

Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*

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SUMMARY

Ustilago maydis is a dimorphic fungus with a yeast-like non-pathogenic form and a filamentous (hyphal) pathogenic form that induces tumor formation in maize. Within mature tumors, hyphae give rise to teliospores, which are round, diploid cells surrounded by a specialized cell wall. Here we describe the time course of fungal development in the plant with a focus on the morphological changes in the hyphae and the pathway of teliospore formation. We confirm and extend earlier observations that *U. maydis* hyphae branch extensively on the leaf surface and intracellularly before induction of tumors. We observe that at later stages the filaments undergo a series of discrete morphogenetic changes leading to teliospore formation. In particular, we show that the hyphae become embedded in a mucilaginous matrix within the tumor cells and the hyphal

tips become modified. The hyphae then undergo fragmentation to release individual cells that exhibit a variety of shapes on their way to becoming rounded. Finally, a specialized cell wall is deposited. Support for the existence of such a pathway comes from analysis of a mutant defective in the *fuz1* gene: inactivation of *fuz1* blocks production of the mucilaginous matrix and fragmentation of the hyphae, leading to a defect in teliospore formation. The different morphological changes that occur while in the plant but not in culture suggest that plant inputs play a key role in fungal development.

Key words: fungal dimorphism, teliospore formation, *U. maydis*, tumor induction

INTRODUCTION

The pathogenic form of the dimorphic fungus *U. maydis* is a dikaryotic filament (hypha), which consists of elongated cylindrical cells separated by septa. The non-pathogenic form is haploid and yeast-like; the cells are cigar-shaped and daughter cells arise by budding (reviewed by Christensen, 1963; Banuett, 1992). The transition from yeast to hyphal form entails several steps: formation of conjugation tubes by haploid cells of opposite mating type, fusion of cells to form a dikaryon, and finally filamentous (hyphal) growth of the dikaryon. This transition is regulated by two mating type loci, *a* and *b*, and requires that mating partners carry different alleles at both of these loci. The *a* locus encodes components of a pheromone response pathway, and the *b* locus encodes a homeodomain protein that appears to be a major regulator of genes for filamentous growth and for pathogenicity (reviewed by Banuett, 1992, 1995; Bölker et al., 1995; Kahman et al., 1995).

In nature, the mating process normally occurs on the leaf surface (see Holliday, 1961; Christensen, 1963), specifically on leaves of its two hosts, maize (*Zea mays*) and teosinte (*Zea mexicana*). Conjugation yields vigorously growing dikaryotic hyphae (Snetselaar and Mims, 1992; Snetselaar, 1993), which are branched (Knowles, 1898; Sleumer, 1932; Mills and Kotzé, 1981; Snetselaar and Mims, 1992), and which proliferate inter- and intracellularly leading to tumor induction on different plant

parts (Hanna, 1929; Christensen, 1931; Sleumer, 1932; Callow and Ling, 1973; Mills and Kotzé, 1981; Snetselaar and Mims, 1994). The dikaryotic hyphae appear to undergo nuclear fusion around the time of tumor formation (Sleumer, 1932; Ehrlich, 1958). Within the tumors, the fungus produces a specialized spore, the teliospore, which is competent to undergo meiosis to produce the yeast-like haploid form when it germinates. Many of the events observed in planta do not occur in culture: hyphal branching is absent, hyphae are short-lived, nuclear fusion is rare, and teliospores are not produced (Holliday, 1961; Day et al., 1971; Banuett and Herskowitz, 1989; 1994b). The mating process, which in culture can take place on a variety of media (Rowell and DeVay, 1954; Puhalla, 1968; Day and Anagnostakis, 1971; Snetselaar and Mims, 1992; Snetselaar, 1993; Banuett and Herskowitz, 1994b), appears to be similar both in culture and in planta (Snetselaar and Mims, 1992; Snetselaar, 1993; Banuett and Herskowitz, 1994b). The differences in fungal behavior in planta and in culture have led us to propose (Banuett, 1992; Banuett and Herskowitz, 1994a) that the plant provides some signal that triggers different aspects of fungal development.

The formation of teliospores within tumor cells entails a morphological change from cylindrical hyphal cells to rounded teliospores. Although several different ways by which this change might occur can be imagined, earlier studies (Lutman, 1910; Rawitscher, 1912; 1922; Christensen, 1931; Sleumer,

1932) indicate that teliospores form by a process of hyphal fragmentation. These studies described the occurrence of hyphal fragments that undergo morphological changes before the production of teliospores (see for example, Rawitscher, 1922; Sleumer, 1932; Christensen, 1931).

In the present study we have carried out a time course of hyphal development and teliospore formation in the plant. Our goal was two-fold. First, we wished to reexamine fungal development in planta after infection with haploid strains having different *a* and *b* alleles using light microscopy. Our observations confirm, expand, and clarify several aspects of fungal growth in planta. Second, these observations on development of dikaryons provide a reference for analysis of the genetic control of fungal development. In particular, we show that the presence of different *a* alleles is not required for hyphal growth in planta and that the *fuz1* gene is required for progression through the proposed pathway of teliospore formation.

