Transcriptome Analysis of a *Ustilago maydis* *ust1* Deletion Mutant Uncovers Involvement of Laccase and Polyketide Synthase Genes in Spore Development

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Ustilago maydis, causal agent of corn smut disease, is a dimorphic fungus alternating between a saprobic budding haploid and an obligate pathogenic filamentous dikaryon. Maize responds to *U. maydis* colonization by producing tumorous structures, and only within these does the fungus sporulate, producing melanized sexual teliospores. Previously we identified *Ust1*, an APSES (*Asm1p*, *Phd1p*, *Sok2p*, *Efg1p*, and *StuAp*) transcription factor, whose deletion led to filamentous haploid growth and the production of highly pigmented teliospore-like structures in culture. In this study, we analyzed the transcriptome of a *ust1* deletion mutant and functionally characterized two highly upregulated genes with potential roles in melanin biosynthesis: *um05361*, encoding a putative laccase (*lac1*), and *um06414*, encoding a polyketide synthase (*pks1*). The Δ *lac1* mutant strains showed dramatically reduced virulence on maize seedlings and fewer, less-pigmented teliospores in adult plants. The Δ *pks1* mutant was unaffected in seedling virulence but adult plant tumors generated hyaline, nonmelanized teliospores. Thus, whereas *pks1* appeared to be restricted to the synthesis of melanin, *lac1* showed a broader role in virulence. In conclusion, the *ust1* deletion mutant provided an in vitro model for sporulation in *U. maydis*, and functional analysis supports the efficacy of this in vitro mutant analysis for identification of genes involved in planta teliosporogenesis.

Ustilago maydis, the causal agent of corn smut disease, is a heterothallic member of the phylum Basidiomycota with a dimorphic life cycle consisting of a saprobic budding asexual

phase and a filamentous parasitic phase (Christensen 1963; Holliday 1974). In the parasitic phase, filamentous fungal cells penetrate maize tissue, primarily the silks (stigmas), and enter developing ovaries. During infection, hyphae proliferate, inducing plant cell division and expansion, eventually forming massive smut galls. Inside the galls, darkly pigmented teliospores form, which give the maize ear a burnt, sooty appearance, hence, the disease name “smut.” Galls rupture and the spores are spread by wind and rain. In the spring, spores germinate and yield saprobic budding cells. Compatible budding cells are then able to mate and produce infectious filamentous cells, completing the life cycle (Christensen 1963; Holliday 1974).

The *U. maydis* dikaryon is an obligate biotroph; teliospore production occurs exclusively in planta tumors. Efforts have been made to elucidate the distinctive fungal development program leading to the formation of mature diploid teliospores. The morphological events during this process have been analyzed in detail, especially in galls formed in leaves and stems (Banuett and Herskowitz 1996). Fungal proliferation within plant tumors is associated with an increase in branching. Increasingly shorter branches formed especially at the hyphal tip appear to signal the switch from vegetative to sporogenous hyphae (Banuett and Herskowitz 1996). At this late stage of tumor development, the fungus is mostly found in aggregates embedded in a mucilaginous matrix (Banuett and Herskowitz 1996; Christensen 1963), which accumulates in enlarged apoplastic regions (Doehlemaun et al. 2009). The tips of the hyphae become lobed, followed by hyphal fragmentation that produces segments of one to several cells. At this stage, karyogamy has taken place and 4',6-diamidino-2-phenylindole staining shows a single nucleus per cell (Banuett and Herskowitz 1996; Snetselaar and Mims 1994). Following fragmentation, cells undergo a process of rounding and deposition of the secondary cell walls. Cell walls are hyaline at first, becoming yellow-brown and finally dark brown, and exhibit a characteristic echinulation in mature teliospores. As the number of mature teliospores increases, tumors darken.

Although a number of genes and pathways that influence teliospore development in planta have been identified, a general understanding of the molecular mechanisms and cellular activities involved in teliosporogenesis, as well as the signals that induce the process and what makes it strictly dependent on the host plant, is lacking. Because sporogenesis takes place only in planta, experimental problems are encountered in the analysis of the process. Teliosporogenesis is not well synchro-

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nized in different tumors and, even in different parts of the same tumor, teliospores at different stages of development are encountered (Banuett and Herskowitz 1996).

In a previous study, we identified *Ust1* as the closest *U. maydis* ortholog of the *Aspergillus nidulans* regulatory protein StuA, a member of the fungal-specific APSES (Asm1p, Phd1p, Sok2p, Efg1p, and StuAp) family of transcription factors. We showed that deletion of *ust1* in budding strains resulted in constitutive filamentous growth. What is more, $\Delta ust1$ strains produced large numbers of teliospore-like structures in culture over time (García-Pedrajas et al. 2010). Therefore, we postulated that this previously generated $\Delta ust1$ mutant could provide a synchronous, host-free genetic system to begin to decipher some aspects of the sporulation-associated transcriptome. We hypothesized that differential gene expression analysis in the $\Delta ust1$ mutant in culture would reveal genes playing roles in planta teliospore development.

A critical step of teliospore development is pigmentation. Two main pathways of melanin synthesis are found in fungi. A few fungi synthesize melanin via L-3-4-dihydroxyphenylalanine (L-DOPA), in a pathway similar to that of mammalian melanin biosynthesis. L-DOPA molecules are oxidized by laccase and polymerized into melanin (Eisenman et al. 2007). Analysis of fungal wall pigmentation in the basidiomycete *Cryptococcus neoformans* (Eisenman et al. 2009) suggests a model of vesicle-associated melanization of the cell wall. In this model, small vesicles containing L-DOPA are released in the cell wall, oxidized by laccase, and polymerized into melanin. Laccase mutants were unable to produce darkly pigmented cell walls. Many other fungi produce melanin via the DHN pathway. In this pathway, the precursor is acetyl coA or malonyl coA and the first step is catalyzed by a polyketide synthase (PKS). PKS genes involved in DHN melanin biosynthesis have been characterized in a number of Ascomycota phytopathogenic fungi such as *Bipolaris oryzae* (Moriwaki et al. 2004) and *Magnaporthe oryzae* (Oh et al. 2008). Deletion of the PKS gene in *B. oryzae* produced fully virulent but melanin-deficient mutants while deletion in *M. oryzae* generated nonmelanized appressoria incapable of penetrating rice leaves.

The identification of genes encoding proteins homologous to laccase and PKS as highly induced in cultures of the *ust1* mutant by microarray analysis confirmed the utility of this strain as a tool to investigate aspects of sporulation. In this work, we focused on the functional characterization of two of those genes: a laccase-encoding gene (*um05361*; here named *lac1*) and a PKS-encoding gene (*um06414*; here named *pks1*). In mutants deleted for *lac1*, pigmentation was reduced but not fully abolished. Gene *lac1* also played a broader role in virulence because mutant strains induced reduced disease severity in seedlings. Deletion mutant analysis showed that the role of *pks1*, on the other hand, was restricted to dramatic effects on pigmentation both in culture and in teliospores produced in the host.

