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The fungus *Ustilago maydis*, from the aztec cuisine to the research laboratory

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Summary *Ustilago maydis* is a plant pathogen fungus responsible for corn smut. It has a complex life cycle. In its saprophitic stage, it grows as haploid yeast cells, while in the invasive stage it grows as a mycelium formed by diploid cells. Thus, a correlation exists between genetic ploidy, pathogenicity and morphogenesis. Dimorphism can be modulated in vitro by changing environmental parameters such as pH. Studies with auxotrophic mutants have shown that polyamines play a central role in regulating dimorphism. Molecular biology approaches are being employed for the analysis of fundamental aspects of the biology of this fungus, such as mating type regulation, dimorphism or cell wall biogenesis.

Key words *Ustilago maydis* · Corn smut · Dimorphism · Polyamines · DNA methylation · Mating type

Introduction

Ustilaginales constitute a group of important plant pathogens which produce a variety of diseases in monocotyledons worldwide. These pathogens have the potential of causing severe disease outbreaks despite chemical treatment of the seeds and the plants, the existence of partially resistant cultivars, and the different plough methods employed. Among the Ustilaginales pathogenic for oat the following can be mentioned: *Ustilago hordei* ("covered smut"), *Ustilago nuda* ("loose smut"), and *Ustilago nigra* ("black loose smut"). Among the wheat pathogenic Ustilaginales we may cite *Tilletia indica*, *Tilletia caries* and *Tilletia controversa*.

Of all Ustilaginales, *Ustilago maydis* is the best known member of the group. *U. maydis* is a pathogen specific for corn (*Zea mays*) and teozinte (*Zea mexicana*), which is considered to be the ancestor of cultivated corn. *U. maydis* is the agent responsible for corn smut, a worldwide distributed disease, which under some conditions may cause severe economical losses in the agriculture. On the other hand, in Mexico smut invading sweet corn ("huitlacoche") is considered a delicacy. This is not surprising since domesticated corn originated in Mesoamerica. The fungus has been prepared in typical dishes since the pre-Columbus period in the central part of México

Fig. 1 A traditional Mexican dish made of *Ustilago maydis* galls. This particular dish is called "sopa de huitlacoche" (huitlacoche soup). A variety of dishes where *U. maydis* is used are typical of central México

(Fig. 1), and has been recently introduced into the "noveau cuisine" of luxury restaurants with notable success, not only among natives, but tourists also.

This hemibasidiomycete displays a complex life cycle which requires the host in order to be completed [17, 29]. In its saprophytic stage the fungus grows in the form of haploid budding yeasts (sporidia). Sporidia of opposite mating types are able to fuse and give rise to the dikaryotic phase which is the infective stage of the fungus. In the host tissues *U. maydis* grows in the form of mycelium, which eventually septates to form teliospores (also called chlamydospores). At this stage, karyogamy occurs. Millions of dark teliospores fill the galls characteristic of the disease. Upon germination of teliospores in the form of the so-called promycelium, meiosis and mitosis occur with the formation of basidiospores. Basidiospores bud off the so-called sporidia, which reproduce by budding, starting the life cycle again.

In recent times *U. maydis* has become an interesting model to study certain fundamental aspects of biology, such as the genetic control of mating, dimorphism and pathogenesis. This is because it can be analyzed by classical and molecular genetics. In the following pages we will describe some of the salient points of the biology of the fungus which makes it so attractive for researchers as well as gourmets.

## Life cycle of *Ustilago maydis* in nature

The life cycle of the fungus is depicted in Fig. 2 [for review see 3, 5, 17, 29]. During its saprophytic phase, *U. maydis* grows yeast-like. Cigar-shaped cells reproduce by budding, buds appearing in the zone of the cells with maximal curvature, similarly to *Saccharomyces cerevisiae* and characteristically at an angle of 30°–45° with the mother cell. However, in contrast to haploid strains of *S. cerevisiae*, the presence of bipolar budding may be a common event in *U. maydis*. The nucleus which is normally present at the center of the mother cell moves toward the bud, and divides mitotically. One daughter nucleus then migrates to the bud, and the other returns to the center of the mother cell [55]. Whether *U. maydis* cells in nature are haploid or diploid, remains uncertain. It appears that *U. maydis* is not a very successful colonizer of natural niches, since the range of carbon sources that it is able to utilize is very limited. Thus, the fungus does not metabolize most organic acids nor polysaccharides [13, 34]. Different aspects of the cell cycle, including mating, are under the complex control of two loci, *a* and *b*. Mating and pathogenicity require that both partners share compatible *a* and *b* loci. Microscopic studies of compatible cells resupended in plain water have revealed the initial stages of the mating reaction [55]. It has been observed that after about 1 h of incubation, budding is inhibited, and cells start to make narrow filaments instead, which are referred as mating hyphae or conjugation tubes.

