the gene expression of SG200 and SG200Δtup1 strains grown on MM-charcoal array plates for 48 hours (see Methods). We identified a total of 115 genes (around 2 % of the covered genes) with altered expression in the tup1 mutant strain. Of these, 59 were upregulated and 56 downregulated. Within this list appear the bE and bW genes together with 34 genes that have also been described as b regulated genes [63], and 17 genes described as pheromone regulated [64] (Table S3). Thus, around 36% of the genes directly or indirectly regulated by kinase pathway). Upon induction, this strain promotes the expression of a and b loci genes via the Prf1 transcription factor. After deleting tup1 from this strain, we checked for a and b loci gene expression under inducing conditions. As expected, increased transcription for genes at both loci was observed in the wild-type strain; however, this was not the case for the tup1 mutant, indicating that Tup1 regulates a and b gene expression downstream of Fuz7 MAPK kinase (Figure 8A). Since Tup1 is involved in regulating the expression of genes related to glucose metabolism, the expression level of Fuz7 under the control of the erg1 promoter was also examined. No difference in fuz7DD expression was observed between the wild-type and the Δtup1 strains (Figure 8A).

Apart from its effect on the expression of the previously mentioned genes, induction of the fuz7DD in the tup1 deletion strain could also induce conjugation tube formation. As shown in Figure 8B, tup1 mutants in this background were able to form conjugation hyphae at similar levels to wild-type fungi in inducing conditions (Figure S9 for quantification). This result makes it unlikely that Tup1 is regulating conjugation tube formation downstream of the MAP kinase cascade and, at the same time, strongly suggest that tup1 regulates mating-type genes downstream of Kpp2 MAP kinase.
tup1 are also regulated upon bE/bW heterodimer and/or pheromone/fuz7DD induction, in agreement with our earlier results and supporting the quality of our dataset. Additionally, in order to experimentally validate our microarray data, the differential expression of some of the genes was confirmed by Northern blot analysis (Figure 9A).

All the 115 Tup1-regulated genes were classified in functional categories using the Blast2Go tool [65]. Enrichment analysis of genes up-regulated by the deletion of tup1 did not reveal a significant over-representation in any of the GO categories (Table S4). The genes down-regulated upon tup1 deletion our analysis revealed a significant over-representation in two GO categories: “Carbohydrate metabolic process” (GO:0005975; 8 genes) and “Antioxidant activity” (GO:0016209; 3 genes) (Table S4). 4 of the 8 genes belonging to the first category were also b-regulated genes, with two of them defined as strictly b-dependent (Table S3). The second category comprises proteins involved in the inhibition of dioxygen or peroxide-induced reactions and could be related to pathogenicity since production of these compounds is a well-characterized plant defense mechanism [66,67], and H2O2 detoxification is required for U. maydis virulence [68].

Interestingly, several tup1-regulated genes are associated with processes that could be related to the morphological switch from yeast-like to filamentous growth. Almost 10% of these genes are potentially involved in cell wall synthesis or modification, revealing that the altered yeast-to-hypha transition, promoted by deletion of tup1, results in a different cell wall composition.

Significantly, we found that rop1, that encodes a direct activator of Prf1, was down-regulated in the tup1 deletion strain (Table S3). This suggests an indirect role for rop1 in controlling prf1 expression. Rop1 has been described as being required for the mating of compatible strains on charcoal containing media, with a post-fusion role, due to the inability of SG200Δrop1 to form white fuzzy colonies on charcoal plates. It is essential for conjugation tube formation upon pheromone stimulation, and for expression of pheromone-responsive genes [61]. These phenotypes clearly resemble the situation described for tup1 mutants; however, rop1 mutants are fully pathogenic, with no mating or filamentation defects described on the plant leaf surface [61].

In addition to rop1, we identified an interesting candidate gene, um15096, that could be related to the tup1 mutant phenotypes. In Schizosaccharomyces pombe, a homologue of um15096, named pac2, has been shown to be a repressor of ste11 (the putative functional homologue of prf1) [69]. Interestingly, um15096/pac2, herein referred to as pac2, appeared over-expressed in the tup1 deletion strain. To check whether this putative prf1 repressor could also be

