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Review

Metamorphosis of the Basidiomycota *Ustilago maydis*: Transformation of yeast-like cells into basidiocarps

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ABSTRACT

Ustilago maydis (DC) Cda., a phytopathogenic Basidiomycota, is the causal agent of corn smut. During its life cycle *U. maydis* alternates between a yeast-like, haploid nonpathogenic stage, and a filamentous, dikaryotic pathogenic form that invades the plant and induces tumor formation. As all the members of the Subphylum Ustilaginomycotina, *U. maydis* is unable to form basidiocarps, instead it produces teliospores within the tumors that germinate forming a septate basidium (phragmobasidium). We have now established conditions allowing a completely different developmental program of *U. maydis* when grown on solid medium containing auxins in dual cultures with maize embryogenic calli. Under these conditions *U. maydis* forms large hemi-spheroidal structures with all the morphological and structural characteristics of gastroid-type basidiocarps. These basidiocarps are made of three distinct hyphal layers, the most internal of which (hymenium) contains non-septate basidia (holobasidia) from which four basidiospores develop. In basidiocarps meiosis and genetic recombination occur, and meiotic products (basidiospores) segregate in a Mendelian fashion. These results are evidence of sexual cycle completion of an Ustilaginomycotina *in vitro*, and the demonstration that, besides its quasi-obligate biotrophic pathogenic mode of life, *U. maydis* possesses the genetic program to form basidiocarps as occurs in saprophytic Basidiomycota species.

Contents

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1. Introduction

Ustilago maydis (DC) Cda. is the causal agent of corn smut. During its life cycle, *U. maydis* displays three different cell types: (i) a saprophytic yeast-like, haploid, unicellular form that divides by budding, (ii) a filamentous (hyphal), dikaryotic, pathogenic form, the mating product of sexually compatible haploid cells that invades the host plant and induces tumor (sori) formation, and (iii) a diploid spore, the teliospore, formed within the tumors that germinates outside the host and undergoes meiosis to produce septate basidia (phragmobasidia) from which haploid basidiospores are borne (Holliday, 1974). *U. maydis* belongs to a fungal group (subphylum Ustilaginomycotina; James et al., 2006) that diverges from other Basidiomycota groups in not developing basidiocarps (Alexopoulos and Mims, 1979). Basidiocarps are the fruiting bodies of members of subphylum Agaricomycotina in which spore-producing hymenia are borne and form the typical mushroom-shaped structures commonly seen in nature (Moore, 2005).

Previously we described partially successful experiments devised to reproduce the sexual cycle of *U. maydis* outside its host by growing the fungus on permeable membranes placed over maize embryogenic calli. Under these conditions, mycelial growth of the fungus was induced, but a non-Mendelian segregation of meiotic products was observed (Ruiz-Herrera et al., 1999). In the present manuscript we describe improved growth conditions that allowed development of basidiocarps by *U. maydis*, their structure and morphology, and the occurrence of a complete sexual cycle *in vitro* of the fungus. These results constitute the first report of an Ustilaginomycotina completing its sexual cycle *in vitro* and forming basidiocarps.

2. Materials and methods

2.1. Strains and culture media

The following *U. maydis* strains were used: diploid strain Uid1 (*a1b1Δpan*/*a2b2Δodc::Hyg*^{r}) (Ruiz-Herrera et al., 1999), eight different haploid strains: parental strains FB1 (*a1b1*) and FB2 (*a2b2*), and auxotrophic mutant FB1-49 (*a1b1Δpan*) (Banuett and Herskowitz, 1989); auxotrophic mutant LG4 (*a2b2Δodc::Hyg*^{r}) (Guevara-Olvera et al., 1997), and Cl1(*a1b1Δodc*), Cl2(*a2b2Δpan*, Cl3(*a1b1ΔpanΔodc*), and Cl4 (*a2b2*), isolated in this work. Strains were maintained in 50% glycerol (v/v) at −70 °C, and were grown in liquid pH 7 minimal (MM) or complete (HCM) media (Holliday, 1974) for 20 h under shaking conditions. In some experiments media were added of 1 mM pantothenate, 5 mM putrescine and/or hygromycin (150 mg/L). MS medium (Murashige and Skoog, 1962) solidified with Gelrite and supplemented with different plant growth regulators and sucrose was used for the growth of calli and in dual experiments.