RESULTS

Transcriptome analysis of the *ust1* deletion mutant shows widespread effects on gene expression.

To identify genes potentially involved in the sporulation program, we carried out microarray analysis comparing a *U. maydis* haploid deleted for *ust1* (strain 14/25) with the wild-type (WT) progenitor (strain 1/2) (Table 1). Previously, we had shown that deletion of *ust1* led to filamentous growth in potato dextrose broth (PDB) cultures and, over time, to production of numerous rounded, highly melanized cells (García-Pedrajas et al. 2010). Similarly, in array medium (Scherer et al. 2006), we found that, at 24 h, *ust1* mutant cells grew filamentously while, at 48 h, rounded cells developed with highly pigmented, thick cell walls (Fig. 1). Total RNA was extracted from strains 14/25 and 1/2 grown in array medium (Scherer et al. 2006) for 24 and 48 h. RNA was then reverse transcribed to cDNA, labeled with Cy3, and hybridized onto custom one color NimbleGen arrays covering all of the then-predicted 6,869 *U. maydis* genes. Consistent with its role as a major developmental transcription factor, 808 genes were found to be differentially expressed by at least twofold when comparing the $\Delta ust1$ strain with the WT at 48 h. Of these genes, 184 showed greater than eightfold change, 105 of which were functionally classified and 79 unclassified via the Munich Information Center for Protein Sequences (MIPS) Functional Catalogue Database (FunCat) (Kamper et al. 2006; Ruepp et al. 2004) for various biological functions (Fig. 2). FunCat currently contains 27 main functional categories covering general cellular processes such as cellular transport, communication, and so on. Our results show that 42% of the genes induced more than eightfold were involved in metabolism. Interestingly, genes involved in protein synthesis were upregulated whereas genes involved in transcription were downregulated, perhaps suggesting elevated production of a limited set of spore-specific proteins.

Of the identified 184 greater than eightfold differentially expressed genes, 111 and 73 genes were up- and downregulated, respectively. As noted previously in Northern blots (García-Pedrajas et al. 2010), spore-specific protein 1 (*ssp1*) (*um12271*), a gene identified as highly expressed in teliospores (Huber et al. 2002), was found on the arrays to be highly induced during production of the teliospore-like structures by the $\Delta ust1$ mutant at 48 h. The most differential 10 upregulated and 10 downregulated genes during formation of teliospore-like structures in vitro are listed in Table 2.

Several genes upregulated in $\Delta ust1$ versus the WT exhibited homology to genes in other fungal species involved in spore development or pigmentation, such as *um05361*, similar to laccase genes; *um06414* and the nearby *um06418*, similar to PKS genes involved in melanin synthesis (Table 2); *um02035* (34-fold), related to the yellowish-green 1 (*ayg1*) gene in *A. fumigatus* involved in conidial pigmentation (Tsai et al. 2001); and *um03522* (18-fold), related to UGA4, GABA permease, in-

Table 1. Strains of *Ustilago maydis* used in this study

Strain	Genotype	Reference
1/2	<i>alb1</i> (521)	Gold et al. 1997
14/25	<i>alb1</i> , $\Delta ust1::cbx$	García-Pedrajas et al. 2010
SG200	<i>mfa1 pra1 mfa2 bW2 bE1</i>	Bölker et al. 1995
SG200-1 $\Delta lac1$	SG200 background, $\Delta lac1::cbx$	This study
SG200-2 $\Delta lac1$	SG200 background, $\Delta lac1::cbx$	This study
SG200-3 $\Delta lac1$	SG200 background, $\Delta lac1::cbx$	This study
SG200-1 $\Delta pks1$	SG200 background, $\Delta pks1::cbx$	This study
SG200-2 $\Delta pks1$	SG200 background, $\Delta pks1::cbx$	This study
SG200-3 $\Delta pks1$	SG200 background, $\Delta pks1::cbx$	This study
SG200-1 $\Delta lac1$ + <i>lac1</i>	SG200 background, $\Delta lac1::cbx$ <i>hygCosmid-lac1</i>	This study
SG200-1 $\Delta pks1$ + <i>pks1</i>	SG200 background, $\Delta pks1::cbx$ <i>hygCosmid-pks1</i>	This study

volved in nitrogen storage during conidiation (Kumar and Punekar 1997). This result indicated that genuine teliospores and the spore-like structures produced by the $\Delta ust1$ mutant in culture share developmental features.

The 10 most dramatically downregulated genes (Table 2) were mostly noncharacterized proteins; however, Hmp1, a cruciform DNA recognition protein (Dutta et al. 1997) encoded by *um00496*, was downregulated 378-fold in $\Delta ust1$ compared with the WT. The function of this protein in *U. maydis* has not been determined by deletion mutant analysis but, in a proteomic approach, it was recently also found to be downregulated 5.4-fold in filamentous versus budding *U. maydis* cells (Martínez-Salgado et al. 2013). Additionally, consistent with the observed severe mating defect shown by the $\Delta ust1$ mutant (García-Pedrajas et al. 2010), the *al* mating factor, *mfa1* (*um02382*) (Bölker et al. 1995), is downregulated 94-fold in the $\Delta ust1$ mutant at 48 h. Finally, *um00371* encodes an opsin-1

related protein. In *Neurospora crassa*, the *nop-1* gene, the likely ortholog to *um00371*, is a late conidiation gene and appears to function as a repressor of some sporulation genes. Although it can bind retinal, becoming a light-absorbing pigment, its repression of *con-13* was demonstrated to be in a light-independent manner (Bieszke et al. 2007).

To confirm that the $\Delta ust1$ mutant could be used to identify genes involved in in planta spore development, in this work, we focused on the functional analysis of two genes, *um05361* and *um06414*, upregulated 119- and 140-fold in $\Delta ust1$ versus the WT, respectively (Fig. 3A). BLAST searches with *um05361* showed that it is highly similar to fungal laccase genes; for example, it is 54% similar to the cell-wall-associated laccase 1 (*CNLAC1*) from *C. neoformans*, which is a virulence factor and affects melanization of spores (Zhu and Williamson 2004). Therefore, we named *um05361 lac1*. A schematic comparison of functional domains of *CNLAC1* and *U. maydis* Lac1 is shown in

Fig. 1. Comparison of cell morphology of *Ustilago maydis* *ust1* deletion mutant and wild-type (WT) strains. Cultures of WT strain 1/2 and *ust1* deletion mutant strain 14/25 were examined microscopically after 24 and 48 h of growth in array medium. SG200 grew as budding cells while the $\Delta ust1$ strain is constitutively filamentous, generating rounded, melanized teliospore-like structures in large numbers after 48 h.