Figure 8. Tup1-dependent regulation of mating-type genes and prf1 transcription factor. (A) mfa1, bE1, and prf1 expression levels upon fuz7DD allele induction. Expression of the fuz7DD allele was induced by a shift from a glucose to arabinose containing CM media. Total RNA was extracted 5 hours post-induction and 10 μg were loaded in each lane. U. maydis actin was used as loading control. Strains (above) and probes (right) are indicated. (B) Conjugation tube formation upon fuz7DD induction. Pictures were obtained by optical microscopy 5 hours post-induction. - (glucose) and + (arabinose) indicate non-inducing and inducing conditions, respectively. Scale bars represent 20 μm. (C) Filamentation of constitutive expressed prf1 strains on charcoal media. SG200, SG200prf1con and their derivatives were spotted alone on charcoal plates and grown at 25°C for 24 hours. White fuzzy colonies appearance indicates formation of filaments. (D) On planta filamentation of constitutive expressed prf1 strains. SG200, SG200prf1con and their derivatives were inoculated into maize plants and their filamentation capacity was determined 24 hours post-inoculation.
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Northern blot. Probes (right) and strains (top) used are indicated. Methylene blue stained rRNA was used as loading control. Total RNA was loaded per lane. (B) Filamentation capacity of the plasmid strains (A) Validation of microarray data by Northern blot. Expression of the pac2 gene in regard to rRNA. doi:10.1371/journal.ppat.1002235.g009

Figure 9. Microarray validation and pac2 mutants filamentation and virulence phenotypes. (A) Validation of microarray data by Northern blot. Probes (right) and strains (top) used are indicated. b-dependent (b) and strictly b-dependent genes (sb) according to [63], are indicated. Methylene blue stained rRNA was used as loading control. Total RNA was extracted from the indicated strains growing on minimal media charcoal-array medium for 48 hours at 25 C. A total of 10 µg of RNA was loaded per lane. (B) Filamentation capacity of the pac2 mutant strains. Strains indicated (left) were spotted alone on PD-charcoal plates and grown for 24 hours at 25 C. pac2 indicates constitutive expression of pac2 from the otet promoter. con indicates strains upon fuz7DD allele induction was measured by Northern blot. Expression of the fuz7DD allele was induced by a shift from a glucose (-) to arabinose (+) containing CM media. 10 µg of total RNA were loaded per lane. rRNA was used as loading control. Numbers indicate the relative signal of prf1 gene in regard to rRNA.

playing a role during filamentation and pathogenic development, we over-expressed pac2 by integrating an extra copy of the gene under the control of the otet constitutive promoter in the bp locus of the SG200 strain. Filament formation of SG200pacz2 was reduced on charcoal containing media (Figure 9B) and, more importantly, pathogenicity was reduced to levels comparable to tup1 mutants (Figure 9C). The fact that pac2 is over-expressed in tup1 mutants together with the observation that ectopic pac2 expression decreases filamentation and virulence in the wild-type strain, strongly suggest that pac2 expression contributes to the filament formation and pathogenic defects of Δtup1 cells. Consistent with this, the deletion of pac2 from SG200 resulted in wild-type filamentation and infection rates (Figure 9C). When prf1 expression was induced by constitutively activating the MAPK pathway at Fuz7 level, overexpression of pac2 abolished its expression, while deletion of tup1 did not apparently affect it. Similar results were observed for mfa1 and ΔE1 genes. The double Δtup1Δpac2 mutant showed the same level of expression as the single Δtup1 strain (Figure 9D); probably as consequence of the tup1Δpac2 mutant showed the same level of expression as the single Δtup1 strain (Figure 9D); probably as consequence of the deletion strain phenotypes.

In summary, our microarray data reveal that at least 36% of the genes whose expression is affected by deletion of tup1 seems to be a consequence of tup1-dependent regulation of a and b loci genes through prf1. Moreover, the role of Tup1 in the control of prf1 expression could be explained by the altered expression of rop1 and pac2 observed in the tup1 mutant strain.

Tup1 affects the expression of the prf1 transcriptional regulators rop1 and hap2 but not crk1

As Tup1 seems to have an indirect effect on prf1 transcription level through Rop1 and, putatively, Pac2, we wondered whether the expression of other known prf1 regulators could be affected in tup1 deletion strains. Apart from Rop1, prf1 is known to be directly regulated by Hap2 [70] and indirectly through the MAP kinase Crk1 [71]. Northern blot assays of SG200 and SG200Δtup1 grown on charcoal media showed that the expression level of crk1 was unaffected in tup1 deleted strain. In contrast, the levels of rop1 and hap2 were reduced in comparison to the wild-type strain (Figure 10). However, as Crk1 acts on prf1 indirectly, and since it has been previously reported that the effect of Crk1 on prf1 depends on the prf1 promoter UAS [71], we tested whether Tup1 could regulate prf1 via its UAS. For this purpose, we used the HA232 strain, which harbors a GFP reporter gene under the control of the prf1 promoter UAS (see [33] for details). In this strain, GFP is strongly expressed when grown on glucose-
containing media, while its expression is reduced on a maltose containing media [33]. As is shown in Figure S10, the expression levels of the reporter gene were indistinguishable in Δtup1 mutants from the wild-type in all the conditions tested. This indicates that Tup1 is unlikely to act via the prf1 promoter UAS, in contrast to Crk1. Thus, the effect of Tup1 on prf1 expression seems to be mediated via Rop1 and Hap2 but not through the Crk1 pathway.