These grow towards each other, probably by a chemotropic reaction, fusing normally at their tips, giving rise to an infectious hypha containing two elongated nuclei (Fig. 2a). A septum is made at the base of the hypha. Once the two compatible strains have mated, the filamentous dikaryon continues growing. Completion of the life cycle depends on the invasion of a susceptible host; otherwise, karyogamy occurs with formation of diploid cells which grow yeast-like in a form indistinguishable from haploid cells.

In the presence of a susceptible host, the mycelial dikaryon invades the plant (Fig. 2b). The route of invasion has been widely discussed. Apparently the fungus does not penetrate digesting the normal epidermis of the plant, but through natural openings such as stomata and floral organs. It has been suggested that stigma (silks) may be an important route of infection since ear tumors are usually the most prominent ones (Fig. 3). Experimental infection of stigmas showed that compatible haploid cells fused before infection and formed apresoria, from which invasive hyphae emerged and entered stigmas between the cells of the epidermis [56]. Haploid strains are unable to invade the plant, whereas diploid strains are invasive. Inoculation of stigmas with strains carrying compatible *a* alleles, but incompatible *b* alleles proved that the cells fused, but were unable to invade the host; whereas strains carrying non compatible *a* alleles, but compatible *b* alleles were unable to mate, and did not infect the plant [56]. Nevertheless, it has been observed that diploids containing compatible *b* alleles, as well as non compatible *a* alleles obtained by gene substitution, were infectious, demonstrating the *a* locus is not involved directly in pathogenesis [21]. Similar results were obtained with diploids containing incompatible *a* loci obtained by electrofusion [61]. Once within the plant, the fungus grows extra cellular and intracellularly in the form of irregular branched and contorted hyphae, but causing little harm to the host cells. Hyphae penetrate the cell wall of host cells, but their plasma membrane remains intact [56]. Hyphal cells are binucleate or multinucleate. No clear clamp connections have been reported. Most of the growth of the fungus occurs in meristematic tissues of the plant, inducing morphological changes in the plant, with the final formation of galls or tumors. In these tissues sporulation of the fungus occurs. The hyphae acquire a round shape, and a thick cell wall is formed (Fig. 2c). The spore wall is initially smooth and hyaline, but as the spore matures it becomes pigmented, and its surface turns rough or echinulated. Finally the mature spores (named teliospores or chlamydospores) separate and are liberated when the galls dry out and crack open (Fig. 2d). Internally of the spores a dramatic change occurs, as karyogamy takes place with the formation of the diploid stage. Teliospore formation has never been induced in vitro. It is therefore assumed that factors from the plant are necessary for maintenance of the dikaryon and for teliospore formation.
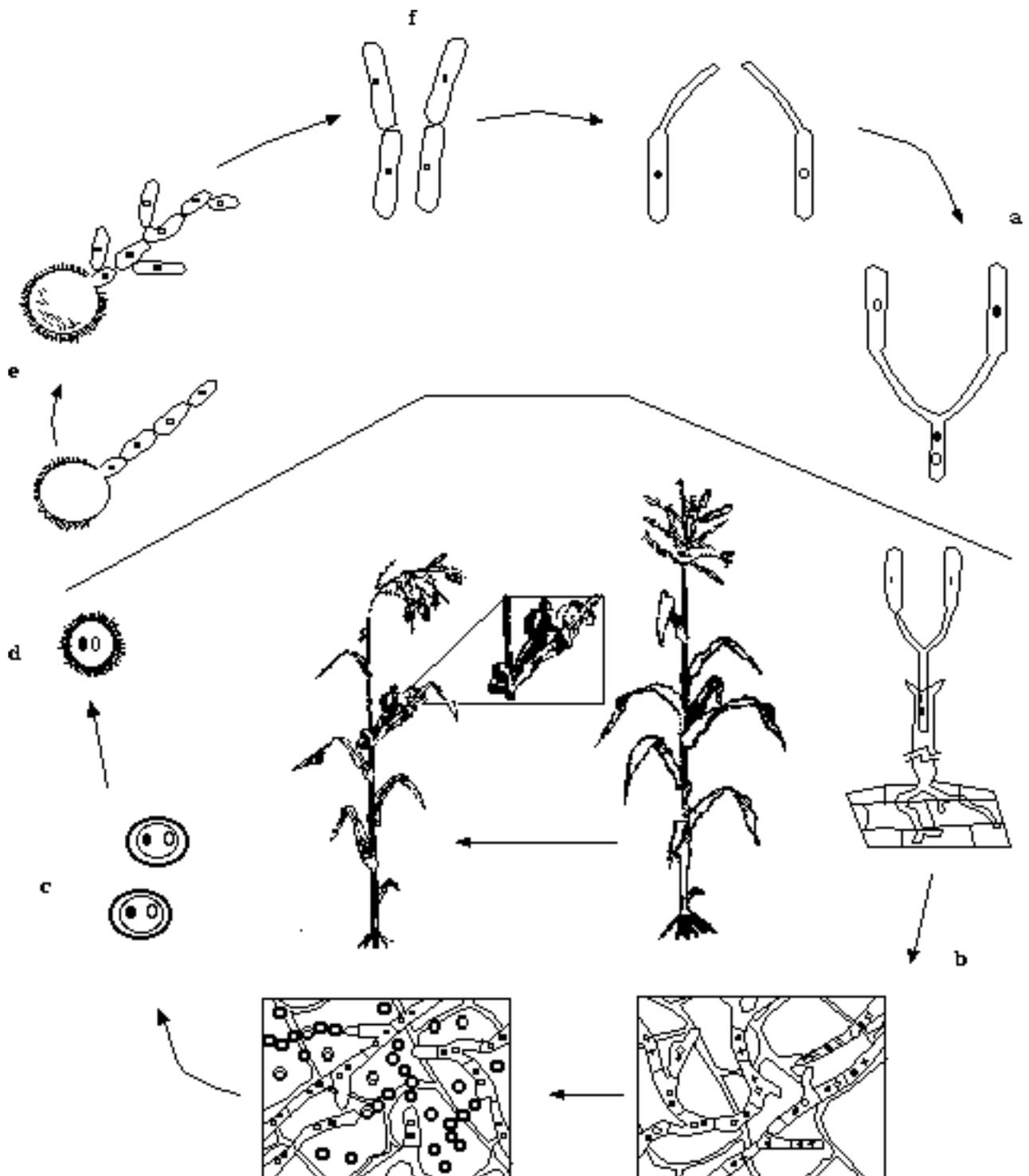
Fig. 2 Life cycle of *Ustilago maydis*. (a) Growing of conjugation tubes towards each other and fusion in compatible sporidial strains. (b) Invasion of the plant by the dikaryotic mycelium. (c) Teliospore formation. (d) Karyogamy and teliospore maturation. (e) Teliospore germination and formation of promycelium. (f) Meiotic products named sporidia which reproduce by budding
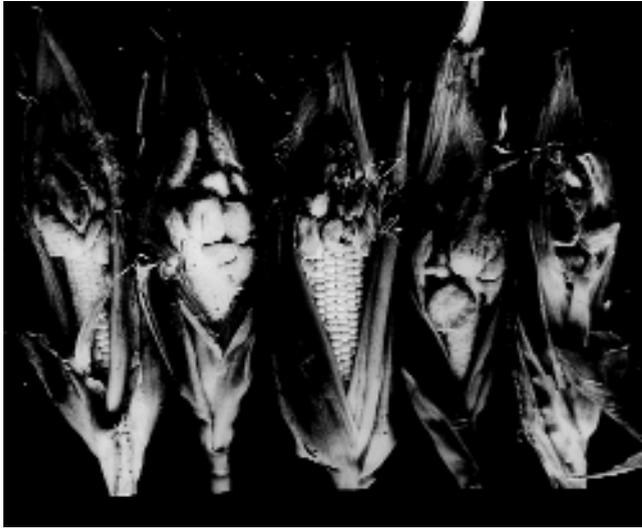
Fig. 3 Maize ears infected by *Ustilago maydis*. Note the large, amorphous tumors replacing the normal grains