Fig. 2. Functional classification of genes highly differentially expressed (more than eightfold) during teliospore-like structure formation in the *Ustilago maydis* *ust1* deletion mutant. Differentially regulated genes were classified into functional groups using the MIPS FunCat database. Many genes were assigned to multiple functional categories. Solid bars represent genes that were upregulated while open bars represent those downregulated.

Figure 3B. According to the FunCat analysis, predicted functions of laccase are extracellular lignin degradation (P value = $8.76\text{e-}04$), metabolism of melanin (P value = $4.09\text{e-}03$), and biogenesis of cellular components (cell wall) (P value = $6.84\text{e-}02$). Secretion of laccases has been observed in other fungi (Eisenman et al. 2007; Litvintseva and Henson 2002) and the *U. maydis* Lac1 protein contains a 23-amino-acid signal peptide typical of extracellular enzymes, as predicted by the Neilsen et al. algorithm ($P = 0.985$) on the SignalP 3.0 server and Secre-tome 2.0 server. Using INTERPRO, laccase domains are located in *U. maydis* um05361 similar to those of *CNLAC1* from *C. neoformans* (Fig. 3B). BLAST searches with um06414 indicated strong similarity with PKS genes involved in the biosynthesis of DHN melanin and, therefore, we named this gene *pks1*. INTER-PRO identified two PKS domains in the predicted protein encoded by the *U. maydis* um06414 gene. A comparison of the predicted domain structure of Pks1 with PKS proteins from two other plant pathogens, *Colletotrichum heterostrophus* and *Botrytis oryzae*, is shown in Figure 3B. Gene um06414 (*pks1*) is one of only three genes found in the *U. maydis* genome that encode proteins containing the minimal PKS domains ketoacyl CoA synthase (KS) and thioesterase (TE). Gene *pks1* also possesses two putative acyl carrier domains. Genes involved in the synthesis and modification of these secondary metabolites are often clustered together and are co-expressed (Khaldi et al. 2008). Consistently, *pks1* appears to be part of a secondary metabolism gene cluster which contains a second highly upregulated PKS gene, um06418 (Table 2), which, in addition to the KS and TE domains, contains an acyl transferase (AT) domain but lacks the critical phosphopantetheine binding domains. It was previously speculated that, because neither um06414 nor um06418 individually possess all the critical domains but, in combination, they do, they likely act together (Bölker et al. 2008).

Quantitative real-time polymerase chain reaction (qPCR) was used to confirm and quantify differential expression of *lac1*, *pks1*, and the previously reported spore-specific protein gene *ssp1* (um12271) (Huber et al. 2002), earlier shown to be induced in the *ust1* mutant by Northern blot analysis (García-Pedrajas et al. 2010) and found to be upregulated 68-fold in the microarray data reported here. Expression was quantified after 24 and 48 h of growth in PDB (Fig. 3C) and normalized to the frequently used constitutive reference gene um03726, encoding a probable CPR1 cyclophilin, generally referred to as

peptidylprolyl isomerase (*ppi*) (Scherer et al. 2006). All three genes were highly induced in the *ust1* mutant as compared with their expression in the WT 1/2 strain. Interestingly, although formation of melanized teliospore-like structures in the Δ *ust1* strain is only prominent after 48 h of incubation (Fig. 1) (García-Pedrajas et al. 2010), these genes were already highly upregulated in 24-h cultures (Fig. 3C). For example, *ssp1* was 43-fold upregulated at 24 h and 38-fold upregulated at 48 h according to the quantitative real-time PCR. The *pks1* and *lac1* genes were upregulated 82- and 55-fold, respectively, at 24 h, and their expression roughly doubled at 48 h. Because of the consistency between 24- and 48-h harvest array data and the mature developmental phenotype observed at 48 h of growth, we focus here on the 48-h results.

Mutant analysis confirms a role for *lac1* and *pks1* in cell wall pigmentation.

Using the DelsGate method (García-Pedrajas et al. 2008), the entire *lac1* and *pks1* open reading frames (ORF) were individually replaced by a plasmid containing a carboxin resistance gene (Keon et al. 1991) in the *U. maydis* haploid solo-pathogenic strain SG200 (Bölker et al. 1995). Deletion of *lac1* and *pks1* was confirmed by PCR and Southern blot analysis (Fig. 4) in three independent transformants for each gene (Table 1). All mutant strains exhibited no obvious altered phenotypes in culture; cell shape and cell growth rate in PDB medium was not notably different from the parental strain (data not shown). Solopathogenic strain SG200 is a merodiploid able to generate an active b heterodimer (Table 1) and, therefore, mimics the dikaryon and grows filamentously on charcoal-amended plates. We found that this filamentation was not impaired in either Δ *lac1* or Δ *pks1* strains (data not shown).

It has been observed that, under stress conditions, including prolonged incubations, cultures of *U. maydis* become darkly pigmented (Kusch and Schauz 1989). Because we anticipated that *lac1* or *pks1* could play a role in cell pigmentation, we investigated whether the mutations would affect this process in vitro. For that, we grew the parental and mutant strains in yeast extract-peptone-sucrose (YEPS) until mid- to late-logarithmic phase and then transferred them at several different cell concentrations to PDB and incubated them for an additional 96 h at 34°C to enhance stress (discussed below). The Δ *lac1* and Δ *pks1* strains complemented with autonomously replicating

Table 2. Top 10 most up- and downregulated genes in Δ *ust1* after 24 h of growth in array medium

Locus	<i>Ustilago maydis</i> annotation	Fold change ^z
Upregulated genes		
um05439	Related to chitin-binding protein	+187
um06414	Related to polyketide synthase	+140
um06370	Conserved hypothetical protein	+121
um05361	Related to laccase I precursor	+119
um06418	Related to polyketide synthase	+119
um12304	Hypothetical protein	+116
um03138	Hypothetical protein	+103
um05436	Conserved hypothetical Ustilago-specific protein	+98
um02489	Putative protein	+69
um12271	Putative dioxygenase Ssp1	+68
Downregulated genes		
um00496	Hmp1; cruciform DNA recognition protein Hmp1	-378
um11020	Conserved hypothetical protein	-152
um02416	Conserved hypothetical protein	-95
um02382	Mfa1; mating factor a1	-94
um12056	Probable ENA2' P-type ATPase	-62
um04482	Conserved hypothetical protein	-52
um02161	Conserved hypothetical protein	-45
um01187	Conserved hypothetical protein	-38
um00371	Related to opsin-1	-38
um05325	Conserved hypothetical protein	-36

^z Representative fold change from three biological replications.