To sum up, although other factors may be implicated in tup1 mutant phenotypes, Tup1 seems to control the dimorphic transition and participates in the virulence program of U. maydis by indirectly regulating prf1 expression via altered rop1 and hap2 expression levels, and possibly also through aux2, which would lead to a down-regulation of prf1-dependent expression of a and b loci genes and their related phenotypes.

Discussion

In the basidiomycete phytopathogen U. maydis, the switch from non-infective yeast-like growth to an infective filament formation occurs in response to different environmental cues, and is tightly controlled by complex genetic pathways in order to ensure the coordination and timing of the different processes associated with dimorphism. In this work, we have shown that the highly conserved general transcriptional repressor Tup1 plays a central role in controlling the proper expression of the genes implicated in the genetic control of mating, filamentation, and pathogenic development of this corn smut fungus.

Tup1 has been shown to be important during growth of vegetative cells in other fungi such as S. cerevisiae, C. neoformans or P. marneffei [12,27,72]. In the case of Ustilago maydis, differences could be observed in the tup1 mutants, although none of these were statistically significant. Interestingly the normal growth of Δtup1 strains contrasts with the poor growth capacity described for U. maydis strains harboring a partial deletion of sgl1, the functional homolog to S. cerevisiae SSV6. However because these strains were not stable, the role of Sgl1 could not be completely analyzed [34]. Thus a comparison between Tup1 and Sgl1 of their growth capacity on U. maydis vegetative cells cannot be properly established. In other fungi, single deletions of tup1 and sgl1 have been reported to result in different phenotypes [73-76].

For example, the deletion of SSV6 but not of TUP1 homologues is lethal in S. pombe [75] and Aspergillus nidulans [76]. Moreover, Tup1 and Snf6 have been shown to regulate different sets of genes [74] and to form independent complexes in C. albicans [77].

A central question in this study was whether tup1 is involved in the infectious process of plant pathogenic fungi. We have observed that infections with Δtup1 cells lead to a reduction in tumor formation, plant death, and a failure of spore formation, indicating that Tup1 is required for full pathogenic development in U. maydis, and making tup1 mutants unlikely to cause damage in natural environments. Thus, tup1 seems to play a conserved role in virulence of animal and plant fungal pathogens.

The next key question was to try to understand the mechanism by which tup1 is required for normal tumor formation. Our results suggest that the virulence phenotype of Δtup1 cells has two main causes: (i) a recognition problem between compatible partners, due to the inability of tup1 mutants to form conjugation hyphae upon pheromone stimulation, and (ii) a filamentation defect, due to the inability of SG200 to form filaments at wild-type levels both on PD-charcoal plates and on the plant leaf surface. Additionally, the fact that the differences on conjugation hypha formation between FB1Δtup1 and FB2Δtup1 strains, though not statistically significant, together with the differential filamentation showed by crosses of these strains with their respective compatible wild-type strains on charcoal plates, suggest also a role for Tup1 in cell fusion, at least in the FB2 background. These defects result in tup1 mutants being unable to properly undergo dimorphic transition. These findings suggest that the impaired pathogenicity of tup1 mutant animal and plant fungi may also depend on a conserved role in the yeast-to-hypha transition.