In regards to the symptoms developed in the plant, the most important one is the formation of tumors, but also chlorosis, stunting, and anthocyanin pigments accumulation are characteristic of the disease. Other common symptoms are the development of female flowers in tassels, and tassel-like structures in the ears. Tumors can be formed in almost any meristematic tissue of the host, but the most impressive ones are those appearing in the ears. These are the ones which are used for dish preparation in México. These tumors liberate millions of black teliospores to the medium. Christensen [16] reported that their numbers per cubic centimeter of gall tissue amounted to 2.5–6 billion. Viability of teliospores is variable, from weeks to months. We have observed that washed dry teliospores kept at 4°C loose their viability to less than 10% after one month. When kept within the tumors they are viable for longer periods (J. Ruiz-Herrera and C. León, unpublished results).

Tumor induction probably involves the action of phytohormones and cytokinins, as it occurs during the infections of different plants by some bacterial pathogens, such as *Pseudomonas savastanoi* and *Agrobacterium tumefaciens*. *U. maydis* is able to synthesize cytokinins [36], and indoleacetic acid [62], but no evidence exists that they are responsible for tumor induction during maize infection by the pathogen.

Germination of teliospores occurs readily when placed in a nutrient medium, without special requirements or starving conditions as it occurs in some ascomycetes. Moreover, germination in the absence of nutrients is very poor. Caltrider and Gottlieb [13] described that disaccharides were more efficient carbon sources for teliospore germination, monosaccharides were less efficient, and organic acids and polysaccharides poor substrates. The authors provided evidence that sugar metabolism occurred mostly through the glycolytic

pathway. During germination teliospores produce a germ tube denominated promycelium (Fig. 2e). The nucleus of the spore migrates to the promycelium, and it is at this stage that meiosis occurs. Meiosis of haploid cells has not been observed in vitro, suggesting that predisposition for this phenomenon is acquired in the plant. This, and the above mentioned features occurring during the development of the pathogen in the plant, illustrate the intimate exchange of signals between the host and the parasite. From the promycelium, normally four basidiospores are formed in an acropetal progression. These basidiospores bud off to produce yeast-like cells called sporidia, which reinitiate the life cycle of the fungus (Fig. 2f). It has been described that teliospores produced under natural conditions may form from 1 to 8 basidiospore-like cells, instead of the normal 4 [17]. Whether this is due to problems brought about by differences in the genetic background of the conjugants, remains unknown.

Genetic control of the mating reaction

Mating is probably the event in the cell cycle of *U. maydis* that has received more attention. This is probably due to the fact that only dikaryotic or diploid cells are pathogenic. The control of the mating process in *U. maydis* is a complex phenomenon. Evidence was presented by Rowell [44, 45] that the sexual reaction of the fungus was controlled by two factors, now called *a* and *b*, the first one with two alleles, and the second one with multiple alleles. Only mating of haploid cells with different *a* and *b* loci results in pathogenicity.

The *a* locus It has been demonstrated that the *a* locus governs mating and cell fusion [59]. Only cells bearing different *a* loci can fuse. When cells carrying the same *a* locus are mixed, no mating hyphae are formed, and no cell fusion occurs [55]. Analysis of the behavior of diploids unable to grow in the form of mycelium, suggests that heterozygosis at the *a* alleles is also necessary for maintenance of filamentous growth of the fungus [6]. The two alleles of the *a* locus (*a1* and *a2*) have been cloned, and it has been demonstrated that they are idiomorphs [21], differing in size and DNA sequence, 4.5 kbp, and 8 kbp for *a1* and *a2* respectively [10]. Analysis of the DNA sequence of both alleles revealed the presence of two ORFs. Two of them coded for small proteins made of 40 amino acids for *a1*, and 38 amino acids for *a2*. Both of them displayed at their C-terminus the sequence Cys-A-A-X, characteristic of prenylated proteins, such as yeast pheromones. Accordingly, it was concluded that both genes, named *mfa1* and *mfa2*, coded for the pheromone precursors of *U. maydis*. Both pheromones were purified by the use of a biological assay, and demonstrated to be farnesylated and carboxy-methylated at the C-terminal cysteine residue [58]. Importance of this modification was revealed by the extremely reduced activity of the native polypeptide.

A second ORF coding for a polypeptide 357 amino acids in length was identified at the *a1* locus. This revealed homology (ca. 20%) with *STE3* gene product, the a-pheromone receptor from *S. cerevisiae*, and Map3, the M-factor receptor of *Schizosaccharomyces pombe*. A similar ORF coding for a polypeptide made of 346 amino acids was identified at the *a2* locus [10]. The similarity between both *U. maydis* receptors was of 24%. These results prompted the authors to identify both genes named *pra1*, and *pra2*, as the probable receptors for the pheromone produced by the opposite mating type. Two other ORFs of unknown function have been identified in the *a2* locus: *lga2* and *rga2* [60].

The genes coding for both the pheromone and the receptor are positively regulated when cells carrying compatible mating types are mixed. Moreover, *a1a2* diploids produce at all times increased amounts of the transcripts from the six genes: *pra1, pra2, mfa1, mfa2, lga2,* and *rga2* [60]. With these data a model has been elaborated, according to which haploid cells normally produce basal levels of the pheromone and the receptor. When cells of opposite mating types come together, a self-sustained circuit is turned on. Accordingly, binding of the pheromone produced by cells of a mating type to the receptor located on the surface of the cells of the opposite mating type triggers a reaction which leads to the stimulation in transcription of all the genes present in the a locus: *mfa, pra*, and *lga2* and *rga2* in the case of the *a2* locus [60]. The orchestrated transcriptional activation is mediated by a motif made of 9 nucleotides: ACAAAGGGA, located in the 5' upstream regulatory region of all the stimulated genes. The overproduced pheromone in turn, by the same mechanism, stimulates the expression of the genes present in the *a* locus of their mating partners. This autocrine mechanism is apparently responsible for the formation of conjugation tubes and the initial mycelial growth of heterokaryons [8]. This hypothesis was confirmed by the observation that purified *a1* pheromone triggered the formation of conjugation tubes in *a2*, but not in *a1* cells [58].

