Molecular Mechanisms Involved in the Multicellular Growth of Ustilaginomycetes

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Abstract: Multicellularity is defined as the developmental process by which unicellular organisms became pluricellular during the evolution of complex organisms on Earth. This process requires the convergence of genetic, ecological, and environmental factors. In fungi, mycelial and pseudomycelium growth, snowflake phenotype (where daughter cells remain attached to their stem cells after mitosis), and fruiting bodies have been described as models of multicellular structures. Ustilaginomycetes are Basidiomycota fungi, many of which are pathogens of economically important plant species. These fungi usually grow unicellularly as yeasts (sporidia), but also as simple multicellular forms, such as pseudomycelium, multicellular clusters, or mycelium during plant infection and under different environmental conditions: Nitrogen starvation, nutrient starvation, acid culture media, or with fatty acids as a carbon source. Even under specific conditions, Ustilago maydis can form basidiocarps or fruiting bodies that are complex multicellular structures. These fungi conserve an important set of genes and molecular mechanisms involved in their multicellular growth. In this review, we will discuss in-depth the signaling pathways, epigenetic regulation, required polyamines, cell wall synthesis/degradation, polarized cell growth, and other cellular-genetic processes involved in the different types of Ustilaginomycetes multicellular growth. Finally, considering their short life cycle, easy handling in the laboratory and great morphological plasticity, Ustilaginomycetes can be considered as model organisms for studying fungal multicellularity.

Keywords: Ustilaginomycetes; smut fungi; multicellularity; filamentous growth; mycelial growth

1. Introduction

Multicellularity is considered one of the major transitions in the evolution of complex organisms on Earth. It is defined as the developmental process by which unicellular organisms became pluricellular early and repeatedly during the evolution of life, involving genetic, ecological, and environmental factors [1–5]. Multicellularity has different levels of complexity. In microorganisms, such as bacteria and fungi, simple multicellularity has been observed as a mechanism that increases the affinity for substrates and the obtainment of nutrients, defense against predators, tolerance to environmental stress, host colonization, and may ensure offspring [6–13]. Interestingly, two possible hypotheses have been proposed to explain the development of simple multicellularity: (i) Clonal multicellularity, where the
cells are held together after mitosis and share the same genotype; and (ii) aggregative multicellularity, when unrelated cells come together to form a chimeric structure [5,14,15]. However, it has been observed that the multicellularity developed in filamentous fungi can be different from that of the previously described fungi. This is because their vegetative mycelium rarely adheres to each other except in fruiting bodies—an example of complex multicellularity, which is defined as a three-dimensional structure with a differentiated organization in time and space and a genetically determined developmental program [16,17]. Comparative genomic studies have shown that both filamentous fungi and yeasts conserve the genetic machinery for multicellular growth [16,18]. Fungal mycelium has been described as a model of a simple multicellular structure, showing a polarized syncytium that expands via tip elongation and somatic cell-cell union [19,20]. Likewise, the snowflake phenotype developed by yeasts and some fungi is considered a model of undifferentiated multicellularity [10,11,13,16].

Some fungi show multicellular growth under specific environmental conditions or development stages, e.g., *Neurospora crassa* filamentous growth, *Candida albicans* and *Yarrowia lipolytica* mycelial growth, *Saccharomyces cerevisiae* pseudomycelium and multicellular clusters, *Coprinus cinereus* fruiting bodies, *inter alia* [20–25].

Ustilaginomycetes are a group of approximately 1,185 of Basidiomycetes species, many of which are pathogens of economically important plants [26]. *Ustilago maydis* is probably the most studied species. Like other Ustilaginomycetes, *U. maydis* can grow unicellularly as saprophytic yeast-like cells (sporidia), or multicellularly as mycelium during plant infection and colonization. On the maize plant surface, sexually compatible sporidia mate to form a dikaryotic filament that penetrates the plant by forming appressorium. Mating is regulated by the *a* and *b* independent alleles. The *a* allele encodes a pheromone-receptor system and the *b* allele a *bE/bW* heterodimer transcription factor, which regulate the development of the infective dikaryotic filament [27–32]. In some of these fungi, simple multicellularity has also been observed during their growth under different environmental conditions, such as nitrogen or nutrient starvation, acid culture media, or using fatty acids as the carbon source [33–37]. For example, *Sporisorium reilianum* shows several types of multicellular growth: (i) As mycelium during the plant infection or under nutrient starvation conditions [29,31,33,38]; (ii) as pseudomycelium by deleting certain genes [39]; or (iii) as multicellular clusters in acidic culture medium [13]. In *U. maydis*, basidiocarp formation has also been described [40,41].

There are several mechanisms described as important for multicellular growth in Ustilaginomycetes, for example, the Mitogen-Activated Protein Kinase (MAPK) and Protein Kinase A (PKA) signaling pathways [32,42–45]; the requirement of polyamines [46,47]; epigenetic regulation [48–51]; and cell wall synthesis and degradation (a cellular structure directly involved in fungal multicellularity) [52–54].

Considering that the Ustilaginomycetes can grow unicellularly and multicellularly, and the importance of their morphology during development, in this review we will discuss the signaling pathways, the epigenetic regulation, the polyamine requirements, the polarized cell growth, and the synthesis or degradation of the cell wall during the multicellular growth of this class of fungi, highlighting the experimental advantages of the Ustilaginomycetes as model organisms for studying multicellularity.

### 2. Signaling Pathways Involved in the Ustilaginomycetes Multicellular Growth

Fungi, like all living organisms, grow and develop under fluctuating environmental conditions. Therefore, they have developed mechanisms to sense, respond, and adapt to conditions that favor their growth, or to deal with adverse conditions (Figure 1).
Multicellular growth is induced in Ustilaginomycetes: During plant infection and colonization, due to nitrogen or nutrient starvation, in acidic culture media and using fatty acids as carbon sources. *U. maydis*, for example, can grow multicellularly as basidiocarp in the presence of maize embryogenic calli (Figure 2, Figure 3, and Figure 4B–G,J–L) [13,29,32–36,40].

**Figure 1.** Schematic representation of the signal transduction and metabolic pathways involved in the Ustilaginomycetes multicellular growth. Several authors have suggested that the mitogen-activated protein kinase (MAPK) and cyclic AMP (cAMP)/protein kinase A (PKA) pathways are necessary for mycelium development (a simple multicellular structure observed in Ustilaginomycetes) (reviewed by References [32,45,55,56]). Polyamines, especially spermidine, are essential for the expression of the genes involved in the multicellular growth and the response to stress conditions [47,57–59]. Although the Rim pathway is not involved in the fungal multicellular growth, it is required to sense stressful environmental conditions such as pH [60–62], which is one of the most important inducers of multicellular growth in Ustilaginomycetes [13,35].