cosmids containing full-length WT *lac1* or *pks1* alleles, respectively, were also included in this analysis. Under these conditions, pigment production was induced and cultures of parental strain SG200 became moderately dark, whereas this

pigmentation was reduced in the $\Delta lac1$ strain and fully abolished in the $\Delta pks1$ strain (Fig. 5A). Reintroduction of the *lac1* or *pks1* genes restored cell pigmentation to near-WT and WT levels, respectively (Fig. 5A). Cells from these cultures were

Fig. 3. Expression analysis and functional domains of *lac1* and *pks1*. **A**, Heat map of microarray gene expression data. The relative fold change in expression levels of the 36 >20-fold upregulated genes in the $\Delta ust1$ mutant strain is compared with the wild-type (WT) strain 1/2 at 48 h. Microarray data show the average of three biological replicates. Fold changes are indicated by increasing color saturation, with bright red denoting highest upregulated fold change. Spore-specific protein 1 gene (*ssp1*), upregulated in mature teliospores (Huber et al. 2002), is highly upregulated in the $\Delta ust1$ mutant strain (arrow). Two enzyme-encoding genes typically involved in secondary metabolism, laccase 1 (*lac1*) and polyketide synthase 1 (*pks1*), are also upregulated in the $\Delta ust1$ mutant strain (arrows). **B**, Comparison of positioning of laccase (multicopper oxidase) domains in *Ustilago maydis* (*U.m*) *lac1* (um05361) and laccase 1 (*CNLAC1*) from *Cryptococcus neoformans* (*C.n*) (gray boxes) and comparison of positioning of polyketide synthase domains in *U. maydis* *pks1* (um06414), *Colletotrichum heterostrophus* (*C.h*) *pks1*, and *Bipolaris oryzae* (*B.o*) *pks1* (black boxes). **C**, Validation of differential expression by quantitative real-time polymerase chain reaction (qRT-PCR) of *lac1*, *pks1*, and *ssp1* in $\Delta ust1$ compared with the WT after 48 h of growth in potato dextrose broth. Fold change was calculated with the $2^{-\Delta\Delta CT}$ method (Applied Biosystems) and in relation to the constitutively expressed *ppi* gene. Shown here are the mean values and standard error of four biological replicates.

Fig. 4. Multiplex Southern blot confirmation of deletion of *lac1* (um05361) and *pks1* (um06414) open reading frames (ORF). Approximately 500 ng of total DNA from each strain was restriction digested with *NarI* and resolved on a 0.8% agarose gel. The blot was hybridized to digoxigenin-labeled ORF probes according to the manufacture's protocol.

centrifuged and it was noted that the supernatant remained clear, whereas pellets of parental WT SG200 were dark, indicating pigment retention within the cell walls (Fig. 5B). When observed microscopically, cells from these cultures exhibited aberrant morphology even in the case of WT SG200. However, whereas SG200 had darkly pigmented cell walls, SG200 $\Delta pks1$ cell walls remained hyaline (Fig. 5C).

Gene *lac1* but not *pks1* plays a dramatic role in seedling virulence.

The role of *lac1* and *pks1* in virulence to maize was analyzed. We first performed pathogenicity assays on 7-day-old maize seedlings and observed that deletion of *lac1* resulted in severely reduced disease symptoms. Thus, whereas, after 14 days, many plants infected with SG200 had formed galls,

Fig. 5. Effects of *lac1* and *pks1* genes on in vitro stress-induced cell pigmentation. **A**, Parental strain (SG200), mutant strain $\Delta lac1$, mutant strain $\Delta pks1$, *lac1* mutant complemented with a *lac1* containing cosmid ($\Delta lac1 + lac1$), and *pks1* mutant complemented with a *pks1* containing cosmid ($\Delta pks1 + pks1$) were grown in yeast extract-peptone-sucrose at 30°C until mid- to late-logarithmic phase optical density of 3 to 6.4, then transferred to potato dextrose broth at 1:2, 1:4, 1:8, and 1:16 dilutions and incubated for 96 h at 34°C. **B**, Centrifuged cell cultures of SG200 and $\Delta pks1$. **C**, Microscopic view of pigmentation of SG200 and $\Delta pks1$ cells.

Table 3. $\Delta lac1$ pathogenicity at 14 days postinoculation^y

Strains	Number of plants	0	1	2	3	4	5	Rating ^z
SG200	25	1	10	2	3	8	1	2.40 a
SG200-1 $\Delta lac1$	31	17	14	0	0	0	0	0.45 b
SG200-2 $\Delta lac1$	35	19	16	0	0	0	0	0.46 b
SG200-3 $\Delta lac1$	34	18	16	0	0	0	0	0.47 b
Noninoculated	23	20	3	0	0	0	0	0.13 b

^y These results correspond to one representative pathogenicity experiment. Similar results were obtained in three independent experiments. The disease ratings are as follows: 0 = no symptoms, 1 = anthocyanin or chlorosis, 2 = small leaf galls, 3 = small stem galls, 4 = basal stem galls, and 5 = plant death. SG200-1 $\Delta lac1$, SG200-2 $\Delta lac1$, and SG200-3 $\Delta lac1$ are three independently generated *lac1* deletion mutants.

^z Values followed by different letters are significantly different ($P \leq 0.05$) in Duncan's multiple range test.

ΔlacI strains primarily generated chlorosis and, dramatically, produced no galls (Table 3). In contrast, deletion of *pksI* did not lead to a reduction in disease severity to seedlings (Table 4).

Genes *lacI* and *pksI* play minor and major roles, respectively, in teliospore pigmentation in adult ears.

To more closely examine the role of *lacI* and *pksI* in genuine teliospore development, developing maize ears in adult plants were inoculated. Infected ears were examined 21 days postinoculation to analyze spore development. Both mutants induced extensive tumors comparable with those produced by the WT (Fig. 6). However, the quantity and pigment intensity of teliospores was reduced in galls infected with the *ΔlacI* mutant and, strikingly, teliospore pigmentation was completely absent in the galls from plants infected with the *ΔpksI* mutant.

Table 4. *ΔpksI* pathogenicity at 14 days postinoculation^y

Strains	Number of plants	0	1	2	3	4	5	Rating ^z
SG200	38	6	5	21	4	2	0	1.76 ab
SG200-1 <i>ΔpksI</i>	41	3	12	15	9	2	0	1.87 ab
SG200-2 <i>ΔpksI</i>	42	0	15	21	5	1	0	1.80 ab
SG200-3 <i>ΔpksI</i>	37	0	5	16	12	4	0	2.40 a
Noninoculated	34	18	16	0	0	0	0	0.47 b

^y Results from one representative pathogenicity test. Similar results were obtained in three independent experiments. SG200-1 *ΔpksI*, SG200-2 *ΔpksI*, and SG200-3 *ΔpksI* are three independently generated *pksI* deletion mutants.