The b locus In contrast to the existence of only two *a* loci, the number of *b* loci in nature has been calculated in as many as 25 [40]. Compatibility of the *b* locus controls filamentous growth and pathogenicity independently of the *a* locus [32, 61]. Several alleles of the *b* locus have been cloned. Each one of them consists of two separate genes which are transcribed divergently, designated as *bE* and *bW*. They code for two different polypeptides, 410 and 626 amino acids in length respectively. Both polypeptides contain a central homeodomain region which separates a variable domain, and a constant region with over 90% homology between the different corresponding alleles [3, 4, 33, 53]. In contrast, *bE* and *bW* corresponding to the same *b* allele have no sequence similarity between themselves. The products from *b* genes are not essential for growth of either haploid or diploid strains. This result suggests that their only role is mating. Pathogenicity tests employing strains carrying deletions at either allele revealed that pathogenic behavior requires the presence of at least one *bE* and one *bW* heterozygous genes [23]. It has been suggested that the protein products associate in the form of heterologous aggregates which switch on the genes responsible for the mycelial and pathogenic phenotype [23]. Although the rationale by which so many different possible aggregates are active remains unknown, results obtained by use of the two-hybrid technique have game some insight into the problem [31]. The authors demonstrated that *bE* and *bW* products were able to form dimers only when they belonged to different alleles. This dimerization involved the N-terminal region, and was independent of the homeodomain. Point mutations in the former domain gave products affected in their ability to form dimers. With these data the authors elaborated a model, according to which dimerization occurred through binding involving sequential polar and hydrophobic interactions which matched only if the polypeptides belonged to different alleles.

Important for the understanding of the mating reaction is the observation that expression of both *bE* and *bW* is stimulated by the pheromone synthesized by cells of the opposite mating type [60]. This regulation is apparently mediated by the existence in *bE* and *bW* genes promoters of the same motif (ACAAAGGGA) present in genes from the *a* loci (see above). In turn, increase in the levels of *b* genes brought about a decrease in transcription in *a* genes. These result indicates that a fine regulation in the synthesis of products involved in mating takes place as the mating process and heterokaryon growth proceed.

Dimorphism

Conditions for the dimorphic transition in vitro As described above, haploid cells grow in the form of budding yeasts, both in nature and in culture media. The mycelial form is displayed only *in planta* by dikaryotic or diploid cells. Nevertheless, transient mycelial growth may be obtained on solid complex medium when two compatible haploid strains are mixed [19, 20]. This reaction, named "fuzz" by the appearance of the aerial mycelium [6–8], is a useful test for mating compatibility. However, no mycelial growth had been obtained in liquid culture. The possibility to reproduce the dimorphic transition under these conditions appeared extremely attractive, since it might allow a detailed analysis of the factors involved in the process and the reactions that take place during its initial period. Besides, the possibility to obtain mutants affected at selected steps would thus be feasible. Using a similar approach to the one described in *S. cerevisiae* [7, 24] demonstrated that *a1/a2b1/b2* diploids of *U. maydis* produced long mycelial cells when transferred from a rich medium to a nitrogen-deficient medium. This mycelial growth, resembling the formation of conjugation tubes was controlled by the *a* loci, since diploids carrying equal *a* alleles remained growing in the form of budding yeasts under the same conditions.

Dimorphism of haploid cells in vitro Recent results from our laboratory [50] demonstrated that the dimorphic transition can be obtained in vitro by control of the pH of the media. Cells grown at neutral or near neutral pH grow yeast-like, whereas at pH below 5.0 mycelial growth is displayed. Most interesting was the observation that mycelial growth under these experimental conditions was displayed not only by diploid and mero-diploid strains, but also by haploid cells. Mycelial growth at acid pH is thus independent of the heterodimers produced by the products of the *b* locus, and even from the presence of a functional *b* locus. *bE, bE* and null *b* mutants, all were able to form mycelium. It was suggested that acid pH somehow bypasses the requirement for the products of the *b* locus. These results suggests that the effect of the heterodimer is to turn off a repressor, rather than activate the expression of genes involved in mycelial growth [50]. On this basis, we have elaborated a simple model for the first stages in the transcription of genes involved in dimorphism, and the possible effect of pH (Fig. 4).

In these studies it was observed that the nitrogen source played an important role in morphogenesis at acid pH. Whereas ammonium was an efficient inducer of mycelial growth, nitrate was not. This difference was pinpointed to the existence of an ammonium-proton antiport, coupled to a proton pump [50]. Also important was the stage of the cells. Whereas mycelium-to-yeast transition could be induced at all times, the opposite was restricted to the initial periods after cell inoculation in fresh pH 3 medium. Since starvation and cold shock favored the yeast-to-mycelium transition, it was hypothesized that this occurred only in cells at the G_0 stage, before the cells accumulated the theoretical inhibitor (Fig. 5).