**Figure 2.** Multicellular growth of Ustilaginomycetes is induced during infection and colonization of the host plants. (A) Multicellular growth of *Ustilago maydis* as a filament is induced during the maize and *Arabidopsis* experimental host infection [27,63,64]. The different colors in sporidia nuclei represent different yeast-like cells that are sexually compatible. (B) Similarly, *Sporisorium reilianum* multicellular growth is induced during maize, sorghum, and *Arabidopsis* infection [29,31,38].
Figure 3. The environmental conditions induce the multicellular growth of Ustilaginomycetes. (A) Nitrogen starvation [34], acid culture media [35], and fatty acids as a carbon source [36] induce multicellular growth as mycelium in *Ustilago maydis*. (B) *U. maydis* grows multicellularly as gastroid-type basidiocarps when it is cultivated in dual cultures with maize embryogenic calli [40]. (C) Host plant extracts or nutrient starvation induce multicellular growth as mycelium in *S. reilianum* [33]. (D) Acid pH in culture medium induces multicellular cluster growth in *S. reilianum* [13].
Although pH is one of the environmental factors that induce multicellular growth in some Ustilaginomycetes (Figure 3A,D and Figure 4B,J–L), the Pal/Rim pathway, the main mechanism fungi use to sense pH (Figure 1) [61,65], is not involved in U. maydis multicellular growth [60–62], and may not be involved in other Ustilaginomycetes growth either (the Pal/Rim pathway is conserved in smut fungi, see Table 1). We will discuss the MAPK and PKA signal transduction pathways involved in Ustilaginomycetes multicellular growth in response to the different conditions or factors previously mentioned.
| Pathway | Gene | Ustilago maydis | Ustilago hordei | Ustilago bromivora | Sporisorium reilianum | Sporisorium graminicola | Testicularia cyperi |
|---------|------|----------------|----------------|-------------------|---------------------|----------------------|-------------------|
| PKA     | RAL2 | UMAG_00884     | UHOR_01334     | UBro_01334        | sr12177             | EX895_006321         | BCV70DRAFT_173605 |
|         | GPA3 | UMAG_04474     | UHOR_06981     | UBro_06981        | sr15360             | EX895_002426         | CE102777_5455     |
|         | BPP1 | UMAG_00703     | UHOR_01085     | UBro_01086        | sr11991             | EX895_000934         | CE92962_8479      |
|         | PKA  | UMAG_06450     | UHOR_09003     | UBro_09003        | sr16888             | EX895_004218         | CE31316_43035     |
|         | UAC1 | UMAG_05232     | UHOR_03218     | UBro_03218        | sr13141             | EX895_004629         | BCV70DRAFT_200755 |
|         | UBC1 | UMAG_00525     | UHOR_00805     | UBro_00805        | sr10199.2           | EX895_000753         | BCV70DRAFT_164254 |
|         | ADRI | UMAG_04456     | UHOR_06957     | UBro_06957        | sr15343             | EX895_002409         | BCV70DRAFT_103006 |
|         | SGL1 | UMAG_05501     | UHOR_07888     | UBro_07888        | sr16182             | EX895_002861         | BCV70DRAFT_212144 |
|         | HGL1 | UMAG_11450     | UHOR_00981     | UBro_00981        | sr11921             | EX895_000866         | BCV70DRAFT_202631 |
|         | CRK1 | UMAG_11410     | UHOR_04041     | UBro_04041        | sr10962             | EX895_005166         | BCV70DRAFT_157158 |

| MAPK    | RAS2 | UMAG_01643     | UHOR_01498     | UBro_02437        | sr12711             | EX895_004437         | CE98444_29301     |
|         | SQL2 | UMAG_10803     | UHOR_02247     | UBro_02247        | sr12585             | EX895_004314         | BCV70DRAFT_231562 |
|         | SGL2 | UMAG_11476     | UHOR_04466     | UBro_04466        | sr13877             | EX895_001846         | BCV70DRAFT_223969 |
|         | UBC2 | UMAG_05261     | UHOR_08070     | UBro_08070        | sr16309             | EX895_003017         | BCV70DRAFT_197656 |
|         | MAPKK| UMAG_04238     | UHOR_06391     | UBro_06391        | sr15150             | EX895_002006         | BCV70DRAFT_154027 |
|         | MAPKK| UMAG_11453     | UHOR_01494     | UBro_00992        | sr11928             | EX895_000873         | CE125301_8671     |
|         | MAPKK| UMAG_00721     | UHOR_01105     | UBro_01105        | sr12009             | EX895_000953         | BCV70DRAFT_71012  |
|         | MAPK | UMAG_03305     | UHOR_05144     | UBro_05144        | sr14305             | EX895_005556         | CE107062_12366    |
|         | MAPK | UMAG_02331     | UHOR_05144     | UBro_05144        | sr14305             | EX895_005556         | BCV70DRAFT_107063 |
|         | CRK1 | UMAG_11410     | UHOR_04041     | UBro_04041        | sr10962             | EX895_005166         | BCV70DRAFT_157158 |

| RIM     | RIM101| UMAG_10426     | UHOR_06410     | UBro_06410        | sr15166             | EX895_002022         | CE711_11906       |
|         | RIM9  | UMAG_00581     | UHOR_00899     | UBro_00899        | sr11859             | EX895_000793         | CE73867_14878     |
|         | RIM20 | UMAG_11510     | UHOR_05885     | UBro_05885        | sr14775             | EX895_001317         | BCV70DRAFT_202631 |
|         | RIM13 | UMAG_02075     | UHOR_03447     | UBro_03447        | sr13297             | EX895_003420         | BCV70DRAFT_198765 |
|         | RIM23 | UMAG_04392     | UHOR_06683     | UBro_06863        | sr15280             | EX895_002350         | BCV70DRAFT_161062 |
|         | RHO4  | UMAG_10663     | UHOR_01182     | UBro_05736         | sr14670             | EX895_001201         | CE23374_4310      |

ID genes according to NCBI (a), ExPASy (b), e!EnsemblFungi (c), and JGI MycoCosm (d), and considering the genomic data published for U. maydis [66], U. hordei [67], U. bromivora [68], S. reilianum [69], S. graminicola [70], and T. cyperi [71]; the six Ustilaginomycetes with the best-annotated genomes. Genes identified based on their homology with U. maydis. The sequences analyzed were deposited in https://github.com/lucilaortiz/Multicellularity-associated_proteins.
2.1. MAPK Pathways

In eukaryotic organisms, including Ustilaginomycetes, the MAPK signaling pathway is one of the most conserved and important mechanisms for the transduction of information from the exterior to the interior of the cell (Table 1). In the MAPK pathway, stimuli are perceived by receptors and proteins anchored in the cell membrane. The receptors can be G-protein-coupled to transmembrane receptors, receptor tyrosine kinases (RTKs) or two-component signal transduction systems (TCS). When the receptors perceive the stimuli, a sequential activation is induced by phosphorylation of serine/threonine residues in the three protein kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK) (Figure 1). The MAP kinase activates the transcription factors that translocate to the nucleus to regulate the expression of the genes in response to the external stimuli [45,72,73].

In fungi, the MAPK signaling pathway regulates physiological processes, such as the cell cycle, multicellularity, sporulation, mating, cell wall synthesis and integrity, response to stress, and virulence [45,74,75]. The \textit{Saccharomyces cerevisiae} and \textit{Candida albicans} MAPK signaling pathways have been extensively studied, so there is more information available. The \textit{S. cerevisiae} Kss1 MAPK signaling pathway is necessary for multicellular pseudomycelium growth. The \textit{C. albicans} homologous pathway is known as Cek1, and \textit{U. maydis} homologous pathway is known as PMM (pathogenesis, mating, multicellularity, or morphogenesis). In \textit{U. maydis}, the MAPK PMM pathway controls the multicellular growth in vitro [induced by different stress conditions (Figure 3A,B and Figure 4B)], and in vivo [during host plant infection, (Figures 2A and 4C–E)] [32,45]. The deletion of \textit{C. albicans} and \textit{U. maydis} MAPK pathway components suppresses the multicellular formation of pseudomycelium and mycelium, respectively [22,32,44,76–80].

In \textit{U. maydis}, the proteins of the MAPK core are known as: Ubc4/Kpp4 MAPKKK, Fuz7/Ubc5 MAPKK and Ubc3/Kpp2 MAPK (Figure 1). They are important for mating, pathogenicity, and the formation of multicellular mycelium. They show homology with the protein kinases \textit{STE11, STE7, and KSS1} of the \textit{Saccharomyces} Kss1 MAPK pathway [81–86].