Consistent with the conjugation and filamentation phenotypes of tup1 mutants, the expression of a and b loci mating-type genes was reduced in tup1 deletion strains, most likely as a consequence of Tup1-dependent regulation of the prf1 transcription factor. Microarray analysis of SG200Δtup1 during filamentation on charcoal media revealed a number of mis-regulated genes whose expression was also affected upon b-compatible heterodimer and/or pheromone/aux7DD induction, including the b locus genes themselves, supporting the proposed role for tup1 during U. maydis mating and dikaryotic filament formation. On the other hand, in our microarray analysis we did not detect tup1-dependent changes in gene expression for any of the b-dependent genes previously described as being essential for pathogenicity [60,63,78], which is consistent with the ability, albeit reduced, of tup1 mutants to induce tumors in maize.
Interestingly, the main effector that links tup1 to the control of dimorphism seems to be conserved between U. maydis and C. albicans. In contrast, the genetic pathways by which tup1 acts on filamentation seem to differ, depending on the genetic control of hypha-specific genes in each organism. In C. albicans, Tup1 is proposed to control filamentous growth through the repression of hypha-specific genes by forming complexes with the transcriptional repressors Rgl1 and Nrg1, rather than affecting the elements in the Cph1-mediated MAPK and Elg1-mediated cAMP pathways [7,54–56]. Moreover, expression analysis of filament-specific genes in Δphl1/Δphl1, Δgfi1/Δgfi1 and Δtup1/Δtup1 strains revealed common and divergent target genes [7]. Thus, Tup1 integrates into the network system proposed for the control of filament-specific genes in this fungus [7,10]. On the other hand, in U. maydis, Tup1 controls infective filament-specific gene expression via a central regulatory, the Prf1 transcription factor, which is transcriptionally and post-translationally regulated by the cAMP and MAPK pathways [47,51–53]. Interestingly, U. maydis Prf1 is a High Mobility Group (HMG) transcription factor, similar to C. albicans Rgl1. Thus, an analogous mechanism, implicating a Tup1-Prf1 complex, could explain the roles of Tup1 in the regulation of hypha specific genes in U. maydis. Moreover, in S. cerevisiae, a complex between Tup1p and the HMG-transcription factor Rox1p has also been proposed [19,79–81]. S. cerevisiae ROX1, whose deletion can be complemented by C. albicans RFG1 [33], is known to control hypoxic gene expression in a TUP1 dependent manner [19,79–81]. Additionally, the deletion of TUP1 increases the expression of ROX1 [82,83], but Rox1p itself is also able to regulate its own expression [83]. In aerobic conditions these observations can be explained by the proposed Tup1p·Smn6p·Rox1p complex which would regulate ROX1 expression and Rox1p-dependent hypoxic gene expression. In anaerobic conditions, however, the regulation of ROX1 expression seems to implicate an anaerobic repressor that requires Tup1p for its function [83]. Similarly, in U. maydis, the expression of prf1 is dependent on Tup1p and prf1 is also self-regulated [52]. However, when we analyzed the effect of Tup1 on prf1 expression level more deeply, we observed that at least two direct activators of Prf1 were also down-regulated upon tup1 deletion, rop1 and hap2. This finding, although not excluding a putative Tup1-Prf1 complex, points to an indirect effect of Tup1 on the expression of prf1 and its regulated genes. Rop1 is required for pheromone response and for fuzzy colony formation on charcoal-containing plates, but is dispensable for mating and filamentation on the plant leaf surface. In the case of hap2, it is known to be essential for the pheromone response and has also an effect on the filamentation capacity of SG200 that seem to be conserved on planta. Thus, we propose that the effect of Tup1 on prf1 is the sum of the effects of Tup1 in both rop1 and hap2 on artificial media, while only the effect on hap2 would be responsible for the on planta phenotypes. The drastic effect of tup1 deletion on prf1 expression levels on charcoal plates may be diminished on the plant leaf surface as rop1 is dispensable in this situation.

In this work, we have also described a new gene, pac2, which is likely to be playing a role in the tup1 mutant virulence phenotype, since its over-expression causes a decrease in the pathogenic capacity of U. maydis SG200 strain and its expression is increased in the SG200Δtup1 strain. Since the homologue of this gene in S. pombe is a repressor of ste11 [69], the putative functional homologue of pac1, we analyzed the relationship between Pac2 and Prf1 in U. maydis. We found that over-expression of pac2 in a FB1pac2,fuz7DD strain abolished the prf1 expression observed in the wild type strain establishing Pac2 as a repressor of Prf1. Accordingly, the deletion of pac2 in a SG200Δtup1 strain partially restored its filamentation and virulence defects. However, the double Δtup1Δpac2 mutant in the FB1pac2,fuz7DD background shows the same prf1 expression level than the single Δtup1 strain, probably because of Tup1 control of rop1 and hap2. Nevertheless since prf1 regulation on charcoal plates or during virulence integrates several inputs besides the MAPK pathway the relationship between pac2 and prf1 in the regulation of filamentation and pathogenicity cannot be fully established. Thus, the final role of tup1 in U. maydis virulence is also likely to be linked to its control of hap2 and pac2 mRNA levels (Figure 11).

Surprisingly, although Tup1 is described as a general transcriptional repressor, the deletion of tup1 from U. maydis leads to the down-regulation of the genes that control the dimorphic transition, suggesting an activator role for tup1 in controlling them. On the other hand, determining how Pac2 controls prf1 gene expression would help to determine the role of tup1 as an activator and/or repressor during dimorphism. The way Tup1 seems to control the expression of the prf1 transcription factor, through hap2 and rop1 and, putatively, pac2, clearly reflects the complex genetic regulation that prf1-related processes require.