The relationship between pH and dimorphism in *U. maydis* is similar to the one described for *Candida albicans*, where acid pH inhibits mycelial growth [57]. In this fungus a pH-controlled gene (*PHR1*), whose disruption affects cell polarization has been described [52]. A likely hypothesis is that similar products whose synthesis is pH-controlled are involved in cell polarization exist in *Ustilago*. In this regard it is important to mention that transcription factors which respond to changes in pH have been described in *Aspergillus* [37] and *Yarrowia* [18].

Mutants affected in dimorphism Several mutants affected in dimorphism have been isolated utilizing different approaches. These have been extremely useful for the identification of the probable mechanism involved in the morphogenetic pathways existing in *U. maydis*. Mutants defining three genes involved in mycelial growth of diploid cells were isolated by replica plating of mutagenized cells on lawns of the strain of the opposite mating type [2]. Diploids unable to grow as filaments form smooth, and not fuzzy colonies on charcoal-containing plates [19, 20 see above]. These genes named *fuz1, fuz2*, and *rtf1*, are independent of *a* and *b*. Besides its role in mycelial growth, *fuz1* was found to be involved in teliospore formation, and *fuz2* in teliospore germination. On the other hand *rtf1* appears to code for a tumor repressor, since *rtf1* mutants carrying identical *b* loci surprisingly induced tumor and teliospore formation in corn plants.

A completely different approach gave results suggesting that cAMP and cAMP-dependent kinases are involved in mycelial growth. Mutants that displayed mycelial growth in their haploid stage were identified as fuz colonies when UV-irradiated cells were grown on charcoal-containing plates of complex medium [9].

Another gene involved in mycelial groth, *fuz7,* was cloned by means of PCR using oligonuclotides made from conserved sequences of MAP kinases [7]. This genes showed homology with the MAP kinases kinases (MEK/MAPK) family. Deletion of *fuz7* affected mycelium formation and conjugation. It was shown that the gene was involved in several processes on the life cycle of *U. maydis*. It is necessary for conjugation tube formation, filament formation and maintenance of the dikaryon, as well as tumor induction and teliospore germination [7, 8]. The description of the role of protein kinases in filament formation points out the importance of the signal transduction pathway in dimorphism.

Using merodiploids, a strain partially affected in mycelial formation and pathogenesis was isolated by insertional mutagenesis [22]. Complementation of the mutant allowed cloning of the gene. This codes for a protein of unknown function made of 1150 amino acids.

We have recently taken advantage of a procedure developed in our laboratory (see above) to induce the dimorphic transition, to isolate mutants unable to grow as mycelium (*myc* mutants) when incubated at an acidic pH [35]. Genetic analysis of the mutants revealed that most of them were recessive when crossed with the wild type of the opposite sex. Complementation analysis has permitted to
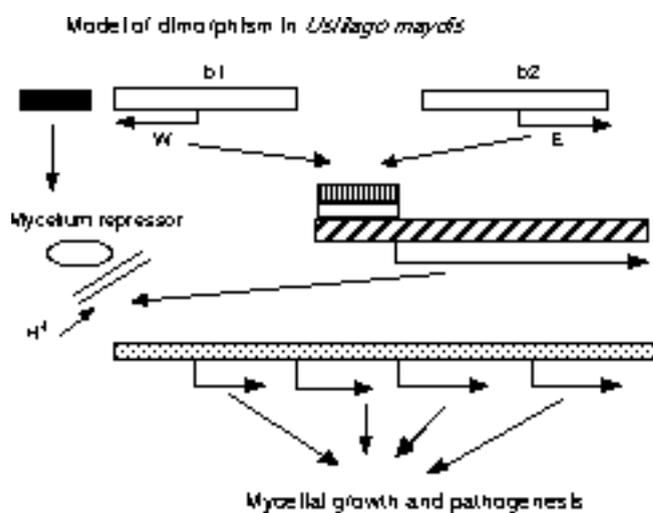
Fig. 4 Model of dimorphism in *Ustilago maydis*. *bW1* and *bE2* products interact to form a heterodimer that activates a gene whose product inhibits the transcription of genes specifically involved in mycelial growth and pathogenesis. Acid pH bypasses the effect of the *b* alleles, and turns off the same repressor
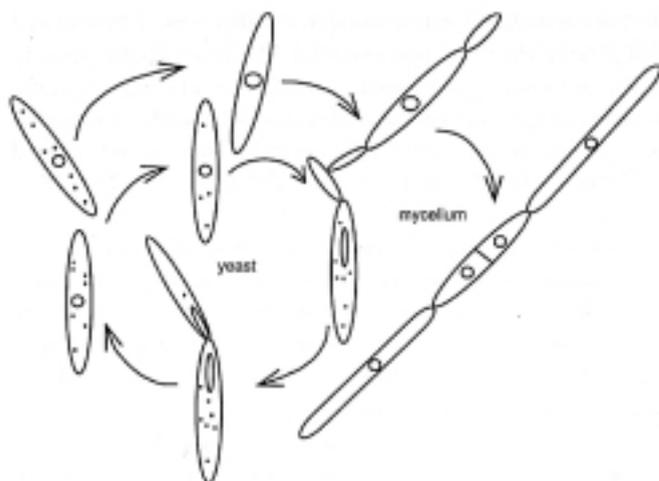
Fig. 5 *Ustilago maydis* cell cycle. Yeast cells reproduce by budding. When taken to stage G_0 and acid pH, the effect of the hypothetical inhibitor (represented by small dots) is bypassed permiting mycelial growth