In the \textit{Ustilago esculenta} and \textit{Sporisorium scitamineum} Ustilaginomycetes, the protein kinase Kpp2 has the same function as its ortholog in \textit{U. maydis} [87,88]. Phylogenetic analysis showed that the Kpp2 sequence is highly conserved among smut fungi [87]. In \textit{U. esculenta}, the UeKPP2 gene is up-regulated in multicellular filamentous growth, and UeKpp2 interacts with Uefuz7 and UePrf1, the same as in \textit{U. maydis} [88]. Interestingly, \textit{Δskpp2} showed a decrease in mating and filamentation, which was partially restored by adding tryptophol, a quorum-sensing (QS) aromatic alcohol secreted by yeasts that stimulates \textit{S. cerevisiae} and \textit{C. albicans} mycelium or pseudomycelium growth, respectively. In \textit{S. cerevisiae}, production of these aromatic alcohols is regulated by extracellular nitrogen and cell density. For example, high concentrations of nitrogen sources repress its synthesis, while low concentrations activate it. However, no multicellularity regulation by QS has been observed in \textit{U. maydis} or other Ustilaginomycetes [87,89,90].

In Ustilaginomycetes, upstream of the MAPKs PMM core is the Ubc2 adaptor protein (Figure 1), an orthologue of Ste50 from \textit{S. cerevisiae}. Ubc2 contains the domains: SAM (sterile α motif), RA (Ras homology 3), and in two SH3 C-terminals (Src homology 3). All of them are important for protein-protein interaction. For example, through the SAM domain, Ubc2 interacts with ubc4/kpp4 (MAPKKK) protein kinase [79,91]. \textit{UBC2} is necessary for \textit{U. maydis} multicellular mycelial growth. The \textit{Δubc2} strains that were affected in the MAPK PMM pathway showed a severe reduction in the formation of dikaryotic filamentous and haploid mycelium, which was induced under in vitro conditions [44,91]. Interestingly, the transcriptomic analysis of \textit{Δubc2} showed that 939 genes [≈14.0% of the \textit{U. maydis} genome (6883 genes)] are directly or indirectly regulated by the MAPK PMM pathway [80]. Among the genes natively regulated by the inactivation of MAPK PMM, there were genes encoding proteins involved in membrane synthesis or cell wall synthesis [chitin synthases, chitin deacetylases, Kre6 (glucan synthase), Rot1 (involved in cell wall function), chitin-binding proteins,exo-1,3-beta-glucanase precursors]; genes encoding proteins involved in multicellular growth (actin cytoskeleton organization,
myosins, kinesins); genes encoding protein kinases and serine/threonine kinases, with CRK1 between them; and genes encoding the GTPases, such as RAS and SQL2 [80].

The Ras proteins are small monomeric GTPases that act as molecular switches in the signaling pathways that alternate between the GTP and GDP-bound forms in response to extracellular stimuli. In U. maydis, the SQL2 gene encoding a CDC25-like protein is involved in the activation of Ras2 [92]. The constitutive activation of Ras2 or the overexpression of SQL2 promotes the filamentous multicellular growth of this fungus, even in haploid strains [92,93]. S. cerevisiae Ras2 acts in the same way in the MAPK pathway during pseudomycelial growth [94].

The MAPK pathway is conserved in Ustilaginomycetes (Table 1). As occurs in U. maydis, the MAPK pathway may interact antagonistically with the cAMP/PKA pathway to regulate unicellular (Figure 4A,H,I) or multicellular growth (Figure 4B–G,J–L) [32,45,95] in other Ustilaginomycetes. MAPK PMM is required for multicellular growth as mycelium, and PKA for unicellular growth as yeast. The mutant strains in the MAPK cascade genes, e.g., UBC2, UBC3, UBC4, and UBC5, showed a constitutive yeast growth [81,83–85,91]. The PKA and MAPK signal transduction pathways converge with Crk1, a Ser/Thr kinase protein. The ∆crk1 mutant strains, unlike the wild type strains, grow constitutively as unicellular yeasts on acidic culture medium and under nutrient starvation conditions [95]. However, the CRK1 gene is also necessary for the filamentous growth induced by defects in the cAMP/PKA pathway [95]. Likewise, in S. scitamineum, the MAPK and PKA pathways show an antagonistic interaction during multicellular or unicellular growth regulation. The ∆skspp2 mutant strains show a reduction in filamentation, which is partially restored by adding cAMP [87].

As mentioned above, U. maydis can form large hemo-spherical structures with gastroid-type basidiocarp characteristics (a complex multicellular structure) when grown in solid medium supplemented with auxins in dual cultures with maize embryogenic calli (Figure 3B) [46]. In this fungus, the MAPK PMM pathway is involved in the formation of basidiocarps. The ∆fuz7 mutant strains did not develop basidiocaps, and several genes encoding serine/threonine kinases were up-regulated during the early stages of basidiocarp formation, e.g., Kpp2 and Kpp6 were 2.2 and 11.4 times overexpressed, respectively. Similarly, this occurred during the development of Coprinopsis cinérea fruiting body, when the MAPK pathway genes are up-regulated [96]. Finally, during the development of multicellular clusters of Sporisorium reilianum (Figures 3D and 4J–L), the important role of several MAPK proteins and serine/threonine kinases was suggested by transcriptional network analyses [13].

2.2. cAMP/PKA Pathway

In eukaryotic cells, the secondary messenger, cyclic adenosine monophosphate (cAMP) is produced in response to extracellular stimuli. When the receptors perceive a stimulus, a dissociation of the α-subunit of the G-protein is induced in order to activate or repress the cAMP synthesis by the adenylate cyclase enzyme (Uac) (Figure 1). When the levels of cAMP are low, the PKA is an inactive tetramer compound of two catalytic and two regulatory subunits. However, when the levels are high, cAMP binds to a regulatory subunit causing a holoenzyme dissociation in a dimeric regulatory subunit and two active monomeric catalytic subunits which phosphorylate transcription factors and metabolic enzymes [97–99].

In fungi, the cAMP/PKA signaling pathway has a function in mating, sporulation, dimorphism, response to stress, and virulence [77,100,101]. In S. cerevisiae and C. albicans, both cAMP/PKA and MAPK pathways participate in the shift from unicellular growth as yeast to multicellular growth in the form pseudomycelium or mycelium, respectively [22,77,78,102]. In contrast, and as described above, cAMP/PKA and MAPK PMM are antagonistic in U. maydis [32,45,55]. In this fungus, the inactivation of the cAMP/PKA pathway by deleting the genes that encode adenylate cyclase (Uac1) or a regulatory subunit of PKA (Adr1) showed a constitutive phenotype of multicellular haploid mycelium. In addition, the unicellular budding growth was restored when exogenous cAMP was added to the culture medium [42,43]. The same phenotype was observed in ∆gpa3 and ∆bpp1 mutants, that were affected in the α- and β- heterotrimeric GTPase subunits, respectively. The cAMP/PKA pathway in Ustilago
hordei acts in the same way as in U. maydis. The deletion in the heterotrimeric GTPase α-subunit (Δfil1) produced constitutive mycelial growth in a solid and liquid medium, and the addition of exogenous cAMP restored the budding growth [103].