^z Values followed by different letters in the rating column are significantly different (*P* ≤ 0.05) in Duncan’s multiple range test.

DISCUSSION

Our knowledge of molecular signaling underlying teliospore development in *U. maydis* is modest, hindered by the impossibility of inducing this process outside the host plant. In this work, we took advantage of a *ΔustI* strain which, in culture, develops rounded and darkly pigmented structures resembling teliospores produced in plant galls (García-Pedrajas et al. 2010) to gain new insights into the regulation of this process. Employing a custom microarray, we identified several hundred genes that were differentially expressed when comparing *ΔustI* and WT 48-h in vitro cultures. We identified 808 more than twofold differentially expressed genes, 184 of which had more than eightfold change. Of these 184 genes, 111 genes were up-regulated and 73 downregulated in the *ΔustI* strain. Of the 111

Fig. 6. Teliospore development in maize galls induced by the wild-type (WT), *ΔlacI*, and *ΔpksI* mutant strains. Tumors (left) induced by the WT, *ΔlacI* (LAC), and *ΔpksI* (PKS) mutant strains were photographed 22 days after inoculation. Teliospore production and pigmentation (right) are shown in hand-sectioned specimens derived for the pictured galls.

upregulated genes, 36 genes were >20-fold upregulated during the filamentation or sporogenesis phase. A gene previously reported as highly expressed in teliospores (Huber et al. 2002), *ssp1*, was in the group of genes we identified as highly induced during production of the spore-like structures by the $\Delta ust1$ mutant. As noted in the Results, other genes upregulated in $\Delta ust1$ versus WT (e.g., *um05361*, *um06418*, *um02035*, and *um03522*) exhibited homology to genes in other fungal species involved in spore development or pigmentation. This result indicated that the spore-like structures produced by the $\Delta ust1$ mutant in culture share developmental features with genuine fungal spores. To further explore the roles these genes played in teliosporogenesis or pigmentation, here, we functionally characterized two of the identified highly upregulated genes. For this functional analysis, we focused on laccase (*lac1*, *um05361*) and PKS (*pks1*, *um06414*), upregulated 119- and 140-fold, respectively, compared with the WT. Bölker and associates (2008) identified three putative PKS-encoding genes in the *U. maydis* genome. The nearby highly induced *pks*-related gene, *um06418* (upregulated 119-fold) (Table 2), was not a target of functional analysis in this study, in part because of the dramatic null pigmentation phenotype obtained with the single *pks1* (*um06414*) deletion. A third identified PKS (*um04105*) (Bölker et al. 2008) was not induced in the *ust1* deletion mutant. Although a member of the Basidiomycota like *Cryptococcus neoformans*, the fact that *pks1* appears to play the predominant role in melanin biosynthesis indicates that, unlike its relative, *U. maydis* is not restricted to production of L-DOPA-derived melanin. It is possible that *lac1* could play a role in this pathway by polymerizing the DHN melanin subunits synthesized by *pks1* activity into melanin, as has been suggested for melanin biosynthetic clusters in which both PKS and laccase enzymes are present (Eisenman and Casadevall 2012). However, *lac1* is unlinked to *pks1* and, thus, could potentially be involved in L-DOPA-derived melanin. Both DHN and L-DOPA melanin pathways are found in many fungi. The presence of catechol melanin (AKA plant melanin) reported by early biochemical characterization in *U. maydis* (Piattelli et al. 1963) is not supported by our results. This type of melanin is reportedly synthesized through the activity of polyphenol oxidase enzymes potentially using dopamine as a substrate. Our finding that the $\Delta pks1$ mutant was essentially devoid of spore pigmentation suggests that *U. maydis* depends on DHN melanin production and that there are not alternative pathways that mask the effects of this deletion. Thus, we propose that *lac1* plays a relatively minor role in pigmentation but depends on production of substrate through *pks1* via the DHN melanin pathway, as has been reported for several fungi of the phylum Ascomycota, including plant pathogens (Lin et al. 2012; Sugareva et al. 2006). However, some DHN pathway enzymatic functions are not detected in the *U. maydis* genome, including hydroxynaphthalene dehydratase and scytalone dehydratase (Langfelder et al. 2003). Although not tested here, we speculate that deletion of the linked PKS (*um06418*) would generate a phenotype similar to that of $\Delta pks1$. As noted by Bölker and colleagues (2008), the proteins encoded by *um06414* and *um06418* together but not individually possess all necessary PKS functional domains and, thus, would be expected to act by complementary gene action.

In this work, we characterized a gene with a potential role in melanin synthesis, *lac1* (*um05361*), highly similar to other fungal laccase genes. As discussed above, a few fungi synthesize melanin via a pathway utilizing L-DOPA as substrate. In this pathway, L-DOPA is oxidized by laccase and then polymerized into melanin (Eisenman et al. 2007). That is the case of *C. neoformans*, where laccase is a cell wall-associated virulence factor and functions in melanin biosynthesis of the spore

wall (Zhu and Williamson 2004). *CNLAC1* and *CNLAC2* are the only two apparent laccase genes in *C. neoformans*. They are 5.3 kb apart in the same orientation, with 72% amino acid sequence identity. However, only deletion of *CNLAC1* generated nonmelanized mutants (Zhu and Williamson 2004). In the *U. maydis* genome, there are also two laccase genes, *um05361* (*lac1*) and *um05861* (*lac2*), which are not linked; however, both were highly upregulated in $\Delta ust1$ (119- and 48-fold, respectively). We deleted *lac1*, which is more similar to *CNLAC1* than to *CNLAC2*, in the solopathogenic strain SG200 and observed that the $\Delta lac1$ mutant strain induced galls with a reduced number of teliospores and reduced teliospore pigmentation, whereas WT SG200-generated galls contained abundant melanized teliospores. Because melanin production in *C. neoformans* requires laccase (Eisenman et al. 2007), a similar phenomenon may operate in *U. maydis* because both are members of the phylum Basidiomycota. However, although laccases play a critical role in the synthesis of melanin via L-DOPA, they or similar multicopper oxidases could also play a role in the synthesis of DHN melanin. For example, the gene cluster for conidial pigment biosynthesis in *A. fumigatus* in addition to PKS (*alb1*), SCD (*arp1*), and THN-reductase (*arp2*) genes also contains a multicopper oxidase (*abr1*) and a laccase gene (*abr2*) (Tsai et al. 1999). Interestingly, in seedlings but not adult plants, virulence was reduced in $\Delta lac1$ mutant strains. However, tumors induced by the $\Delta lac1$ mutant in ears contained fewer and less darkly pigmented teliospores compared with the WT. Although not tested here, we speculate that deletion of both *lac1* and *lac2* might have a more dramatic effect on teliospore pigmentation. Lac1 likely has a function of extracellular lignin degradation and, because Lac1 has a 23-amino-acid signal peptide typical of extracellular enzymes, it is likely that laccase is secreted following infection in order to degrade the plant cell wall, of which 5% is lignin (Marita et al. 2003), and allow fungal invasion. The differential effects on seedlings versus adult plants of the $\Delta lac1$ mutant potentially could be due to differential induction in these tissues, as was reported for many *U. maydis* genes (Skibbe et al. 2010). Alternatively, and perhaps more likely, seedlings contain more lignin than developing ovaries and, thus, the fungus requires laccase for cell wall breakdown in order to efficiently colonize these structures but not to produce ear galls.