allocate the mutants in at least two complementation groups. We also found out that the mutants were not affected in either *a* or *b* alleles or in their ability to mate with strains of the opposite mating type. Characterization of the pathogenic behavior of the mutants against corn seedlings produced striking results. Mutations that were recessive for pathogenicity were also recessive for morphogenesis in vitro. Our results led to the conclusion that some of the genes affected in *myc* mutants were involved in both dimorphism and pathogenicity [35], establishing a direct correlation between these two processes, and adding further information to previous observations on this issue [2, 7–9, 25].

Polyamines and dimorphism Previous studies from our laboratory have revealed that polyamines are involved in cell differentiation in fungi [47]. In general, we have observed that preceding each differentiative step, there occurs the elevation in the levels of polyamines and ornithine decarboxylase (ODC), the first and the most controlled enzyme in the biosynthetic route of the polyamines. If ODC activity is blocked or polyamine pools are depleted by the action of the specific ODC inhibitor 1,4 diaminobutanone (DAB), cell differentiation is prevented without serious alterations in cell growth. Specificity of the inhibitor is supported by the observation that putrescine addition reverses the effect of DAB [27, 41, 48]. Addition of DAB also inhibited teliospore germination and dimorphic transition in *U. maydis* [28].

We reasoned that in addition to these conclusive evidences, a decisive proof for the specific role of polyamines would be provided by controlling their concentrations in auxothophic mutants. Towards this end, the gene coding for the ornithine decarboxilase from *U. maydis* was cloned in our laboratory. Deduced protein sequence showed high degree

of homology to other fungal ODC genes. The regulatory region of the gene presented two PEST regions and a leader sequence, characteristic of high rate protein turnover and a complex regulation of the transcript, respectively [28]. Only one transcript was detected. The phenotypic alterations brought about by its disruption were valuated in vivo [28]. Null mutants displayed no ODC activity and behaved as polyamine auxotrophs, indicating that a single ODC gene is present in *U. maydis,* and that the only mechanism for polyamine biosynthesis is the ODC pathway. In polyamine-containing media mutants grew normally, and when changed to polyamine-free media their polyamine pool could sustain growth for up to 12–16 h. More important was the observation that when putrescine concentrations were lower than 0.5 mM null mutants grew normally but they were unable to carry out the dimorphic transition under conditions favorable for mycelial growth. Reversion to normal dimorphic conditions requiered high concentrations of putrescine or spermidine [28]. These results conclusively demostrates that polyamines play an important role on cell differentiation on *U. maydis*.

Differential methylation of DNA during the dimorphic transition DNA methylation in higher organisms is involved in several cellular processes including formation of inactive chromatin, timing of chromosome replication, inactivation of the X chromosome, genome imprinting, carcinogenesis and differential gene expression [several authors cited on 42]. In fungi, DNA methylation has been demonstrated to occur only in a few species [1, 47] and some appear to be correlated with different growth stages [30, 51]. High levels of methylation are associated to low transcriptional activity, whereas low methylation levels are associated with high trancriptional activity. In *Neurospora crassa* and *Ascobolus immersus* DNA methylation has been correlated with gene activity [43, 54]. One area of research of our laboratory is to elucidate the role of DNA methylation in fungal development, supported by the evidence that spore DNA from *Mucor rouxii* contains more 5 mC than growing hyphae [14], and that methylation is involved in selective gene expression during spore germination [15]. An adaptation made to the technique named Amplified Fragment Length Polymorphism (AFLPs) was used to determine how general was the phenomenon of differential methylation on fungi. It was found that DNA methylation in fungi is a more general phenomenon than previously thought [42]. Two classes of methylated fragments were identified in our studies, some whose methylation pattern did not change during the dimorphic transition, whereas others were differentially methylated during the dimorphic transition. In *U. maydis* 49 differentially methylated fragments were found [42]. The observed changes in DNA methylated patterns during the dimorphic transition suggests a strong relathionship between DNA methylation and differentiation in fungi.