MATERIALS AND METHODS

Strains used

The strains used were: FBD12 = *a1/a2 b1/b2*; FBD11-7 = *a1/a1 b1/b2*; FB1 = *a1 b1*; FB2 = *a2 b2*; FB12-3 = *a1/a2 b1/b1* (described by Banuett and Herskowitz, 1989) and FB10-1i = *a1 b1 fuz1⁻*; FB10-3c = *a2 b2 fuz1⁻* (described by Banuett, 1991). Strain FD12-3 (*a1/a2 b1/b1*) does not form filaments in culture or in planta (F. Banuett, unpublished) and served as our negative control.

Media and growth conditions of *Ustilago maydis* strains

Strains were grown in UMC broth (Holliday, 1974) till saturation (approx. 2×10^8 cells/ml). Haploids were mixed in equal amounts just before inoculation and 0.1-0.2 ml of the mixture was injected at the base of each seedling with a 1 ml syringe with a 26½ gauge needle. Diploids were inoculated as pure cultures, using 0.1-0.2 ml of saturated culture for each inoculation.

Plant growth and inoculation conditions

Maize variety B164 was grown in a Conviron chamber under a 14 hour ramp cycle with maximum day temperature of 28°C/10 hour 20°C constant night temperature. Plants were inoculated 5 days after germination and observations were begun 24 hours postinoculation and continued for 15 days, when the tumors were full of teliospores.

Seedlings used for inoculations were chosen to be at the same developmental stage, that is, containing three to four partially unrolled leaves. At least 25 seedlings were inoculated at the same time for each set of strains whose development was to be studied. These procedures were repeated on three separate occasions.

Microscopic observations and photomicroscopy

The surface of the leaf was peeled (epidermal peel) with the use of a sharp razor blade and forceps. These strips usually consist of a single cell layer, which facilitates microscopic observation. Other deeper layers of the leaf were obtained as free-hand sections using a razor blade; these preparations can be one to a few layers thick.

The epidermal peels or sections were not fixed. They were placed in a drop of water or in a drop of Fungifluor (Polysciences) immediately before microscopic observation. Fungifluor specifically stains fungal cell walls, although some background staining of the plant cell walls can occur. Fungal cell walls stained with Fungifluor emit bright fluorescence when excited with ultraviolet light (UV).

DAPI (4',6-diamidino-2-phenylindole) staining was performed immediately before observation. 5 µl of the dye solution (1 µg/ml) were applied to the plant tissue preparation which had been placed in a drop of water. This method of staining the nuclei did not work in

preparations taken very early in infection when the hyphae are mostly on the leaf surface. In this case we observed shrinkage of the hyphae.

Sections of plant tissue or epidermal peels mounted in water or Fungifluor solution on a microscope slide with a coverslip were squashed by gently pressing with the thumb through several layers of paper towels. Squashes allowed better observation of material embedded in the mucilaginous matrix.

Microscopic observations were carried out using a Zeiss Axiophot microscope equipped with an MC100 camera or a Polaroid camera. For photomicroscopy we used 35 mm Ektachrome EPJ 320T or EPT 160T color film, or 35 mm Kodak TMX 100, 400, PX 125 film, or polaroid 557 (for 4×5 photographs) black and white film. Ektachrome 25 or 64 color film was used for macrophotography of plants.

RESULTS

To learn about hyphal development and teliospore formation, we carried out a light microscopic analysis of infected plants beginning 1 day after inoculation and continuing for 15 days until the tumors appeared dark brown or black, which signals the completion of teliospore formation.

We carried out four sets of infections of 5-day-old maize seedlings (see Materials and Methods). The first set of infections was carried out with a mixture of wild-type haploid strains that carry different *a* and *b* alleles (*a1 b1* + *a2 b2*) to produce a dikaryotic infection. These infections were compared with infections by haploid strains defective in the *fuz1* gene (*a1 b1 fuz1⁻* + *a2 b2 fuz1⁻*). The third set of infections was carried out with diploid strains that are heterozygous at both *a* and *b* (*a1/a2 b1/b2*). These infections were compared with diploid strains that are homozygous at *a* and heterozygous at *b* (*a1/a1 b1/b2*).

Early stages of hyphal growth in planta are characterized by extensive branching

Early symptoms of infection: chlorosis

Twenty-four hours after co-inoculation with wild-type haploid strains, we detected very small chlorotic spots (areas of yellowing) on the leaf sheath and leaf blade near the area where the inoculum was injected. The spots, although small at this stage, were very obvious when excised leaves or whole seedlings were examined with a dissecting microscope. Induction of chlorosis is known to be one of the earliest detectable symptoms of *U. maydis* infection (reviewed by Christensen, 1963). Two to three days after infection, chlorosis becomes very extensive and is readily seen by the naked eye (Fig. 1A). The epidermal layer around the chlorotic spots was peeled off and examined under the microscope as described in Materials and Methods, on the assumption that the fungus would be found in or near the chlorotic areas. This expectation was based on previous observations that strains causing tumor induction induce a strong chlorotic response at positions where tumors later develop (F. Banuett, unpublished).