Similarly, the number of genes that we found to be up- or down-regulated following tup1 deletion when cultured on charcoal

Figure 11. Proposed model for the roles of Tup1 in the control of mating-type genes. The MAP kinase pathway is shown in red. Black arrows represent transcriptional control. Components exclusively required in laboratory conditions (charcoal, pheromone stimulation, etc) are shown in green. Components specifically required during pathogenesis are shown in orange. In laboratory conditions the effect of Tup1 on prf1 expression would be mediated via its control of hap2, rop1 and pac2 expression levels. During infection, where rop1 is not required, Tup1 would control prf1 expression through hap2 and pac2. Question marks indicate putative elements or interactions.

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containing media was equivalent. Thus, under the conditions tested, the loss of tup1 causes a similar effect on both the repression and repression of genes. Although this could reflect indirect changes in genes expression resulting from the repression of Tup1-gene targets, it is nevertheless an intriguing observation. Regarding an activating role for Tup1, previous studies have also shown that Tup1 can behave as an activator as well as a repressor of the same target gene in different conditions [84] or different genetic backgrounds [83] in S. cerevisiae.

Finally, we have shown that tup1 seems to be required for spore production inside maize tumors. Roles for Tup1 in sporulation have been previously reported in other fungi. In S. cerevisiae, the sporulation-specific genes DI1 and DI2, which are required for spore wall formation, are regulated by Tup1p [86]; in Neurospora crassa, mutants for tyr-1, the homologue of TUP1, are asexual [87]; and in C. neoformans, tup1 deletion considerably reduces spore production [27].

In summary, our work provides new insights into the complex regulatory circuits for sexual and pathogenic development of U. maydis. We have identified for the first time a requirement for tup1 in several steps of the life cycle of a pathogenic plant fungus, including in the genetic pathways controlling dimorphism and virulence. Our findings contribute to a better understanding of the role of this general transcriptional repressor in pathogenic fungi and of the precise genetic control that these pathogenesis-related processes require. We consider that the roles and mechanisms of action described for U. maydis tup1 in this work will also be extremely valuable for studying the roles of tup1 in the transcriptional regulation of morphogenetic processes in other organisms.

Methods

Strains, growth conditions and plasmids

Escherichia coli DH5x was used for cloning purposes. Growth conditions for E. coli [88] and U. maydis [42,89] and the quantification of appressoria formation on the plant leaf surface [60] have been described previously. Quantification of filaments was performed as for the appressoria. For studies of growth rates and morphology, cells were grown on YEPSL liquid media for 12 hours, then diluted in the same media to an OD600 of 0.05 and grown until an OD600 of 0.8–1. Exponential growth cultures were determined by counting cells at different time-points. For charcoal grown cells, biomass was recovered and transferred to liquid nitrogen pre-chilled mortars. Total RNA was then recovered by centrifugation, washed with cold water, and total RNA was isolated with QIAGEN (Valencia, CA) RNeasy mini kit.

DNA and RNA procedures

Molecular biology techniques were used as described by [88]. U. maydis DNA isolation and transformation procedures were carried out following the protocol of [91]. Deletion constructs were generated according to [36]. To generate single deletion U. maydis mutants for tup1 (Um03280), pac2 (Um15096) and um0407 (genes, fragments of the 3′ and 3′ flanks of their open reading frames were generated by PCR on U. maydis FB1 genomic DNA with the following primer combinations: UmTUP1KO5-1/UmTUP1KO5-2 and UmTUP1KO3-1/UmTUP1KO3-2; UmPAC2KO5-1/UmPAC2KO5-2 and UmPAC2KO9-1/UmPAC2KO9-2; Um0407KO5-1/Um0407KO5-2 and Um0407KO9-1/Um0407KO9-2; (Sequences in Table S2). These fragments were digested with SfiI and ligated with the 1.9 Kb SfiI carboxin resistance cassette, 2.7 Kb SfiI hygromycin resistance cassette, or 1.5 Kb SfiI neosourcein resistance cassette as described previously [33]. Ligation products were then clone into pGEM-T-EASY vector (Promega). PCR generated linear DNA for each construct was used for U. maydis transformation.

For complementation of the tup1 deletion, the p123-tup1 plasmid was generated. p123-tup1 is a p123 [92] derivative in which the eGFP fragment has been substituted with the tup1 open reading frame. For this purpose, the tup1 open reading frame was amplified by PCR with the oligonucleotides Tup1p-Start and Tup1p-Stop, which contain NdeI and NotI restriction sequences respectively. Pluss high fidelity DNA polymerase (Invitrogen) was used. The PCR product was digested with NdeI and NotI, purified, and cloned into a p123 vector digested with the same restriction enzymes. Positive cloning was verified by restriction analysis and sequencing. To generate SG200Δtup1pΔtup1 strain, p123-tup1 was linearized with SfiI and integrated into SG200 wild-type strain ip locus by homologous recombination.