Structure and synthesis of the cell wall of *Ustilago maydis*

Fungi are very succesful organisms to invade and colonize all different ecological niches. This success is due in great part to their cell wall which protects the protoplast during the invasion of hostile environments. The view that the cell wall of fungi is only a rigid outer layer has changed dramatically; now we are aware that walls are active structures, where important biochemical and physiological processes take place. Therefore cell wall nature and mechanisms involved in its synthesis have attracted special attention to investigators wordwide [46].

The knowledge of cell wall composition and synthesis in *U. maydis* is still in its infancy. However recent studies have shed important information. In our laboratory a comprehensive study was done on this subject. It was found that cell walls of the yeast form in *U. maydis* did not stain by osmium tetroxide or $KMNO_4$, apparently due to the presence external compounds that interfere with the fixation process. On the other hand, the mycelial form did stain with $KMNO_4$. In both forms the wall appeared covered by a loose layer which was strongly stained by the salt. *U. maydis* walls were made most of polysaccharides, existing only minor differences between the yeast and the mycelial form. Chitin and phosphate were more abundant in the mycelial phase, but protein and neutral sugars existed in larger amounts in yeast cells. Important differences were found in the relative proportion and nature of the neutral sugars present in the wall. Xylose and mannose were found in yeast cells in higher amounts, whereas glucose and galactose were found in higher amounts in the mycelial form. Fucose, arabinose and ribose were exclusive of yeast cells walls. Our results differed somewhat from those of Prillinger et al. [38] who found larger amounts of glucose, lower amounts of galactose and mannose, and traces of arabinose. We attributed these differences to strains, media and conditions of growth [49]. Those authors had made an exhaustive analysis of the carbohydrate composition of yeast cell walls, and concluded that the so-called *Ustilago* type of walls were characterized by a glucose/mannose ratio larger than 1 [38, 39]. Amino acid composition was similar in both morphologies. Fifty percent of the protein was accounted by only five aminoacids mostly hydrophilic and acidic ones [49].

The most important structural component of the cell wall in fungi is chitin, and *U. maydis* is not the exception. Therefore efforts to unravel the mechanisms involved in the synthesis of chitin have been made, especially in our research group. Two chitin shynthase genes have been cloned [63]. *Umchs1* and *Umchs2* were isolated using oligonucleotides deduced from conserved regions of other fungal *CHS* genes. Their sequence have strong similarity with *CHS* genes from other fungi, with seven domains and a well conserved central part. Transcripts of both genes were detected in the mycelial and the yeast phase,

with higher expression in the mycelial form. *Chs1* appears to be more abundant. Gene disruption at either gene demonstrated that gene products are non-essential. Mating and pathogenicity of the null strains was normal. Nevertheless, rate growth, chitin synthase activity levels and chitin content, mostly of mycelial cells were reduced in both mutants [63]. These results agreed to those of Gold and Kronstad [26] who used two PCR fragments of chitin synthase genes *chs1* and *chs2* [11] to bring about gene disruption. Recently in our laboratory a third CHS gene with homology to *CHS3* of *S. cerevisiae* [12] was cloned (unpublished results). Disruption of this gene revealed that the chitin synthetase it codes for, is also not essential. However, null mutants displayed reduce growth rate and chitin synthetase and chitin levels. Their virulence towards corn seedlings was also reduced. A summary of the phenotypic alterations exhibited by the different *chs* null mutants is shown in Fig. 6. Accordingly, it appears that the several chitin synthetases of the fungus are not specific of function or stage. The presence of several genes coding for different chitin synthases suggests that functions
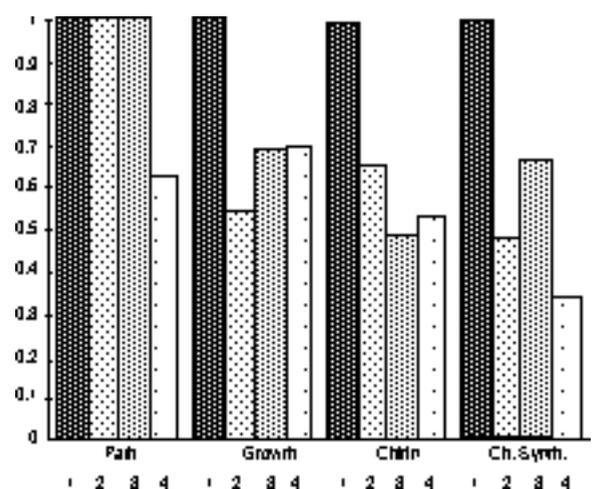
Fig. 6 Comparison of several phenotypic parameters among wild type and *chs* disruptants. 1, wild type; 2, *chs1*; 3, *chs2*; 4, *chs3*. Path, pathogenicity in maize seedlings; growth, cell protein content in liquid media; chitin, chitin content; ch. synth., chitin synthetase activity. Values referred to wild type (1.0)

can be partially substituted.

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