Hyphal morphology: branch primordia, branching, and collapse

Observations of peeled epidermal layers from the chlorotic areas 24 hours after inoculation revealed clusters of yeast-like cells on the epidermal surface (Fig. 1B, C). An appendage can be seen arising at one of the cell poles of some of the yeast-like cells (Fig. 1B) that is similar in appearance to the conjugation tubes observed when haploid strains are co-cultured in

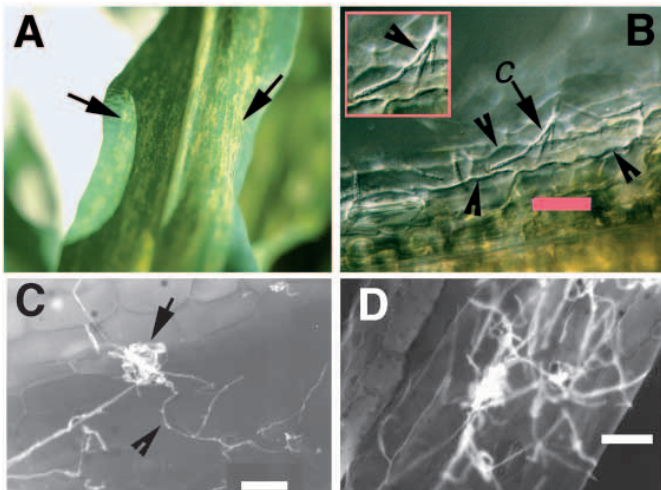


Fig. 1. Early stages of infection. (A) Chlorosis is a yellowing of the green tissue of the leaf (arrows). The leaf shown here was photographed 2.5 days after inoculation with *a1 b1* and *a2 b2* strains. Callow and Ling (1973) showed that there is loss of chloroplasts in the chlorotic region but the reason for this loss is unknown. Chlorosis can be induced by both pathogenic and non-pathogenic strains, though in the latter case the response is not as strong (Christensen, 1963). (B) During the first 24 hours after inoculation, the yeast-like cells attach to the leaf surface in clusters. Conjugation tubes (c) are seen arising from the yeast-like cells (Nomarski optics) and have been shown to mediate cell fusion (Snetselaar and Mims, 1993; Banuett and Herskowitz, 1994b). The fungal cells observed here are at the edge of a horizontal epidermal peel. The inset shows a larger magnification of one of the yeast-like cells with an emerging conjugation tube. Arrowheads mark the boundaries of one epidermal cell. (C) A hypha (arrowhead) is seen arising from a cluster of yeast-like cells (arrow). The outlines of the cells of the epidermal layer are clearly visible in the upper part of the figure. (D) Filamentous network on the leaf surface observed 2 days after inoculation. Epifluorescence optics was used to visualize the hyphae in C and D in peels which had been treated with Fungifluor solution immediately before observation. Scale bar for B-D, 50 μm .

liquid medium or water agar (Snetselaar and Mims, 1992; Snetselaar, 1993; Banuett and Herskowitz, 1994a,b). Formation of these conjugation tubes in the plant has also been documented by Snetselaar and Mims (1992).

By the second day postinoculation, there is an extensive network of filaments on the leaf epidermal surface (Fig. 1D). This network spreads out from the clusters of yeast-like cells, although it is difficult to trace the origin of a given filament to a pair of haploid cells. The process of conjugation tube formation and production of dikaryotic hyphae is not synchronous; clusters of yeast-like cells in various stages of hyphal production are observed during the first 4 days after infection. Thus, in some areas the filament network is more extensive than in others. From the second to the fourth day after inoculation some filaments cluster around the stomata and in some cases penetrate through them (Fig. 2A). Mills and Kotzé (1981) have also reported penetration through stomata.