Although the cAMP/PKA signaling pathway is highly conserved in Ustilaginomycetes (Table 1), in S. scitamineum, and contrary to U. maydis and U. hordei, the cAMP/PKA pathway is involved in multicellular growth as mycelium. The deletion of different pathway components (Δssgpa3, Δssuac1 and Δssadr1) suppresses the formation of dikaryotic mycelium. The production of cAMP in Δssauc1 was blocked, and severely reduced in Δssgpa3 and Δssadr1. The mycelial growth was restored in Δssgpa3 and Δssuac1 by adding exogenous cAMP, but not in Δssadr1. This makes sense because SsGpa3 and SsUac1 act upstream of cAMP production [104]. Moreover, in this smut fungus, the AGC protein kinases (SsAgc1) regulate the signaling pathway involved in mating and dikaryotic filamentous growth; therefore, this protein is crucial for the expression of genes involved in multicellular growth of S. scitamineum [105].

Other cAMP/PKA pathway components and their functions during U. maydis unicellular-multicellular growth have been analyzed. An example of this is the HGL1 gene (hyphal growth locus), which encodes a regulator of the unicellular and multicellular morphologies and is important for mating and teliospore formation during the fungus’ pathogenic process [106]. Moreover, the SQL1 gene encoding a repressor of the U. maydis cAMP/PKA pathway is itself a repressor of unicellular fungus growth [107].

In the early developmental stages of U. maydis basidiocarps (a complex multicellular structure), the gene encoding the cAMP-dependent protein kinase catalytic subunit was 2.3 times overexpressed [41]. In this regard, in C. cinerea, Schizophyllum commune and Volvariella volvacea, the cAMP content is essential for the formation of the fruiting body [108–111], and the genes of cAMP/PKA signaling pathways are up-regulated during the development of the C. cinerea fruiting body [96]. Finally, the participation of the kinase A protein and cAMP-dependent protein kinase in the formation of S. reilianum multicellular clusters was predicted by transcriptional network analyses [13].

3. The Role of Polyamines in the Multicellular Growth of Ustilaginomycetes

Polyamines are aliphatic polycations that are present in all living organisms. They are essential for development and growth [112]. The polyamines modulate the enzymatic activities, gene expression, DNA-protein interactions, and protect DNA from enzymic degradation [113,114]. In fungi, they regulate spore germination, appressorium formation, conidiation and multicellular growth in the form of mycelium [46,57,115–119]. The common polyamines in some fungi are putrescine, spermidine, and spermine. However, Ustilaginomycetes, such as U. maydis do not have spermine [59,120,121], and they conserve the same genes for polyamine synthesis (Table 2, Figure 1). This characteristic, and the fact that these fungi can grow as unicellular and multicellular forms, make them excellent model organisms for studying the metabolism and function of polyamines in fungi multicellularity.

Strains of U. maydis with ODC gene mutated, which encode ornithine decarboxylase (Odc) and is responsible for putrescine synthesis, are unable to grow as mycelia under acidic pH conditions with limiting concentrations of putrescine [46]. In basidiomycota fungi, such as smut fungi, the spermidine synthase (Spe) is encoded by a chimeric bifunctional gene that also encodes saccharopine dehydrogenase (Sdh), an enzyme involved in lysine synthesis [121]. The U. maydis Δspe-Δsdh mutant strains are auxotrophic for lysine and spermidine and require high concentrations of spermidine in order to grow multicellularly as mycelium under acidic conditions in culture medium [57]. Interestingly in Ustilaginomycetes, there is an alternative mechanism for putrescine synthesis, through the polyamine oxidase enzyme involved in the retroconversion of spermidine to putrescine. U. maydis double mutants (Δodc/Δpao) in the genes encoding Odc and Pao grow multicellularly as mycelium with the addition of spermidine only and in complete absence of putrescine [58]. This indicates that spermidine is the most important polyamine for multicellularity in Ustilaginomycetes [58,59,119,122].
Table 2. Genes involved in the polyamine synthesis pathway of Ustilaginomycetes.

| Gene | Ustilago maydis | Ustilago hordei | Ustilago bromivora | Sporisorium reilianum | Sporisorium graminicola | Testicularia cyperi |
|------|----------------|----------------|-------------------|--------------------|-----------------------|----------------------|
| OAT  | UMA0_05671     | UHO_07330      | UBR0_07330        | sr15996            | EX895_002715          | BCV70DRAFT_201257    |
| ODC  | UMA0_01048     | UHO_01580      | UBR0_01580        | sr12348            | EX895_006099          | BCV70DRAFT_212462    |
| PAO  | UMA0_05850     | UHO_08347      | UBR0_08347        | sr16473            | EX895_008244          | BCV70DRAFT_173429    |
| SPE  | UMA0_05818     | UHO_08297      | UBR0_08297        | sr16440            | EX895_003211          | BCV70DRAFT_199161    |
| SAMDC| UMA0_10792     | UHO_01520      | UBR0_01520        | sr12300            | EX895_006201          | BCV70DRAFT_205658    |
| SSAT | UMA0_00127     | UHO_00200      | UBR0_00200        | sr11469            | EX895_000152          | BCV70DRAFT_169192    |

ID genes according to NCBI (\(^a\)), ExPASy (\(^b\)), e!EnsemblFungi (\(^c\)), and JGI MycoCosm (\(^d\)), and considering the genomic data published for U. maydis [66], U. hordei [67], U. bromivora [68], S. reilianum [69], S. graminicola [70], and T. cyperi [71]; the six Ustilaginomycetes with the best-annotated genomes. Genes identified based on their homology with U. maydis. The sequences analyzed were deposited in https://github.com/lucilaortiz/Multicellularity-associated_proteins.
4. Epigenetic Regulation of Multicellular Growth in Ustilaginomycetes

In fungi, as in all eukaryotic organisms, DNA is wrapped and highly compacted around histone proteins forming the nucleosomes within the nucleus. This organization can be altered by post-translational modifications of histones that relax or compact the nucleosomes: Acetylation by histone acetyltransferases (HATs), or deacetylation by histone deacetylases (HDACs), respectively. Nucleosome relaxation allows accessibility of DNA to different proteins involved in replication and gene transcription in response to environmental signals.

Although Ustilaginomycetes have homologous genes encoding HATs and HDACs (Table 3), the epigenetic regulation of multicellular growth has only been studied in U. maydis [48–50]. In this fungus, the GCN5 HAT was deleted. The mutant strains were slightly more sensitive to different stress conditions than the wild type, but they grew multicellularly as mycelium and fuzz-like colonies (constitutive mycelial growth) under all the growth conditions analyzed. Also, the virulence was dramatically reduced [48]. Through transcriptomic analysis of mutant and wild type strains, it was observed that in the mutant strain (Δgcn5) a total of 1203 were differentially regulated. Of these, 574 were repressed and 629 were overexpressed. Interestingly, 67 genes described as important for yeast growth (unicellularity, Figure 4A) and 66 described as important for mycelial growth (multicellularity, Figure 4B) [123], were down-regulated and up-regulated, respectively in the Δgcn5 mutant [49]. Among the differentially expressed genes, the following phenomena stand out: Genes involved in cell wall biogenesis and genes related with pathogenesis were down-regulated; genes required for mycelium development were overexpressed. For example, the REPI gene that encodes a repellent protein was 33.2 times up-regulated in the mutant strain [49].

Contrary to the deletion of GCN5 HAT, the deletion of HOS2 and CLR3 HDACs affected U. maydis multicellular growth. In the Δhos2 mutant, the formation of the conjugation tube and the mating of compatible single-cell strains were altered. In addition, the multicellular dikaryotic mycelium and virulence were reduced [50].

In summary, the HATs and HDACs play an indispensable, but opposed, role in U. maydis multicellular growth and possibly in other Ustilaginomycetes fungi. While the histone acetylation by Gcn5 allows the expression of genes involved in unicellular growth as yeast (Figure 4A), histone deacetylation by Hos2 andClr3 allows multicellular growth as mycelium (Figure 4B). Histone deacetylation has been usually associated with gene repression. However, these findings demonstrate that the HDACs also function as transcriptional activators [51]. Considering that these genes are evolutionarily conserved in eukaryotic organisms such as Ustilaginomycetes (Table 3) [124–129], and that most eukaryotic organisms are multicellular, it is tempting to speculate about the importance of this histone acetylation or deacetylation switch during the developmental process by which unicellular organisms became multicellular during evolution.