In this work, we also examined the role of *pks1* by analysis of deletion mutants. The roles in virulence of PKS genes in different fungal species vary. In some animal pathogens, melanization appears to contribute to virulence by reducing the susceptibility of melanized fungal structures to host defense mechanisms and antifungal drugs. For example, in *A. fumigatus*, mutants deficient for the PKS, PksP, produce white conidia instead of the gray-green conidia typical of the WT and are strongly attenuated in virulence in a mouse model (Langfelder et al. 2001). Another similar case is *Penicillium marneffeii*, in which deletion of the PKS gene *alb1* leads to the lack of melanin and reduced ornamentation in conidia and reduced virulence toward mice (Woo et al. 2010). In plant pathogens that produce highly differentiated melanized appressoria to penetrate plant tissue such as *M. oryzae*, deletion of its respective melanin synthetic PKS gene generated nonmelanized appressoria incapable of penetrating rice leaves (Oh et al. 2008). Appressoria of *U. maydis*, on the other hand, are weakly differentiated and nonmelanized. In this species, the role of *pks1* appears to be restricted to melanin synthesis because we did not detect any role in pathogenic development. Our analysis of the *pks1* promoter by using the Kyoto University Bioinformatics Center network database (GenomeNet) identified two StuA consensus-fitting binding sites, 10 bp per site: CAACGCGCTT at 207 bp and CATCGCGAAT at 350 bp upstream of the *pks1*

ORF. Because the DNA-binding domain (APSES domain) of StuA from *A. nidulans* is 70.1% identical to APSES domain of Ust1 from *U. maydis* (García-Pedrajas et al. 2010), we predicted that Ust1 may directly bind StuA sites in the *pks1* promoter. Our data are consistent with the prediction that Ust1 is a transcriptional repressor of genes induced during teliosporogenesis in *U. maydis* (García-Pedrajas et al. 2010). Consistent with this hypothesis, in microarray experiments not reported here, we found that *ust1* was downregulated 1.97-fold in late, actively sporulating galls (21 days postinoculation) versus early (14 days postinoculation) galls (E. Islamovic, unpublished results). In the absence of Ust1, repression is abolished and genes induced during teliosporogenesis are upregulated, such as the *pks1* gene. However, in recent work, we did not find evidence that Ust1 binds the canonical StuA response elements in vitro (Baeza-Montañez et al. 2015) and, therefore, we are uncertain whether the regulation of these genes involved in melanin synthesis by *ust1* is direct or indirect.

Transcript levels of the genes previously shown to be involved in development leading to teliospore formation varied in our microarray experiments (Fig. 7). During teliospore-like structure formation and teliospore development, transcript levels of *hda1* (*um02065*) and *gpa3* (*um04474*) increased, while transcript levels of *fuz1* (*um02587*) and *rum1* (*um02582*) decreased. In this study, we characterized two genes involved in teliospore development in *U. maydis* and we propose their possible genetic temporal involvement during teliosporogenesis (Fig. 8). This development begins with the filamentous pathogenic dikaryon located inside a maize gall undergoing extensive morphological alterations such as filament fragmentation and cell wall thickening to produce round, dark teliospores (Banuett and Herskowitz 1996; Snetselaar and Mims 1994). Genes shown to play developmental roles leading to teliosporogenesis are *hda1* (*um02065*), *gpa3* (*um04474*), *fuz1* (*um02587*), *rum1* (*um02582*), and *hgl1* (*um11450*). Mutation

of each gene results in arrest in teliospore development at a specific stage. The $\Delta hda1$ deletion mutant, the *gpa3*_{Q206L} constitutively active mutant, and $\Delta fuz1$ inactivation mutant are all arrested after branching but prior to filament fragmentation (Banuett and Herskowitz 1996; Kruger et al. 2000; Reichmann et al. 2002). The $\Delta rum1$ deletion mutant is arrested after filament fragmentation but prior to spore structure formation (Quadbeck-Seeger et al. 2000). The $\Delta ust1$ mutant itself does not release its spore-like structures from the mycelium, which is superficially consistent with downregulation of *fuz1* and *rum1*. The $\Delta hgl1$ deletion mutant is arrested after hyphal fragmentation and produced only a few hyaline, rounded structures reminiscent of immature teliospores (Durrenberger et al. 2001). Our results suggest that both $\Delta lac1$ and $\Delta pks1$ deletion mutants are arrested at a late spore developmental stage where teliospores have developed but are not fully mature. The *hgl1* mutant phenotype suggests that it may be immediately upstream and a potential regulator of *lac1* and *pks1*. Our microarray experiments in plant galls in an *hgl1* deletion mutant showed significant decrease in both *lac1* (257-fold) and *pks1* (11-fold) expression compared with the WT (data not shown), which is consistent with an upstream requirement for and potential regulation by *hgl1*.

In summary, this work has provided evidence that the in vitro “sporulation” phenotype of the $\Delta ust1$ mutant is related to in planta teliosporogenesis. Through functional analysis of two genes highly upregulated in the *ust1* mutant, we made key insights into their roles in melanin biosynthesis both in vitro and in planta in *U. maydis*. Additionally, distinctly different effects were observed for *lac1* and *pks1* mutants. Mutant *lac1* plays a relatively weak role in pigmentation but a significant role in virulence in seedlings. On the other hand, *pks1* appears to play no role in virulence but is required for teliospore pigmentation. This suggests that, unlike its fellow Basidiomycota relative, *C. neoformans*, *U. maydis* employs a modified DHN pathway for spore melanization. Additionally, the deposited

Fig. 7. Relative fold change of sporulation related genes in the $\Delta ust1$ mutant compared with the wild type at 48 h in vitro via microarray. Identified proteins involved in tumor/spore development are: *Ssp1* (*um12271*), *Mfa1* (*um02382*), *Hda1* (*um02065*), *Gpa3* (*um04474*), *Fuz1* (*um02587*), *Rum1* (*um02582*), *Hgl1* (*um11450*), *Pks1* (*um06414*), and *Lac1* (*um05361*). Data are from three biological replicates.

array data provide hundreds of additional candidate genes for further exploration of sporogenesis in *U. maydis*.