Branch primordia appear as bumps at irregular intervals on the surface of the hypha and arise at various angles to the main hypha (Fig. 2B,C). A Y-shaped septum separates the hyphal branch from the adjacent cells (Fig. 2D,F). Some branches appear to lack a septum separating them from the main axis (as

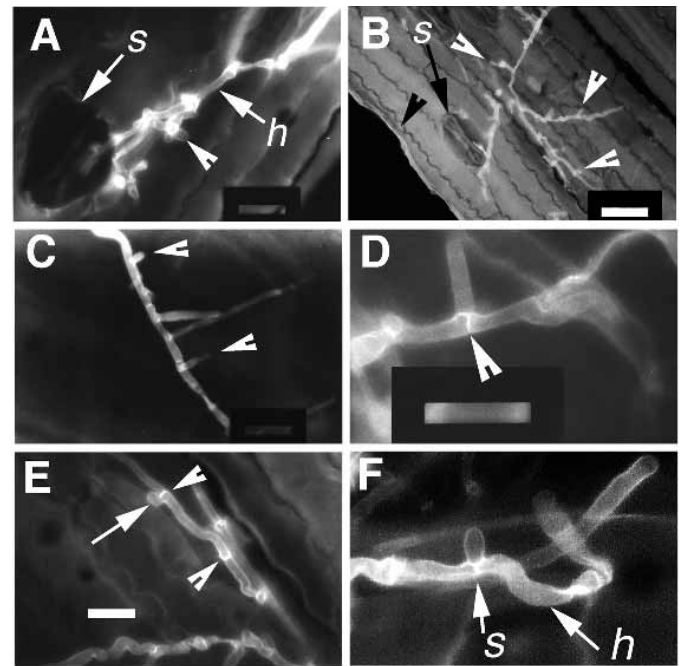


Fig. 2. Hyphal branching. (A) A hypha (h) is seen penetrating a stoma (s) in an epidermal peel. The tip of the penetrating hypha is out of the focal plane. The arrowhead points to a cluster of branches in the hypha. The boundaries of some of the epidermal cells in this horizontal strip can be seen in the bottom right side of the panel. (B) Branch primordia (white arrowheads) are visible in this hypha on the epidermal surface. The cell walls of the epidermal cells are clearly visible here (black arrowhead). A stoma (s) is indicated (black arrow). (C) Branches arise at irregular intervals as shown here (arrowheads). (D) A Y-shaped septum (arrowhead) separates the branch from the main hyphal axis. (E) Cross-walls (arrowheads) separate the long cylindrical cells of the hypha. The arrow points to an emerging branch which shows no Y-septum separating it from the main hypha. (F) A Y-shaped septum (s) separates the emerging branch from the main hypha (h). Compare with the branch in E. Epifluorescence optics was used in all panels to visualize hyphae in epidermal peels treated with Fungifluor. Scale bars, 20 μm in A, C-F; 50 μm in B.

in Fig. 2E); a septum will presumably develop later. The appearance of these branch primordia and their accompanying septa resemble in some respects the clamp connections of other Basidiomycetes (see Discussion). Branching may play an important role in invasion as each new hyphal tip provides a new opportunity for penetration. Cross-walls (septa) at right angles to the main axis of the hypha separate the long cylindrical cells that make up the filaments (Fig. 2E).

Induction of anthocyanin streaking can be observed as early as 2-3 days after infection, although it is more common to see streaks later, after the tumors are formed (5-6 days after infection). Fig. 3A shows anthocyanin streaking before the appearance of tumors. Epidermal peels taken from such pigmented areas revealed cells with anthocyanin and no detectable fungus (Fig. 3B). Similar observations were reported by Hanna (1929), who demonstrated by chemical analysis that this pigment is anthocyanin.

Three to four days postinoculation, the septate hyphae appear to be inside the plant cells (Fig. 4), where they also branch extensively (Fig. 4A,B). Our criteria for concluding that

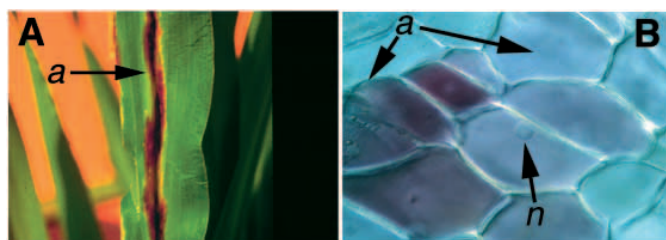


Fig. 3. Anthocyanin streaking. (A) The leaf shows a streak of pigment (arrow) along the midvein and before any tumors are detectable. The leaf shown here was photographed 2.5 days after inoculation. a, anthocyanin. (B) An epidermal peel taken from the pigmented area of the leaf in A shows cells with the characteristic purple-reddish color of the water soluble anthocyanins. No fungus is visible in these cells. a, anthocyanin; n, host cell nucleus.

the hyphae are inside the plant cells are two-fold. First, in epidermal peels consisting of a single cell layer in which single plant cells have been ruptured, hyphae can be seen to be partly outside the ruptured host cell and partly inside it (Fig. 4C). Secondly, the pathway of a single hypha can be followed in peels consisting of a single cell layer by focusing up and down. Such analysis reveals that individual hyphae thread their way from cell to cell and within cells (Fig. 4D). Electron microscopic micrographs (Snetselaar and Mims, 1994) have clearly shown that *U. maydis* hyphae grow intracellularly, but in contrast with many pathogenic fungi, the intracellular growth exhibited by *U. maydis* is characterized by penetration of the cell wall without disruption of the host cell plasma membrane. The hyphae appear to be closely apposed to the host plasma membrane (Snetselaar and Mims, 1994).