For over-expression of pac2, the p123-pac2 plasmid was generated by replacing the EGF fragment from p123 with the pac2 open reading frame. The Pac2 open reading frame was amplified using the oligonucleotides UmPac2ATGSmaXma y UmPac2StopNotI, digested with SmaI and NotI restriction enzymes and ligated into the p123 vector digested with the same enzymes. Successful cloning was verified by restriction analysis and sequencing. To generate SG200pac2, p123-pac2 was linearized with SfiI and integrated into SG200 wild-type strain ip locus.

For constitutive expression of pac2 in FB1, we constructed the plasmid pSHOP2. This plasmid consists in 1 kb fragment of the upstream sequence of pac2 open reading frame (ORF) followed by the osef constitutive promoter, the hygromycin resistance cassette and 1 kb of the pac2 ORF integrated in a pGEM-T-EASY vector. For this purpose 1 kb fragment of the upstream sequence of pac2 was amplified with the primers Umpac2-5UTR-1 and Umpac2-5UTR-2, using FB1 genomic DNA; the osef constitutive promoter followed by 1 kb of pac2 ORF was amplified with the primers Umotefpac2 and Umpac2-1kb, using the plasmid p123-pac2 as template. Both flanks where then digested with SfiI restriction enzyme and ligated with the hygromycin resistance cassette. This construction was ligated to a pGEM-T-EASY vector. FB1p-pac2 was generated by transformation of the wild-type FB1p-pac2 with the mentioned construct.

Single homologous integration of the linear plasmids or PCR products transformed was verified by PCR and Southern blot.

In the expression analysis, cells grown on liquid culture were recovered by centrifugation, washed with cold water, and total RNA was isolated with QIAGEN (Valencia, CA) RNasy mini kit. For charcoal grown cells, biomass was recovered and transferred to liquid nitrogen pre-chilled mortars. Total RNA was then
extracted from the crushed powder with trizol reagent (Invitrogen) and with the QIAGEN RNeasy mini kit. Isolated RNA was separated by formaldehyde denaturing agarose gel electrophoresis, and transferred overnight by capillary action to nylon membranes. Probes were obtained by PCR with the oligonucleotides indicated in Table S6. Radioactive labelling of PCR generated probes was carried out. Radioactive bands were visualized and quantified using a Molecular Dynamics PhosphorImager.

For qRT-PCR first strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s protocol. As a template for the reaction 1 μg of total RNA was used. Samples were incubated at 50°C for 1 hour. Real-time PCR was performed in a ABIPRISM 7000 Sequence Detection System (Applied Biosystems) using the Power SYBR Green PCR Master Mix according to the manufacturer’s protocol. Primers used for detection are shown in Table S6.

**Sequence alignment and domain structure**

*U. maydis* Tup1 sequence was obtained from MIPS *U. maydis* Database [http://mips.gsf.de/genre/proj/ustilago/]. *S. cerevisiae* and *C. albicans* Tup1 sequences were obtained from SGD [http://www.yeastgenome.org/] and CGD [http://www.candidagenome.org/] databases, respectively. The rest of the Tup1 sequences were obtained from the NCBI. Multiple sequence alignments were made with ClustalW2. Domain structure analysis was performed using InterProScan Sequence Search tool from the European Bioinformatics Institute [http://www.ebi.ac.uk/]/ Pfam retrieved domains were used. Schematic representation of the retrieved domains was performed maintaining proportions of each domain with respect to the whole protein sequence length.

**Fluorimetric measurement of GFP**

Cells were grown on nitrate minimal media containing 1% glucose or 1% maltose to an OD<sub>600</sub> of 0.6–0.8, then pelleted and resuspended in sterile water to an OD<sub>600</sub> of 1.0. Fluorescence from 200 μl of cell suspension transferred to a microtiter plate was measured by using a POLARstar Omega fluorescence reader (BMG LABTECH). GFP fluorescence was measured at a wavelength of 485 nm for excitation and 520 nm for emission. Quantification of filament formation on charcoal plates was performed by fluorescence analysis of colony samples. Fluorescence was normalized to OD<sub>600</sub>. At least three independent experiments were performed, each measured in triplicate.

**Microscopy**

Cell morphology of WGA-stained cells, conjugation tube and b-dependent filament formation were analyzed with a Zeiss Apotome microscope.