MATERIALS AND METHODS

Fungal strains and growth conditions.

The *U. maydis* strains used in this study are presented in Table 1. Standard growth of fungal strains was at 30°C on potato dextrose agar (PDA) (Sigma-Aldrich, St. Louis) plates supplemented to 2% agar (2PDA) or in PDB with shaking at 200 rpm. Cultures used for protoplast generation were grown in YEPS medium (1% yeast extract, 2% peptone, and 2% sucrose) and transformants plated on YEPS containing 2% agar supplemented with 1 M sorbitol and carboxin at 3 µg/ml for selection. Array medium and other specialized media used for specific purposes are described in the pertinent sections below.

Microarray analysis and qPCR differential expression confirmation.

To identify genes potentially involved in the sporulation program, we carried out microarray analysis comparing a haploid *U. maydis* WT strain (1/2) and $\Delta ust1$ (14/25) in vitro. Two types of microarray chips (1-plex and 4-plex) containing 6,869 *U. maydis* ORF were designed, produced, and validated by NimbleGen Systems, Inc. (Madison, WI) using the genome sequence of *U. maydis* publicly available at the MIPS *U. maydis* Genome Database. We generated three biological replicates (A, B, and C). For samples from groups A and B, a 1-plex NimbleGen microarray chip was used (manufacturer's website). On the 1-plex chip, each *U. maydis* gene was represented by 14 different probes spread throughout each gene. This was replicated in four blocks (four technical reps per chip). For samples from group C, a 4-plex NimbleGen microarray chip was used. On the 4-plex chip, each *U. maydis* gene was probed using 10 different probes. Averages were taken from the probe sets and reported as expression values. WT and $\Delta ust1$ strains were grown in PDB for 48 h at 30°C. To allow effective comparison with other array data generated by *U. maydis* research-

ers, cells were then transferred to liquid array medium (6.25% Holiday salt solution, 30 mM L-glutamine, and 1% glucose [pH 7.0] and filter sterilized) at 30°C (Scherer et al. 2006). Total RNA samples from WT 1/2 and $\Delta ust1$ mutant strains grown in array medium for 24 h (mutant's filamentous phase) and 48 h (mutant's teliospore-like structure formation phase) were extracted and purified (Sigma Spectrum plant total RNA kit, catalog number STRN50). RNA was sent to NimbleGen, where it was reverse transcribed to cDNA and Cy3 labeled. The cDNA samples were hybridized to custom one color arrays, microarray chips were scanned, and raw data were normalized with appropriate controls and statistically analyzed using standard NimbleGen microarray pipeline. Gene expression patterns were visualized as a scatter plot using ArrayStar v2.0 software. The entire set of supporting microarray data is deposited at Gene Expression Omnibus (accession number GSE54659).

For qPCR, total RNA was extracted from WT and $\Delta ust1$ strains grown for 24 and 48 h in PDB using a Spectrum plant total RNA kit (Sigma-Aldrich). cDNA was synthesized using the SuperScript III first-strand synthesis system for qPCR (Invitrogen, Carlsbad, CA) using oligo-dT priming and according to the manufacturer's recommendations. Transcript abundance was quantified by qPCR using SYBR-GREEN methodology (Bio-Rad, Hercules, CA) with gene-specific primers designed through the Integrated DNA Technologies website. Reactions were performed on a Cepheid SmartCycler I (Cepheid, Sunnyvale, CA). Transcript relative expression levels for *lac1*, *pks1*, and *sspl* were calculated by using Δ cycle threshold (CT) calculations ($2^{-\Delta\Delta CT}$ method) (Pfaffl 2001) with primer pairs *lac1*qPCRf and *lac1*qPCRr; *pks1*qPCRf and *pks1*qPCRr; *sspl*qPCRf and *sspl*qPCRr; and normalized to a reference cyclophilin gene, *ppi* (*um03726*) using primers *ppif* and *ppir* (Table 5), as previously described (Doehlemann et al. 2009). Means of gene expression fold-changes and their corresponding standard error were calculated based on four biological replicates. Comparisons were made regarding positioning of laccase (multicopper oxidase) domains in *U. maydis lac1* (*um05361*) and laccase 1 (*CNLAC1*) from *C. neoformans* (Zhu

Fig. 8. Positioning of *lac1* and *pks1* in a temporal model of gene function affecting *Ustilago maydis* teliosporogenesis. Identified genes involved in teliosporogenesis are *hda1* (*um02065*) (Reichmann et al. 2002), *gpa3* (*um04474*) (Kruger et al. 2000), *fuz1* (*um02587*) (Banuett and Herskowitz 1996), *rum1* (*um02582*) (Quadbeck-Seeger et al. 2000), and *hgl1* (*um11450*) (Durrenberger et al. 2001). Mutation of each gene results in arrest in teliospore development. The $\Delta hda1$ deletion mutant, *gpa3*_{Q206L} constitutively active mutant, and $\Delta fuz1$ inactivation mutant are all arrested after branching but prior to filament fragmentation. The $\Delta rum1$ and $\Delta hgl1$ deletion mutants are arrested after filament fragmentation but prior to spore structure formation. Our results suggest that $\Delta lac1$ and $\Delta pks1$ mutants are defective in late spore development.

and Williamson 2004) and comparison of positioning of PKS domains in *U. maydis pks1* (um06414), *Colletotrichum heterostrophus pks1* (GenBank: U68040.3) (Baker et al. 2006), and *B. oryzae pks1* (GenBank: AB176546.1) (Moriwaki et al. 2004).

Production and complementation of *lac1* and *pks1* deletion mutants.

The *lac1* and *pks1* deletion constructs were prepared using the DelsGate method (García-Pedrajas et al. 2008) with the gene-specific “DGP” primers (Table 5). For *lac1* gene deletion, Lac1DGP1/Lac1DGP2 and Lac1DGP3/Lac1DGP4 primer pairs were used whereas, for *pks1* gene deletion, Pks1DGP1/Pks1DGP2 and Pks1DGP3/Pks1DGP4 primer pairs were used to amplify the 5′ and 3′ gene flanks, respectively. *Escherichia coli* DH5α cells were used for transformation during deletion construct assembly. Low-sodium Luria-Bertani medium containing kanamycin (Research Products International Corp., Chicago) at 50 µg/ml was used for selecting *E. coli* transformants. *U. maydis* transformations were performed using a protocol from Tsukuda and associates (1988), with modification in protoplast generation. For these experiments, we replaced Novozyme 234, which is no longer available, with Vinoflow enzyme (Novozymes, Denmark). The absence of the *lac1* or *pks1* ORF was first confirmed via PCR by using primers lac1F/lac1R or pks1F/pks1R, respectively (Table 5). Transformants with no amplification product were subsequently screened for homologous integration of the deletion construct via PCR using primers lac1 5/cbx out or pks1 5/cbx out (Table 5). Finally, multiplex Southern blotting was used to confirm that putative transformants indeed lacked bands hybridizing to the ORF of *um05361* or *um06414*. Approximately 500 ng of total DNA from each strain was restriction digested with *NarI* and resolved on a 0.8% agarose gel. The gel was stained with ethidium bromide and relative band size versus migration distance was documented with a *HindIII*-digested λ DNA marker, followed by alkaline transfer to a Hybond XL membrane (Amersham Pharmacia Biotech, UK). The blot was prehybridized for approximately 4 h at 42°C followed by simultaneously probing overnight with ORF fragments of both *um05361* (889 bp) and *um06414* (725 bp). The probes were generated by PCR amplification using primers lac1F and lac1R and pks1F and pks1R, respectively (Table 5), labeled with digoxigenin-dUTP using the DIG-high prime kit, and added together at a