At this stage, some hyphae and their branches are full of cytoplasm (Fig. 4D) whereas others are 'vacuolate' or empty (Fig. 4E). (We do not know if these vacuolate compartments consist mostly of a large vacuole with little cytoplasm or if they are totally devoid of cytoplasm). The vacuolate compartments are in many instances collapsed and appear as threads attached to the rest of the hypha (Fig. 4F). The hyphae thus consist of thick segments joined to thin threads (Fig. 4F); in some cases this arrangement alternates and in other cases most of the hypha is completely collapsed (data not shown). Hyphal collapse is also observed in culture (Day and Anagnostakis, 1971; Snetselaar and Mims, 1992; Snetselaar, 1993; Banuett and Herskowitz, 1994b); where, except for the tip cell, most of the hyphae lack cytoplasm.

Appearance of tumors

By the fifth day postinoculation, very small bumps on the leaf blade surface can be detected when the leaf is rubbed between the fingers. Examination of such areas with the dissecting microscope confirms the presence of very small tumors. In some cases, small tumors can be detected at this stage with the naked eye. Factors such as humidity, temperature, and light intensity affect the time at which tumors form (F. Banuett, unpublished). Even though our experiments are carried out under controlled conditions (see Materials and Methods), slight fluctuations of these parameters may occur from day to day.

Tumors can form more or less uniformly on the leaf blade (Fig. 5A) or as clusters on the midrib (Fig. 5B,C). Tumors arising later usually form near the soil line (i.e., at the base of

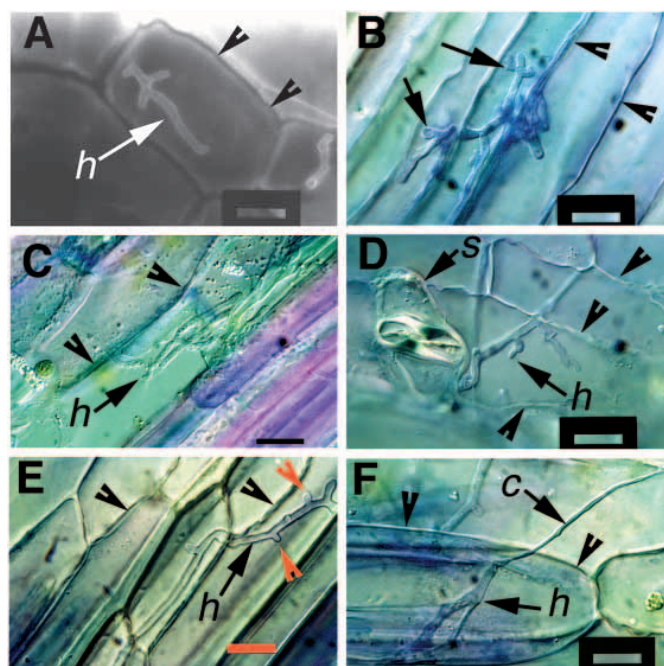


Fig. 4. Hyphal branching within plant cells and hyphal collapse. (A) A hypha (h) with branches is seen inside a plant cell using epifluorescence optics. The black arrowheads point to the cell wall of the host cell. (B) A branching hypha (arrows) is clearly seen spanning three different epidermal cells. Arrowheads point to cell walls of two plant cells. (C) A plant cell ruptured during preparation of a single cell layer epidermal peel shows hyphae (h) partially outside and partially inside the plant cell. Arrowheads point to the cell wall of one plant cell. (D) A single hypha (h) with branches is seen traversing two different cells near a stoma (s). Other branches of this hypha are out of the focal plane. The arrowheads indicate cell walls of three adjacent plant cells. (E) A vacuolate hypha (h) shows clearly the pattern of branching (orange arrowheads) and also the hypha traversing from one cell to another. Black arrowheads point to the cell walls of two non-adjacent plant cells. (F) Part of a hypha has collapsed (c) in one host cell whereas the rest of the hypha (h) in another host cell clearly shows cytoplasmic content. Arrowhead points to the cell wall of one plant cell. Nomarski optics was used for B-F; epifluorescence optics for A. Scale bar, 20 μ m in A,B,D-F; 50 μ m in C.

the plant (Fig. 5D). A single plant may show different distributions of tumors on different leaves (F. Banuett, unpublished). Examination of epidermal peels and free-hand sections of the tumors and the surrounding area (see Materials and Methods) revealed an increase in hyphal branching within the tumor cells (data not shown). The host cells in the tumorous area are larger than those in the surrounding areas, as has been documented by others (see Callow and Ling, 1973).

Seven to eight days postinoculation, branching is profuse and occurs at closer intervals (Fig. 6A), particularly near or at the tip of the hypha, and the branches are short. These changes signal the beginning of teliospore formation.