Another epigenetic mechanism suggested in the regulation of the multicellular growth of Ustilaginomycetes is DNA methylation. Different DNA methylation patterns were observed during the unicellular and multicellular growth of U. maydis wild-type strains [130]. Interestingly the mutants affected in the putrescine synthesis (Δodc), which could not grow multicellularly as mycelia (discussed above) [46], showed DNA methylation patterns similar to those of the wild-type yeast [131]. Knowing that polyamines bind to DNA [132], and that DNA methylation is involved in gene silencing [133], it suggests that polyamines, particularly spermidine, prevent DNA methylation, thus allowing the expression of genes involved in U. maydis multicellular growth [134]. Taking into consideration that the genes involved in the polyamine synthesis are conserved in Ustilaginomycetes (Table 2), it is possible that this regulatory mechanism of multicellular growth is present in this class of fungi.
| Gene | Ustilago maydis a | Ustilago hordei a,b | Ustilago bromivora c | Sporisorium reilianum a,b | Sporisorium graminicolor a | Testalaria cyperi a,b,d |
|------|------------------|--------------------|--------------------|--------------------------|--------------------------|-------------------------|
| GCN5—Histone acetyltransferase | UMAG_05168 | UHOR_03120 | UBRO_03120 | sr13072 | EX95_004562 | BCV70DRAFT_198404 |
| Putative histone acetylase | UMAG_10190 | UHOR_03120 | UBRO_03120 | sr13072 | EX95_004562 | BCV70DRAFT_198404 |
| CLR3—Histone deacetylase | UMAG_02102 | UHOR_03487 | UBRO_03487 | sr13325 | EX95_004958 | BCV70DRAFT_166948 |
| HOS2—Histone deacetylase | UMAG_11828 | UHOR_01015 | UBRO_01015 | sr11943 | EX95_000889 | BCV70DRAFT_70918 |
| TEA4—SH3 domain protein | UMAG_01012 | UHOR_01534 | UBRO_01534 | sr12319 | EX95_006176 | BCV70DRAFT_159133 |
| TEA1—Kelch domain protein | UMAG_15019 | UHOR_01124 | UBRO_01124 | sr12022 | EX95_009966 | BCV70DRAFT_69247 |
| REPI—Repellent | UMAG_03924 | UHOR_05948 | UBRO_05948 | sr14829 | EX95_001361 | BCV70DRAFT_80316 |
| REP4—Repellent | UMAG_04517 | UHOR_06499 | UBRO_06499 | sr15402 | EX95_002086 | BCV70DRAFT_202324 |
| HUM2—Hydrophobin 2 | UMAG_11562 | UHOR_07126 | UBRO_07126 | sr15903 | EX95_002555 | BCV70DRAFT_207191 |
| HUM3— Hydrophobin 3 | UMAG_04433 | UHOR_06926 | UBRO_06926 | sr15320 | EX95_003233 | BCV70DRAFT_189664 |
| RRME—RNA-binding protein | UMAG_03494 | UHOR_05377 | UBRO_05377 | sr14484 | EX95_006466 | BCV70DRAFT_213009 |
| Actin-binding protein | UMAG_05340 | UHOR_08160 | UBRO_08160 | sr16324 | EX95_003081 | BCV70DRAFT_135405 |
| Actin—interactin gprotein; actin patch component | UMAG_05949 | UHOR_08467 | UBRO_08467 | sr16579 | EX95_005119 | BCV70DRAFT_213703 |
| Actin filament organization | UMAG_04613 | UHOR_06638 | UBRO_06638 | sr15500 | EX95_002183 | BCV70DRAFT_202237 |
| LPSI—Nuclear migration | UMAG_03164 | UHOR_04938 | UBRO_04938 | sr14206 | EX95_005820 | BCV70DRAFT_200264 |
| Actin regulating Ser/Thr kinase | UMAG_03081 | UHOR_04824 | UBRO_04824 | sr14140 | EX95_002483 | BCV70DRAFT_200264 |
| Formin; actin nucleation | UMAG_12254 | UHOR_06202 | UBRO_06202 | sr19204 | EX95_001591 | BCV70DRAFT_205747 |
| Formin; actin nucleation. Functionally redundant with Bni1 | UMAG_01141 | UHOR_01723 | UBRO_01723 | sr13440 | EX95_006065 | BCV70DRAFT_12160 |
| F-actin capping protein alpha subunit | UMAG_00243 | UHOR_00664 | UBRO_00664 | sr17788 | EX95_006522 | BCV70DRAFT_196835 |
| F-actin capping protein beta subunit | UMAG_11177 | UHOR_08001 | UBRO_08001 | sr16266 | EX95_002944 | BCV70DRAFT_201290 |
| Chaperonin role for actin and tubulin | UMAG_01279 | UHOR_01918 | UBRO_01918 | sr10401 | EX95_001741 | BCV70DRAFT_197510 |
| Chaperonin role for actin and tubulin | UMAG_06235 | UHOR_08845 | UBRO_08845 | sr16767 | EX95_003720 | BCV70DRAFT_199532 |
| Chaperonin role for actin and tubulin | UMAG_06007 | UHOR_08612 | UBRO_08612 | sr16694 | EX95_003550 | BCV70DRAFT_202419 |
| Chaperonin role for actin and tubulin | UMAG_02571 | UHOR_04113 | UBRO_04113 | sr17312 | EX95_003225 | BCV70DRAFT_198632 |
| Chaperonin role for actin and tubulin | UMAG_03959 | UHOR_06000 | UBRO_06000 | sr14864 | EX95_001395 | BCV70DRAFT_69984 |
| Chaperonin role for actin and tubulin | UMAG_02350 | UHOR_03838 | UBRO_03838 | sr13533 | EX95_004884 | BCV70DRAFT_198957 |
| Chaperonin role for actin and tubulin | UMAG_05605 | UHOR_00911 | UBRO_00911 | sr11943 | EX95_000899 | BCV70DRAFT_72667 |
| Chaperonin role for actin and tubulin | UMAG_04401 | UHOR_06879 | UBRO_06879 | sr15289 | EX95_002355 | BCV70DRAFT_200184 |
| Actin binding and severing protein | UMAG_04314 | UHOR_06753 | UBRO_06753 | sr15202 | EX95_002269 | BCV70DRAFT_211263 |
| Cortical actin cytoskeleton component | UMAG_04417 | UHOR_06902 | UBRO_06902 | sr15303 | EX95_002169 | BCV70DRAFT_200171 |
| Kinesin—Related motor protein (UMAG_Kin7a and Kin7b) | UMAG_11986 | UHOR_06484 | UBRO_06484 | sr15392 | EX95_002076 | BCV70DRAFT_202322 |
| Kinesin—Related motor protein | UMAG_00896 | UHOR_01350 | UBRO_01350 | sr12193 | EX95_006269 | BCV70DRAFT_199338 |
| Kinesin—Related motor protein | UMAG_01560 | UHOR_02319 | UBRO_02319 | sr12632 | EX95_004356 | BCV70DRAFT_199992 |
| Interacts with Myo2—related to motor domain of kinesins | UMAG_04218 | UHOR_06328 | UBRO_06328 | sr15103 | EX95_001669 | BCV70DRAFT_40829 |
| MEP1—Low affinity ammonium transporter | UMAG_04523 | UHOR_06506 | UBRO_06506 | sr15436 | EX95_002121 | BCV70DRAFT_208136 |
| MEP2—High affinity ammonium transporter | UMAG_05889 | UHOR_08388 | UBRO_08388 | sr16507 | EX95_003276 | BCV70DRAFT_199125 |
### Table 3. Cont.