For *on planta* quantification of filament and appressoria formation in co-infection experiments with *U. maydis* GFP and YFP labelled strains, leaf samples were stained with calcofluor white (Sigma) to visualize fungal material and then checked for GFP or YFP fluorescence. Quantification of filament formation on charcoal plates was performed by fluorescence analysis of colony samples from co-spotted YFP and GFP strains. Post-penetration stages were visualized by WGA-AF 488 and Propidium Iodide (Sigma) staining of infected leaf samples as previously described [93]. Samples were examined using a Leica fluorescence microscope, equipped with a PlanApO x 100 lens and a Deltavision widefield microscope (Applied Precision, Issaquah, WA) equipped with 20, 40, 63 and 100 x lens. Image processing was carried out using Adobe Photoshop CS2.

**DNA array**

SG200 and SG200<sub>Δtup1</sub> cells were grown on YEPSL until exponential phase, then washed twice with sterile water and cultured on minimal charcoal array plates (12.5% Holliday salts, 2% vitamins, 30 mM L-glutamine, 2% glucose, 4% agar and 2% charcoal, pH 7) during 48 hours at 25°C. 144 cm<sup>2</sup> plates and a cell density of OD<sub>600</sub> 0.1/cm<sup>2</sup> was used. DNA-array analysis was performed using custom-designed Affymetrix chips (UstilagoA). Probe sets for the individual genes can be obtained from http://mips.helmholtz-muenchen.de/genre/proj/ustilago/. Target preparation, hybridization and data analysis was performed as described before [94], with the following alterations: total RNA was extracted as commented in DNA and RNA procedures for charcoal growing cells; 5 μg RNA were used for first strand cDNA synthesis at 50°C with Superscript II (Invitrogen); an adjusted P-value of ≤0.01 for the false discovery rate [95] and a change in expression of ≥2 was used for filtering. Expression values were calculated as mean of two biological replicates. Array data can be accessed at GEO/NCBI database (accession number GSE29591).

**Accession numbers**

*U. maydis* sequence data can be found in the GenBank/EMBL data libraries under accession numbers XP_759427 for Tup1, XP_762643.1 for Pac2, XP_756724 for bEl, XP_756725 for bW1, XP_758529 for Mfa1, XP_760967 for Acr1, XP_762479 for Egl1, XP_762172 for Rop1, XP_762530 for Hap2, XP_758660 for Cck1, XP_758860 for Prf1, XP_757661 for Fuz7, XP_760954 for um04807, XP_758669 for um11413, XP_756174 for um00027, XP_759558 for um03411 and XP_758874 for um09277. Other sequences used in this study have the following accession numbers: *S. cerevisiae* Tup1p, NP_010007; *C. albicans* Tup1, AAB63195; *C. neoformans* Tup1, XP_570974; *P. marneffei* TupA, AAL99251; *N. crassa* Rco-1, AAB37245; A. nidulans TupA ACD46267; *S. pombe* Tup11, NP_592873; *S. pombe* Tup12, NP_592910.

**Supporting information**

**Figure S1 Sequence alignment of Tup1 proteins from different organisms**. Different conserved domains are indicated. The *U. maydis* Tup1, *S. cerevisiae* Tup1p, *C. albicans* Tup1, *C. neoformans* Tup1p and *P. marneffei* TupA sequences were aligned using ClustalW2. Accession numbers can be found in Methods. (TIF)

**Figure S2 Growth and morphology of tup1 mutants**. In the first row, colony morphology of wild-type and Δtup1 strains grown on YPD plates during 24 hours at 28°C are shown (scale bars represent 1 mm). A magnification of each colony is shown (x2). The second and third rows show differential interference contrast (DIC) and fluorescence images of FITC-labeled wheat germ agglutinin (WGA) cells of each strain during exponential phase growth on rich liquid media (scale bar = 20 μm). (TIF)

**Figure S3 Disease symptoms caused by wild-type and tup1 mutant strains 21 dpi**. Strains and total numbers of infected plants (n) are indicated within the color legend. Seven day old maize seedlings were infected. Symptoms were scored 21 dpi. Tumor categories correspond to: large tumors (>5 mm), medium tumors (1–5 mm) and small tumors (<1 mm). Mean values of three independent experiments and the standard deviation are shown. Asterisk (*) represents statistically significant differences in regard to the wild-type strain. (TIF)