Teliospore formation involves a series of morphological changes in the hyphae

Mucilaginous material, lobed tips and hyphal fragmentation

Nine days postinoculation, the fungal hyphae appear to be sur-

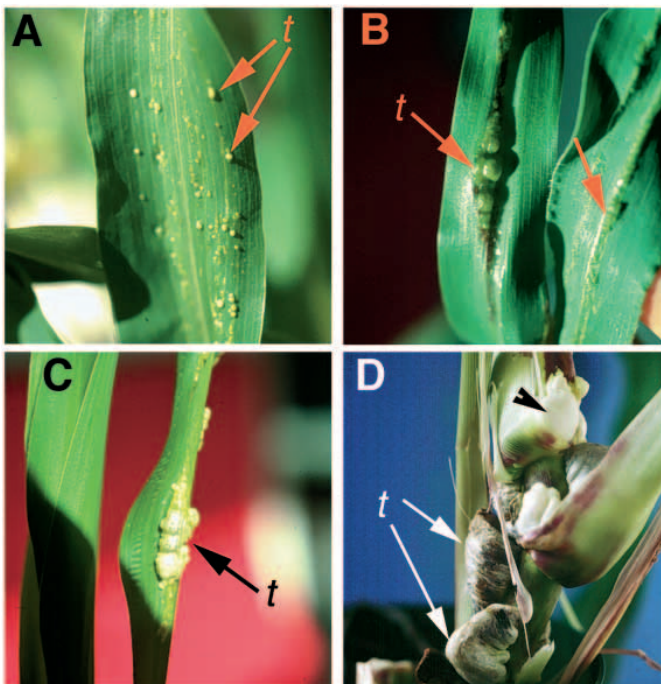


Fig. 5. Tumors on leaf surface. (A) This leaf from a plant 6 days after inoculation shows uniform distribution of small tumors (arrows) on the leaf blade. (B,C) The leaves show clustering of tumors (arrows) along the midrib of the leaf blade. The tumors can be green with specks of anthocyanin, as in B, or they can be white, as in C. The leaves shown here were from plants inoculated 6.5 days earlier. (D) Large tumors (2.5–3 cm in diameter) near the base of the plant are shown here. These tumors arose 3–4 days later than those on the leaves. Tumors with developing teliospores are black (white arrows) due to the dark-brown color of the teliospore cell wall. White tumors (black arrowhead) have not yet started teliospore formation. The tumors shown here were photographed 17 days after inoculation with *al b1 + a2 b2* haploids. t, tumors.

rounded by mucilaginous material. Hyphal outlines become indistinct at this time (Fig. 6B–D), and the hyphae appear bloated and tend to stick together even when squeezed out of the plant cells. The hyphae are no longer straight but twist around each other within the host cells (data not shown). The tip region of many hyphae has a bulbous or lobed appearance (Fig. 6E,F), which may be a consequence of the extensive formation of short branches first observed approximately 8 days postinoculation. Fragmentation of fungal hyphae into segments of one to several elongated cells is also apparent at this stage (Fig. 7A,B). The tumor cells with fungal fragments look like sacs full of worms (Fig. 7C,D). It is clear from these preparations that hyphal fragmentation takes place inside the tumor cells rather than in between cells as reported by others (Mills and Kotzé, 1981; Snetselaar and Mims, 1994). Further evidence that this process occurs intracellularly is that squashes of the plant cells clearly show the fragments embedded in the mucilaginous material emerging from inside the cells (Fig. 7D).

Several explanations could account for the differences between our observations and those by other investigators. The fixation procedures used in the analysis by Snetselaar and Mims (1994) might have caused breakage of the host tumor