| Gene | Ustilago maydis | Ustilago hordei a,b | Ustilago bromivora c | Sporisorium reilianum a,b | Sporisorium graminicola a | Testicularia cyperi a,b,d |
|------|-----------------|---------------------|----------------------|--------------------------|--------------------------|--------------------------|
| Putative arginase—Homology with G6606 of U. esculenta | UMAG_04939 | UHOR_07065 | UBRO_07065 | sr15821 | EX895_005725 | BCV70DRAFT_178133 |
| Homology with SSAGC1 (SPSC_00276) of S. scitamineum | UMAG_11677 | UHOR_02215 | UBRO_02215 | sr12574 | EX895_004280 | BCV70DRAFT_160776 |
| CDC24—Cell division control protein | UMAG_02422 | UHOR_03863 | UBRO_03863 | sr10836 | EX895_004832 | BCV70DRAFT_192816 |
| CLA4—Serine/threonine-protein kinase | UMAG_10145 | UHOR_03893 | UBRO_03893 | sr10814.2 | EX895_004846 | BCV70DRAFT_158032 |
| RHO1—Ras-like GTP-binding protein | UMAG_01032 | UHOR_01539 | UBRO_01539 | sr12536 | EX895_004088 | BCV70DRAFT_166498 |
| RHO1—Ras-like GTP-binding protein | UMAG_05734 | UHOR_12739 | UBRO_07439 | sr16067 | EX895_002745 | BCV70DRAFT_166498 |
| RHO1—Ras-like GTP-binding protein | UMAG_12839 | UHOR_07359 | UBRO_07359 | sr12536 | EX895_004088 | BCV70DRAFT_166498 |
| FUZ1—MYND domain protein | UMAG_02587 | UHOR_04136 | UBRO_04136 | sr13628 | EX895_005246 | BCV70DRAFT_63386 |

Genes mostly analyzed by its deletion. ID genes according to NCBI (a), ExPASy (b), e!EnsemblFungi (c), and JGI MycoCosm (d), and considering the genomic data published for *U. maydis* [66], *U. hordei* [67], *U. bromivora* [68], *S. reilianum* [69], *S. graminicola* [70], and *T. cyperi* [71]; the six Ustilaginomycetes with the best-annotated genomes. Genes identified based on their homology with *U. maydis*. The analyzed sequences were deposited in [https://github.com/lucilaoeitz/Multicellularity_associated_proteins](https://github.com/lucilaoeitz/Multicellularity_associated_proteins).
5. The Cell Wall in the Multicellular Growth of Ustilaginomycetes

The cell wall is the rigid outer structure that covers the cells of prokaryotic and eukaryotic organisms. It protects the cells from osmotic pressure differences between the cytoplasm and the external medium and gives shape to the cell. In fungi, the cell wall is a compound of polysaccharide microfibrils (chitin and β-1,3-glucans) immersed in a glycoprotein matrix [135]. This cellular organelle is a dynamic structure whose composition changes during the cell cycle, depending on the environmental conditions. It is also directly involved in the unicellular or multicellular development of fungi. The cell wall components are synthesized in defined quantities, according to time and space to form a coherent and organized structure [136,137].

Chitin is the characteristic component of the fungal cell wall, giving it rigidity. It is synthesized by the chitin synthase proteins (Chs), which are encoded by the genes known as CHS. Fungi have multiple CHS genes in their genomes, and it can be a compensatory mechanism of the cell in response to the loss of a certain proportion of them. *S. cerevisiae* has three CHS genes: CHS1, which has a repair function during cell separation, allowing unicellular growth [138]; CHS2 is involved in cell division, and it is essential for septum formation during multicellular growth [139]; and CHS3, which is responsible for chitin synthesis in the ring at bud emergence, for the chitin synthesized in the cell wall, and for chitosan synthesis in ascospores [140]. In multicellular fungi, such as *N. crassa* and *Aspergillus nidulans*, the CHS genes are necessary for normal hyphae development [141,142].

Ustilaginomycetes have approximately eight CHS genes (Table 4). In *U. maydis*, the Δchs6 mutants showed morphological alterations that were more notorious during the multicellular growth as mycelium [143]. Chs6, Chs5, Chs7 and Chs8 are localized in the growth zone in the hyphal tip, which indicates that these could contribute to polarized growth during multicellularity in the smut fungus [144]. Interestingly, the CHS genes are differentially expressed during the dimorphic transition of several fungi, e.g., *Mucor circinelloides* and *Paracoccidioides brasiliensis*, showing high levels of expression in the mycelial form [145,146]. In *U. maydis*, its eight CHS genes were expressed in both yeast ( unicellular growth, Figure 4A) and mycelium forms (multicellular growth, Figure 4B), and similarly, as in the previously described fungi, almost all the CHS genes showed high levels of expression in the mycelium [52].

Chitosan is another component of the fungal cell wall, although its function is less understood than that of chitin. Chitosan is synthesized through the partial deacetylation of chitin by chitin deacetylases (Cda). *S. cerevisiae* has two CDA genes, CDA1 and CDA2, that are expressed only during sporulation. CDA1 and CDA2 have redundant functions in chitosan synthesis in ascospores, which contributes to their resistance to lytic enzymes [147]. Of the ten *Magnaporthe oryzae* CDA genes, only CDA1 showed high levels of expression during its multicellular filamentous growth. However, Δcda1 mutat did not show alteration in the chitin content of the wall. Moreover, Cda1 was localized in the cell wall and septa during hyphae multicellular growth, but not in the growth zones. These findings suggest that CDA has an indirect function in hyphae morphology [148]. Ustilaginomycetes have several CDA genes; for example, *U. maydis* has eight CDA genes (Table 4). CDA1 was 40 times up-regulated in the multicellular mycelial form [53], and the Δcda1 mutants developed aberrant hyphae [149].
Table 4. Genes involved in synthesis and degradation of the cell wall, and putatively involved in the Ustilaginomycetes multicellular growth.