concentration of 25 ng/ml each, with washing and detection steps according to the protocol specified by the manufacturer (Roche, Branchburg, NJ, U.S.A.).

Deletion mutants complemented with *lac1* or *pks1* genes were also generated. For that, mutant strains (Table 1) were transformed with cosmids from a WT strain 1/2 *U. maydis* library constructed in the autonomously replicating cosmid vector pJW42 (Barrett et al. 1993) containing the *lac1* or *pks1* gene, respectively. Cosmids from the library harboring full-length genes were identified as described previously (Smith et al. 2004) with primers Lac1DGP1 and Lac1DGP4 or Pks1DGP1 and Pks1DGP4. For complementation, Δ *lac1* and Δ *pks1* deletion mutant protoplasts were generated and transformed with the appropriate cosmid. Transformants were selected with hygromycin at 150 µg/ml for cosmid selection and transferred to PDA plates amended with carboxin at 3 µg/ml and hygromycin at 150 µg/ml. Complemented transformant strains were cultured in PDB liquid with hygromycin at 50 µg/ml to ensure retention of complementing cosmids.

Pathogenicity assays and maize ear inoculations.

Pathogenicity assays were carried out on the susceptible maize ‘Golden Bantam’ (Rich Farm Garden Supply, Winchester, IN, U.S.A.). Seedlings were inoculated by injection just above the soil line 7 days after planting with a suspension of solopathogenic parental strain SG200 or deletion mutant strains at 1×10^6 cells/ml in a SG200 background. Disease symptom data were collected at 7, 10, 14, and 21 days after inoculation. Disease ratings were as described previously by Gold and associates (1997). Briefly, symptoms were rated 0 to 5 as follows: 0 = no disease, 1 = anthocyanin or chlorosis, 2 = leaf galls, 3 = small stem galls, 4 = large stem galls, and 5 = plant death. Maize ear inoculations were carried out on the dwarf ‘Tom Thumb’ (Seed Savers Exchange, Decorah, IA, U.S.A.), as previously described (Chacko and Gold 2012).

Pigment production.

Pigment production was stress induced in PDB cultures by prolonged incubation at temperatures above normal. First, fungal cells of the WT (SG200), Δ *lac1* strain, Δ *pks1* strain, *lac1* deletion mutant complemented with the *lac1* gene-containing cosmid (Δ *lac1* + *lac1*), and *pks1* deletion mutant complemented with the *pks1* gene-containing cosmid (Δ *pks1* + *pks1*)

Table 5. Primer sequences used in this study

Primer ID	Primer sequence
Lac1DGP1	TAGGGATAACAGGGTAATAC AGCATGAAG GTT GTTCCTTGCC
Lac1DGP2	GGGGACAAGTTTGTACAAAAAGCAGGCTAATGTGACAGATCAGACGGCAATCGT
Lac1DGP3	GGGGACCACTTTGTACAAGAAAGCTGGGTAATCCCGTTTGAGTGAAAGCGCAC
Lac1DGP4	ATTACCTGTTATCCCTAGCAGATTGGTCTTGACGAATGGCA
Lac1F	CGGCTTTCTCATGAACGGCAACTT
Lac1R	TGATGTGGTAGCCACTTCGGTCAA
Lac1 5	GATGTGGCAACATGTACACTGCCT
Pks1DGP1	TAGGGATAACAGGGTAATAGAGCCCTGTTTGCTCTCTTCCA
Pks1DGP2	GGGGACAAGTTTGTACAAAAAGCAGGCTAATCTCAGCTGTGAGCTTGAGCTTCT
Pks1DGP3	GGGGACCACTTTGTACAAGAAAGCTGGGTAATCAACATGCGTCGTGCGTGATT
Pks1DGP4	ATTACCTGTTATCCCTAAGGAGTTGGAGTTGATTACAGGCGT
Pks1F	TTGACGCAACCTTGCTCACACTTG
Pks1R	ACACTGCCTTGCTGAAACATGAGC
Pks1 5	ATATTACACGCCATGTTCGGCTTC
cbx out	CGAAAGCGAGACGAGTTG
lac1qPCRf	AAATGCAAAGTACGTGGCTCCTGC
lac1qPCRr	TGAATGCTCTGAGCGAGTGACCTT
pks1qPCRf	GCTCATGTTTCAGCAAGGCAAGTGT
pks1qPCRr	CACGACTCCATGGCAATTTGTGCT
ssp1qPCRf	AGCGCCTCTACGAGGTCAATGAAA
ssp1qPCRr	TGCAATGCCAAACAGCTCCTCATC
ppif	ACGCCGATTCACCTTCGTC
ppir	AACGACGATCCCTCGTAACCGAAA

were grown in 5.0 ml of YEPS at 30°C until mid- to late-logarithmic phase (optical density of 3 to 6.4). Then, each strain was transferred at dilutions of 1:2, 1:4, 1:8, and 1:16 with fresh PDB and incubated at 34°C for 96 h. Cultures in the glass tubes and pelleted in the Eppendorf tubes were photographed with a Nikon digital camera while fungal cells were photographed as previously described (García-Pedrajas et al. 2010).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- GenomeNet motif search: motif.genome.jp
- Institute of Bioinformatics and System Biology MIPS FunCat: www.helmholtz-muenchen.de/en/ibis/resource/services/services/funcat-the-functional-catalogue/index.html
- Integrated DNA Technologies: www.idtdna.com/Home/Home.aspx
- InterPro, Protein classification and analysis server: www.ebi.ac.uk/interpro
- MIPS *Ustilago maydis* genome database: mips.gsf.de/genre/proj/ustilago
- National Center for Biotechnology Information Gene Expression Omnibus: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54659
- NimbleGen: www.nimblegen.com/index.html
- Secretome 2.0 server: www.cbs.dtu.dk/services/SecretomeP
- SignalP 3.0 server: www.cbs.dtu.dk/services/SignalP-3.0