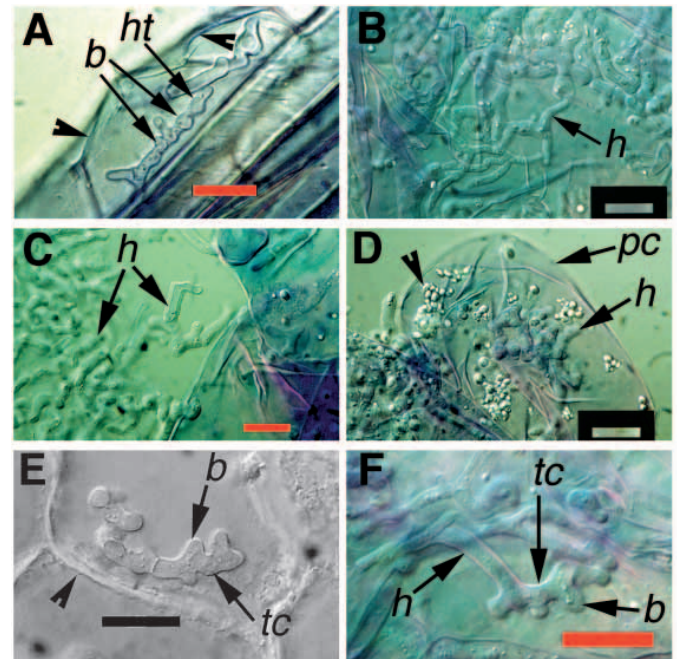


Fig. 6. Mucilaginous matrix and lobed hyphal tips. (A) Branching occurs at closer intervals near the hyphal tip (ht). The branches (b) appear as round bumps because they are emerging towards the plane of focus. The arrowhead points to the cell wall of a plant cell. (B,C) Bloated hyphae (h) with indistinct borders due to the presence of a mucilaginous matrix are observed here. (D) A single tumor cell (pc) with bloated, twisted hyphae (h) is seen here. The white granules (arrowhead) are most likely starch granules. (E) A single tumor cell showing the tip cell (tc) of a hypha. Note the multiple bumps (b) or branches arising from the single tip cell. These modified tips appear approximately 7–8 days postinoculation. The arrowhead points to the wall of the plant cell. (F) A modified hyphal tip (tc) with multiple short branches (b) is shown here. h, hypha. Nomarski optics was used for A–F. The plant tissue sections in all panels were treated with Fungifluor solution. Scale bars, 20 μ m in all panels.

cells containing the maturing teliospores. Material used in our studies was not fixed. Another possibility is that as hyphal fragmentation increases, the tumor cells burst open because of internal pressure due to the mucilaginous matrix and the fragmenting hyphae, thereby releasing the fungal material from the host cell. Perhaps Snetselaar and Mims (1994) examined infected cells after plant cell rupture whereas we examined tumors prior to rupture.

At ten days postinoculation, we observe a continuation of the hyphal fragmentation process and an increase in the lobed appearance of hyphal tips. DAPI staining of fragmented or intact hyphae reveals a single nucleus per cell (Fig. 7E), although it is difficult to assess the boundaries of a cell critically. We presume that karyogamy has taken place in these cells that have a single nucleus (see Discussion). Some tip cells or the short branches arising near the tip cells are rounded (Fig. 7F).

Rounding of cells, cell wall deposition and sculpting

At 11 days postinoculation, large masses of rounded cells and also of triangular, almond-shaped, peanut-shaped, and cells with other unusual morphologies are present (Fig. 8A–C).

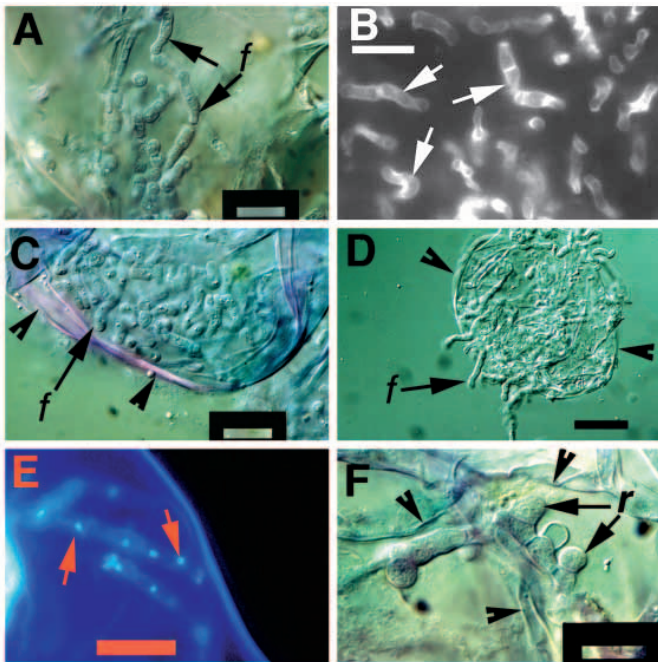


Fig. 7. Hyphal fragmentation and 'worm-like' stage. (A) Fragmentation of hyphae (arrows) becomes evident at approximately 9 days postinoculation. (B) The hyphae fragment into segments of two to several cells (arrows) clearly shown here using epifluorescence optics of sections treated with Fungifluor. (C) A single plant tumor cell (arrowheads point to cell wall) full of hyphal fragments (f) gives the appearance of a sac full of worms, hence the designation 'worm-like' stage. See also D. (D) Single tumor cell that was squashed to show fragments (f) emerging from inside the plant cell. Arrowheads mark the plant cell wall. (E) The fragmented or intact hyphae at this stage contain a single nucleus per cell as indicated by the arrows. (F) Some of the modified tips in the fragments (Fig. 6E,F) contain some cells in the process of rounding (r). Arrowheads mark the cell wall of adjacent plant cells. Nomarski optics was used for A,C,D,F; epifluorescence optics for B and E. Scale bars, 20 μm .

These cells are all embedded in the mucilaginous material (Fig. 8D) and it is nearly impossible to dislodge the cells from this material after squashing the preparation. A single nucleus per cell is revealed by DAPI staining of these masses of cells (data not shown), but not all nuclei stain with DAPI, probably due to interference by the mucilaginous material or perhaps due to changes in permeability of the developing spore wall.