**Figure S4 Infection rates of FBD11 tup1 mutants and crosses between Δtup1 and wild-type strains**. (A) Disease symptoms of plants infected with the indicated strains (color
The total number of plants infected with each strain (n) is indicated above each column. Tumor categories correspond to: large tumors (>5 mm), medium tumors (1–5 mm) and small tumors (<1 mm). Mean values of three independent experiments are shown. B) Representative images of the infections for wild-type and tup1 mutant strains. Scale bars = 1 cm. C) Disease symptoms of plants infected with the indicated strains (color legend). The total number of plants infected with each strain (n) is indicated below the color legend. Tumor categories correspond to: large tumors (>5 mm), medium tumors (1–5 mm) and small tumors (<1 mm). Mean values of three independent experiments and the standard deviation are shown. Statistically significant differences are indicated (*). D) Representative images of maize flowers infected with wild-type or tup1 mutant strains. Scale bars = 1 cm. E) Disease symptoms of plants infected with the indicated strains (color legend). The total number of plants infected with each strain (n) is indicated below the color legend. Tumor categories correspond to: large tumors (>1 cm) and small tumors (<1 cm). Mean values of three independent experiments and the standard deviation are shown. Statistically significant differences are indicated (*).

Figure S5 Infection rates and quantification of appressorium formation in SG200 wild-type and tup1 mutant strains. A) Representative images showing the most prevalent tumor category for wild-type and tup1 mutant infected plants. Scale bars = 1 cm. B) Disease symptoms of plants infected with the indicated strains (color legend). The total number of plants infected with each strain (n) is indicated below the color legend. Tumor categories correspond to: large tumors (>5 mm), medium tumors (1–5 mm) and small tumors (<1 mm). Mean values of three independent experiments and the standard deviation are shown. Asterisk (*) represents statistically significant differences in regard to the wild-type strain. C) Quantification of appressorium formation. A mixture containing equal numbers of SG200CFP and SG200YFPTup1 cells was inoculated on seven day old maize seedlings. Appressorium formation was visualized by fluorescence microscopy of calcofluor stained leaf samples 16 to 24 hours post-inoculation. CFP or YFP fluorescence was used to determine the strain to which each appressorium belonged. The total number of appressoria counted (n) is indicated at the top (three independent experiments; standard deviation is shown).

Figure S6 Quantification of b-dependent filament formation in the AB33 background. A) Filament formation of AB33 and AB33Δtup1 strains in inducing (nitrate) and non-inducing (ammonium) conditions are represented. Color code for each strain (above), media and cell type (below) are indicated. The total number of yeasts/filaments counted for each strain (n) is indicated below the color legend and valid for both media. Quantification was performed 5 hours post-induction. The mean value of three independent experiments and the standard deviation is represented. B) Length of b-dependent filaments produced by wild-type and tup1 deletion strains (2 independent experiments). Measurement refers only to the filament, not to the original yeast cell.

Figure S7 Pathogenicity of solopathogenic tup1 mutant strains. Seven days old maize seedlings were infected with the indicated strains (color legend). Disease symptoms caused by wild-type and tup1 mutant strains were scored 14 dpi. A non-pathogenic FB1 strain was used as control. The total number of infected plants (n) is indicated above each column. Tumor categories correspond to: large tumors (>5 mm), medium tumors (1–5 mm) and small tumors (<1 mm). Mean values of three independent experiments are shown.

Figure S8 qRT-PCR analysis of bEL expression. The indicated strains were grown on charcoal-containing media during 48 hours at 25°C. For normalization act1 gene was used. Expression was calculated relative to the lowest expression value. Shown are the media of three technical replicates. All comparisons are statistically significant, except when comparing CL13 and SG200Δtup1.

Figure S9 Quantification of conjugation tube formation frequency and length in the FB1P_cog1/fuz7DD background. A) Quantification of the number of cells with conjugation tubes, upon induction of fuz7DD allele, in each strain. Expression of the fuz7DD allele was induced by a shift from glucose to arabinoce containing CM media. Quantification was performed 5 hours post-induction. The total number of cells counted (n) is given above each column. Three independent experiments were performed and the standard deviation is shown. B) Length of the conjugation hyphae developed by wild-type and tup1 mutant strains in a FB1P_cog1/fuz7DD background. Total number of hypha counted (n) (top) for each strain (bottom) are indicated (2 independent experiments). Measurement refers only to the filament, not to the original yeast cell.

Table S1 Identity and similarity between U. maydis Tup1 and Tup1 proteins from other organisms.

Table S2 Pfam retrieved domain position of Tup1 proteins.

Table S3 Altered gene expression by deletion of tup1 gene in the SG200 strain.

Table S4 Enrichment analysis for GO-categories of Tup1-regulated genes.

Table S5 List of primers used during this study.

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Author Contributions
Conceived and designed the experiments: AEV AFA JII. Performed the experiments: AEV AFA. Analyzed the data: AEV AFA. Contributed reagents/materials/analysis tools: AEV AFA. Wrote the paper: AEV AFA JII.

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Control of Dimorphism via Tup1 in a Plant Pathogen