| Cell wall | Gene | Ustilago maydis | Ustilago hordei | Ustilago bromivora | Sporisorium reilianum | Sporisorium graminicola | Testicularia cyperi |
|-----------|------|----------------|----------------|-------------------|----------------------|-----------------------|-------------------|
| Synthesis | 1,3-β-glucan synthase | UMAG_01639 | UHOR_02430 | UBRO_02430 | sr12707 | EX995_004433 | BCV70DRAFT_98536 |
| CHS1 | UMAG_10718 | UHOR_07282 | UBRO_07282 | sr16010 | EX995_002642 | BCV70DRAFT_198128 |
| CHS2 | UMAG_04290 | UHOR_06435 | UBRO_06435 | sr15181 | EX995_002037 | - |
| CHS3 | UMAG_10120 | UHOR_00723 | UBRO_00723 | sr10139 | EX995_000691 | CE2605_17934 |
| CHS4 | UMAG_10277 | UHOR_14112 | UBRO_04112 | sr11048.2 | EX995_005224 | BCV70DRAFT_156817 |
| CHS5 | UMAG_05480 | UHOR_07854 | UBRO_07854 | sr16158 | EX995_002841 | BCV70DRAFT_162870 |
| CHS6 | UMAG_03204 | UHOR_04988 | UBRO_04988 | sr11106 | EX995_005864 | BCV70DRAFT_105640 |
| CDA1 | UMAG_11922 | UHOR_04296 | UBRO_04296 | sr13741 | EX995_005345 | BCV70DRAFT_210413 |
| CDA2 | UMAG_00126 | UHOR_00199 | UBRO_00199 | sr11468 | EX995_000351 | BCV70DRAFT_197174 |
| CDA3 | UMAG_00638 | - | - | - | EX995_000863 | - |
| CDA4 | UMAG_01143 | UHOR_01725 | UBRO_01725 | sr12442 | EX995_006063 | - |
| CDA5 | UMAG_01788 | UHOR_06394 | UBRO_06394 | sr15153 | EX995_002009 | BCV70DRAFT_196730 |
| CDA6 | UMAG_00695 | UHOR_01069 | UBRO_01069 | sr10624 | EX995_002316 | BCV70DRAFT_200231 |
| CDA7 | UMAG_05792 | UHOR_04296 | UBRO_04296 | sr16123 | EX995_002800 | BCV70DRAFT_210413 |
| Degradation | CTS1 | UMAG_10149 | UHOR_06394 | UBRO_06394 | sr15153 | EX995_002009 | BCV70DRAFT_196730 |
| CTS2 | UMAG_00278 | UHOR_04393 | UBRO_04393 | sr13813 | EX995_005415 | CE60997_75476 |
| CTS3 | UMAG_06190 | UHOR_08772 | UBRO_08772 | sr11305 | EX995_003673 | BCV70DRAFT_178298 |
| CTS4 | UMAG_05290 | UHOR_08119 | UBRO_08119 | sr10043 | EX995_003444 | BCV70DRAFT_197812 |
| Glucanase 1 | UMAG_04368 | UHOR_06828 | UBRO_06828 | sr15253 | - | - |
| Glucanase 2 | UMAG_05036 | UHOR_07211 | UBRO_07211 | sr15917 | - | CE121843_61034 |
| Glucanase 3 | UMAG_00876 | UHOR_03000 | UBRO_03000 | sr12981 | EX995_004113 | BCV70DRAFT_34433 |
| Glucanase 4 | UMAG_01898 | UHOR_03898 | UBRO_03898 | sr13587 | EX995_004867 | BCV70DRAFT_163992 |
| Glucanase 5 | UMAG_05223 | UHOR_04296 | UBRO_04296 | sr16123 | EX995_002800 | BCV70DRAFT_210413 |
| Glucanase 6 | UMAG_05867 | UHOR_04296 | UBRO_04296 | - | - | - |
| Glucanase 7 | UMAG_00357 | UHOR_06809 | UBRO_06809 | sr15243 | EX995_002306 | BCV70DRAFT_189826 |
| Glucanase 8 | UMAG_05550 | UHOR_06809 | UBRO_06809 | sr15250 | EX995_002316 | BCV70DRAFT_200231 |

ID genes according to NCBI (a), ExPASy (b), e!EnsemblFungi (c), and JGI MycoCosm (d), and considering the genomic data published for *U. maydis* [66], *U. hordei* [67], *U. bromivora* [68], *S. reilianum* [69], *S. graminicola* [70], and *T. cyperi* [71]; the six Ustilaginomycetes with the best-annotated genomes. Genes identified based on their homology with *U. maydis*. CHS, Chitinsynthase; CDA, Chitindefactylase; and CTS, Chitinase. The sequences analyzed were deposited in https://github.com/lucilaortiz/Multicellularity_associated_proteins.
Polysaccharide β-1,3-glucans are the major components of the cell wall, which are synthetized by the β-1,3-glucan synthases (Gls). In *S. cerevisiae*, the genes that encode the catalytic subunit of Gls enzymes are known as *FKS*, due to their sensitivity to FK506 immunosuppressants, but in other fungi, they are known as *GLS*. *N. crassa* has one *GLS-1* gene. *gls-1-RNAi* transformants show a decrease in the β-1,3-glucans synthesis, and abnormalities in the development of the multicellular mycelium; the hyphae were shorter than those of the wild type strain [150]. Gls-1-GFP accumulates at the apex of the hyphae forming a ring structure in the outer region of the Spitzenkörper [151]. In the Ustilaginomycete *U. maydis*, the *GLS* gene showed constitutive expression during multicellular growth like mycelium [52].

On the other hand, in the different stages of fungi development and multicellular growth, the cell wall is remodeled through the synthesis and degradation of their compounds. The cell wall degradation by chitinases and glucanases causes plasticity, which allows the introduction of new synthetized components by forming links between polymers and leading to the expansion of the cellular surface through turgor force [136,137].

The chitinases (Cts) remodel chitin in the cell wall. In *S. cerevisiae*, the endo chitinase Cts1 is necessary for unicellular growth, allowing cellular separation after cytokinesis. The Δcts1 mutants showed abnormal separation during budding growth, forming cellular clusters [152]. During budding (unicellular growth), Cts1 is localized at the mother-daughter neck, contributing to the degradation of the septum, and allowing cell separation [153]. In *C. albicans*, deletion of the *CTS1* and *CTS2* chitinase genes increases the development of pseudomycelium or cellular clusters [154]. Interestingly, the *N. crassa CTS-1* gene is also important for the development of mycelium, which suggests that it has a function in remodeling the cell wall during fungus multicellular growth. The Δcht-1 mutants showed reduced mycelial growth compared to that of the wild- type strains [155]. In Ustilaginomycetes, there are approximately five putative chitinase genes (Table 4). However, they may have redundant functions. In *U. maydis*, only the double mutant Δcts1/Δcts2, but not the single mutants, showed alterations in cell separation. Cts1 and Cts2 are active during unicellular growth as yeast, but only Cts1 showed activity during multicellular growth as mycelium [54]. However, *CTS2* was 6.7 times up-regulated in the *U. maydis* mycelium induced by the acidic pH in the culture medium. [53,123]. Likewise, the β-1,3-end and exo-glucanases have a function in septum separation, and the β-1,3-glucans in elongation and reorganization. *ENG1* encodes a β-1,3-endoglucanase in *S. cerevisiae*. The deletion of *ENG1* shows alteration of the separation of septae and induces growth of mutants as multicellular clusters. Eng1 is mainly localized at the bud neck on the daughter side [156]. Ustilaginomycetes have several putative glucanases (Table 4). However, little is known about their function during unicellular or multicellular growth in these fungi.

Finally, the putative importance of genes involved in cell wall synthesis and structure during development of *S. reilianum* multicellular clusters was determined by transcriptional network analysis [13]. The examples of genes involved include those encoding: 1,3-beta-D-glucan synthase, glucan 1,3-beta-glucosidase, GPI-proteins, UTR2-cell wall proteins, chitin synthase 2 (Chs2), Chs3, exo-1,3-beta-glucanase, Kre6-glucan synthase, chitin deacetylases (Cda), glucan 1,3-beta-glucosidase, and several proteins involved in cell wall biogenesis and architecture. Some of them, such as *UTR2*-cell wall, *CHS3, ECM4*, involved in cell wall biogenesis and architecture, and *CDA*, were down regulated in the multicellular cluster form of the fungus [13]. All these findings demonstrate the direct and critical role of the cell wall in fungi multicellularity. It is tempting to hypothesize that the cell wall is the most important structure involved in this phenomenon in fungi.

6. Polarized Growth during the Multicellularity of Ustilaginomycetes

Mycelia or filamentous growth is one of the best-known forms of simple multicellular structures of fungi. The mycelia are joined and elongated cells in the form of tubes, which extend at the tip and branch out into sub-apical areas involving a coordinated action of the microtubule and actin
cytoskeletons [157]. In Ustilaginomycetes, this phenomenon has been studied most in *Ustilago maydis*, although the genes involved in polarized growth are mostly conserved in this class of fungi (Table 3).