2.2. Embryogenic maize calli

Embryogenic maize calli were initiated from meristems derived from germinated mature seeds of the inbred line B73, cultured on solid MS medium (Murashige and Skoog, 1962) solidified with Gelrite (Sigma, St. Louis, MO) and supplemented with Dicamba, 0.002 mg per ml; adenine, 0.080 mg per ml; sucrose 3%; Gelrite, 2.5 mg per ml, and different plant growth regulators. Germinated seeds were incubated at 26 °C with 50 μmoles m^{-2} s^{-1} irradiance until embryogenic calli developed. These were propagated on the same culture medium under dark conditions at 27 °C.

Table 1
Primers used in PCR analysis of *U. maydis* mating genes.

Gene	Oligonucleotides (5′–3)	Size product (bp)	Tm (°C)
*Pra*1	F: TAGGCTCGTCTGCCCTTTATCGG	250	70
*Pra*1	R: GACTTCTCCGATTTCGTCGAGGT	250	70
Mfa-2	F: CAGGCTTCCAACAACGAGAACCG	250	67
Mfa-2	R: GAATCAAGTGGTCTCGACCTGCC	250	67
*bW*1	F: CCGAACCAACAGGCCCTCAGCCG	250	70
*bW*1	R: GTCGAGAACCATGTGCCAAGTC	250	70
*bW*2	F: CTAAAGGTGGGAGTGCGGTTG	250	66
*bW*2	R: TTCTTGGCAGTGGCGGTAGCATCG	250	66

2.3. Development of U. maydis basidiocarps

U. maydis cells (50 μl, 10^8/ml) were inoculated at variable distance from an embryogenic callus (50 mg fresh weight, see above) on plates of MS solid medium supplemented with auxins and cytokinins (see Section 3). Incubation proceeded at 27 °C, 10 μmoles m^{-2} s^{-1} irradiance and photoperiod of 16 h light and 8 h darkness. At intervals, diameter and fresh weight of the structures (basidiocarps) developed by *U. maydis* were measured, and some were recovered for microscopic observations.

2.4. Purification of DNA and PCR protocol

DNA was extracted using basically the protocol from Fujimura and Sakuma (1993).

PCR amplification was carried using fungal DNA, and specific primers of *Pra1, Mfa2, bW1*, and *bW2* genes (Table 1). The reaction mixture also contained 100 mM dNTPs, 2 mM $MgCl_2$ 1× PCR buffer (Gibco) and 2.5 U of DNA Taq polymerase (Invitrogen). After 30 cycles, products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and observed under ultraviolet light.

2.5. Microscopic observations

Fresh preparations were observed by bright field or by fluorescence microscopy after staining with Calcofluor white. Fixed samples were prepared overnight in FAA (5 mL 37% formaldehyde, 5 mL acetic acid, 90 mL 70% ethanol), dehydrated through an ethanol series (30–100%), followed by a histoclear series 25–100% at 4 °C. They were mounted in Paraplast™ for 3 days, and sections of 8 μm thickness were obtained with a microtome, and stained with Naphtol blue black–PAS (Clark, 1981). Observations were made in a Leica DMRB microscope, and photographed with a Spot camera. For electron microscopy, samples were fixed in paraformaldehyde–glutaraldehyde, postfixed with osmium tetroxide, dehydrated through an ethanol series 30–100%, twice with acetone, and mounted in Epoxy resin. Sections 60 nm in width were obtained with a RMC Mtx ultramicrotome, and contrasted with uranyl acetate (Hayat, 1998). Observations were made with a Jeol JM-1010 electron microscope at 80 kV.

2.6. Genetic recombination analyses

Basidiospores from samples of tissue of basidiocarps formed by the diploid strain Uid1 (*a1b1pan*/*a2b2Δodc*::Hyg^r) were recovered from the hymenium, homogenized in distilled water, purified by differential centrifugation, adequately diluted and inoculated on plates of HMC (Holliday, 1974) to obtain separate smooth colonies, indicating that they were haploid (Banuett and Herskowitz, 1989). Afterwards they were transferred to four different selective media according to the expected auxotrophies, and their phenotype was scored after 48 h. Mating type of *U. maydis* strains was determined by the "fuzz reaction" in charcoal containing MM (Banuett and Herskowitz, 1988) supplemented with panthotenate and/or putrescine, using tester strains of known mating type.