The actin cytoskeleton in *U. maydis* is formed by three structures: Actin cables, actin patches and an actin ring. During the multicellular growth, the hyphae contain an actin cap at the tip, and the actin cables polarize towards the tip [158]. *LI51* is a gene encoding a microtubule plus-end tracking protein required for microtubule cytoskeleton organization, cell wall integrity, septa positioning, and nuclear migration during the filamentous growth. The Δ*li51* mutants showed an aberrant multicellular morphology [159]. Likewise, the *TEA4* gene is involved in the polarized growth, the control of position and the number of septa, as well as the nuclear division in the mycelial form [160]. Also, *FUZ1*, a gene encoding the MYND Zn finger domain protein, is important for the cell wall integrity and dikaryotic filamentous growth after the fusion of compatible sporidia [161].

In Ustilaginomycetes, as in all the filamentous fungi, the kinesin proteins are involved in vesicle transport. *U. maydis* has ten kinesins: Kin1, Kin3, Kin4, Kin6, Kin7a, Kin7b, Kin8, Kin9, and Kin14. Of all these proteins, only Kin1 and Kin3 are important for normal multicellular filamentous growth. Δ*kin1*, Δ*kin3* and the double mutants Δ*kin1/Δkin3* show bipolar growth and short hyphae [162]. Kin1 and Kin3, as well as the myosin *Myo5* and *Tea1*, localize and accumulate at the hyphal apex during multicellular growth [162–164], although *Tea1* is also localized in new sites of the filamentous growth [164]. The mutation of the *MYO5* gene (Δ*myo5*) did not affect the filamentous growth of the fungus. However, it caused irregular growth and irregular chitin deposition [165], thus demonstrating the important relationship between the fungi multicellular filamentous growth and their cell wall.

7. Other Cellular Processes Involved in the Ustilaginomycetes Multicellular Growth

Ustilaginomycetes are model organisms to study the different processes that occur in fungi. Several genes involved in the cellular processes associated with their multicellular growth have been described. Interestingly, these genes are conserved in this class of fungi (Table 3), and it is possible that those genes and the cellular processes work in the same way in Ustilaginomycetes.

In *U. maydis*, it has been observed that *REP1*, *HUM2*, and the *HUM3* genes encode repellent proteins and hydrophobins, a group of small cysteine rich proteins required for the development of multicellular aerial hyphae. The Δ*rep1* and Δ*hum2* mutants showed a drastic reduction of aerial mycelium [165,166]. Moreover, *HUM2* and *HUM3* were overexpressed 9.1 and 14.7 times, respectively, during the formation of the hyphal layer in *U. maydis* basidiocarps [41].

Likewise, the importance of the *PEP4* gene encoding the vacuolar acid proteinase PrA during *U. maydis* mycelial growth under stress conditions and pathogenesis has been reported. The Δ*pep4* mutants showed a severe reduction of multicellular mycelium growth induced by the acidic culture medium or using fatty acids as a carbon source [167]. The GTPases Cdc4 and Rho1 also played essential roles during the polarized growth and filamentation of this fungus [168,169]. In addition, the RNA-binding protein Rrm4 was crucial for the mycelium polarity during infection, because it is involved in the microtubule-dependent transport of mRNAs during fungus multicellular growth [170].

In *S. cerevisiae* and other fungi, a low level of nitrogen in the medium induces multicellular growth of pseudomycelium or mycelium [72]. An important mechanism that links the availability of nitrogen and the unicellular-multicellular growth is are the methyl ammonium permeases (Mep). In *U. maydis*, the *MEP1* and *MEP2* genes, are important for the filamentous development in a medium with low ammonium. The Δ*mep2* mutants did not grow as mycelia, and the Δ*mep1/Δmep2* double mutant showed multicellular aggregates and sediment in liquid media with low ammonia concentrations. Moreover, it was suggested that Mep2 is phosphorylated by the cAMP/PKA signaling pathway in response to low nitrogen conditions [171]. Interestingly, the genes encoding the protein involved in the export of ammonia were up-regulated during *U. maydis* multicellular growth induced under acidic conditions [123]. Similarly, the SSA2 ammonium permease was up-regulated in *Sporisorium scitamineum* during unicellular growth induced by mycophenolic acid, which is a fungal mycelium inhibitor [172]. Also, in *S. scitamineum*, the deletion of the *SSATG8* gene encoding the Atg8 protein
involved in autophagy caused multicellular growth as pseudomyceium and hypersensitivity to oxidative stress [173].

Finally, in the Ustilaginomycete Ustilago esculenta, a pathogenic fungus of Zizania latifolia, the addition of exogenous arginine or the deletion of the UeArginase gene inhibited its multicellular growth as mycelium and reduced the expression of the UeKPP6, UePKAC and UePRF1 genes, thus suggesting that arginine acts as an inhibitor of the cAMP-PKA signaling pathway [174].

All these findings demonstrate that multicellular development in Ustilaginomycetes is a holistic phenomenon, and that it is necessary to deal with the whole cell to the largest possible extent.

8. Conclusions

Multicellularity is defined as the developmental process by which unicellular organisms have become pluricellular during evolution. The Ustilaginomycetes are eukaryotic microorganisms with the capacity to grow unicellularly in the form of saprophytic yeasts or like undifferentiated and simple multicellular structures: Mycelium, pseudomyceium, and cellular clusters. Complex multicellular structures have also been described in the case of U. maydis through the formation of basidiocarps.

The data discussed in this study show that the molecular mechanisms involved in multicellular growth may be conserved in Ustilaginomycetes. These fungi have an important set of genes and regulatory mechanisms involved in their unicellular and multicellular growth. Examples of these mechanisms are: The MAPK and cAMP/PKA signaling transduction pathways, the mechanisms of epigenetic regulation, the metabolic requirements, the polarized cell growth, and the processes of cell wall synthesis and degradation. However, although these fungi are phylogenetically related, they can respond with different morphologies to. For example, U. maydis grows as mycelium in acidic culture media, while S. reilianum grows in the form of multicellular clusters under the same conditions.

Basically, the MAPK and PKA pathways transduce environmental signals to the cell nucleus by the activation of transcription factors, which bind to DNA for the transcription of target genes involved in the Ustilaginomycetes unicellular or multicellular growth. Putatively, the transcription of target genes requires the binding of polyamines (spermidine) to DNA, avoiding its methylation and allowing its transcription. Undoubtedly, the coordinated acetylation or deacetylation of histones is required to access the target DNA and its transcription.

As described above, Ustilaginomycetes multicellularity can be induced in vitro by different kinds of stress or induced in vivo as a fundamental requirement for host plant infection and colonization. Regarding host plant infection, it is well known that mating of compatible sporidia induces filamentous growth in the fungus. In addition, it is well established that many phytopathogenic Ustilaginomycetes have a limited range of plant hosts. However, little is known about the plant compounds that induce the multicellular filamentous development for host plant colonization; or about the plant compounds sensed by Ustilaginomycetes to recognize potential hosts. It would also be important to increase our knowledge of how cell-cell communication occurs in the different multicellular forms developed by the Ustilaginomycetes. It is possible that the communication between cells is also carried out through the MAPK and cAMP/PKA signaling pathways.

There are many cellular processes involved in fungi multicellularity, and it is important to understand that multicellularity is a holistic phenomenon. Therefore, it is necessary to deal with the whole cell. Finally, taking into consideration the morphological plasticity presented by Ustilaginomycetes, the availability of their sequenced genomes, their short life cycle, and easy handling in the laboratory; in this work, we suggest the use of Ustilaginomycetes as model organisms for studying fungi multicellularity.

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