13-14 days postinoculation, the number of cells undergoing rounding has increased. In other parts of the tumor, hyphae in the process of fragmentation, rounding of cells, and lobing are clearly visible. Many rounded cells appear to be in various stages of spore wall maturation, with mature teliospores clearly visible in many of the enlarged host cells (Fig. 8C-E). The teliospore cell wall is echinulate and yellow-brown at first, later becoming dark brown (Fig. 8F). Thus the tumors acquire a dark coloration (Fig. 5D) as masses of teliospores mature inside them. Among the rounded cells, very thin elongated cells are observed (data not shown). It is not clear if these cells are dying or if they later enlarge and round up.

Development of diploids in planta

Diploids are not a natural form in the life cycle of *U. maydis*

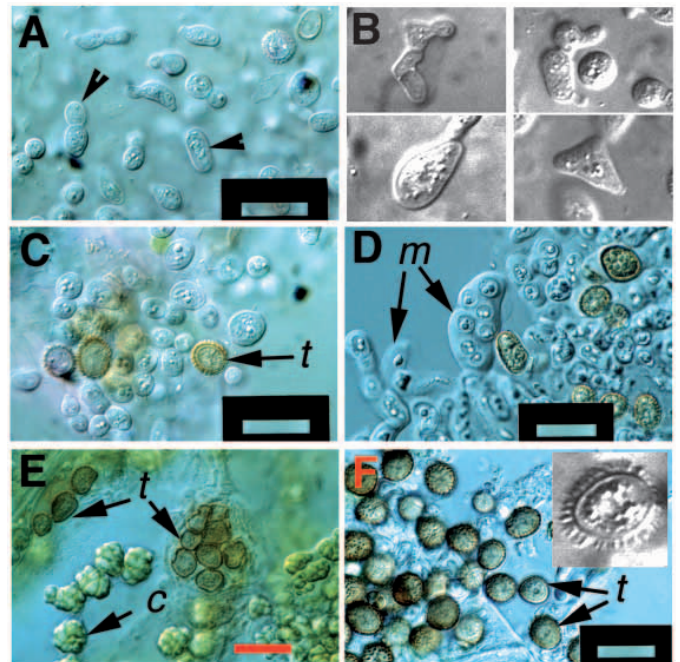


Fig. 8. Different morphologies of cells forming teliospores. (A) Cells that are in the process of forming teliospores exhibit a variety of morphologies, two of which are indicated by the arrowheads. (B) The four different panels show a high magnification of individual cells with triangular, ellipsoidal, and other morphologies. (C) Immature teliospores (t) can be easily discerned because of their yellow color and characteristics of the cell wall. (D) The thick mucilage (m) seen here interferes with observation of the morphological transitions seen in A-C. Treatment with Fungifluor (as in A-C) somehow facilitates observation with Nomarski optics. The preparation in this panel was not treated with Fungifluor. (E) A cluster of maturing teliospores (t) among clusters of abnormal looking chloroplasts (c). (F) Mature teliospores (t) with echinulate cell wall are shown here. The inset shows a higher magnification of a single mature teliospore. Nomarski optics was used for all panels. Scale bars, 20 μm in all panels except B, in which the cells are enlargements of single cells from photographs like those in A and C.

but can be constructed in the laboratory by selection for prototrophy of complementing auxotrophic markers (Holliday, 1974; Banuett and Herskowitz, 1989; 1994a). Diploids heterozygous at *a* and *b* can form yeast-like or mycelial (hyphal) colonies, depending on the culture conditions (Banuett and Herskowitz, 1994b; Ruiz-Herrera et al., 1995). A pure culture of such a strain induces tumor formation (and is said to be solopathogenic) and produces teliospores (Holliday, 1961; Day et al., 1971; Banuett and Herskowitz, 1989).

We showed previously that diploid strains homozygous for *a* but heterozygous for *b* do not form filaments on charcoal agar, indicating that different *a* alleles are necessary for filamentous growth in culture. These strains, nonetheless, induce tumors and form teliospores when pure cultures are used to inoculate plants (Banuett and Herskowitz, 1989). Thus the presence of different *a* alleles is not necessary for tumor induction. The ability of *a1/a1 b1/b2* strains to induce tumors would be an apparent break from the correlation between filamentous growth in culture and tumor induction in planta if the yeast-like form of these strains does indeed induce tumors.

However, the *a1/a1 b1/b2* strain could form filaments in planta, in which case the correlation between filamentous growth and tumor induction would still be maintained.

In order to distinguish between these two possibilities, we inoculated plants with pure cultures of the *a1/a1 b1/b2* diploid strain and followed the time course of infection and teliospore formation for approximately 15 days. Parallel observations were made of plants inoculated with the isogenic diploid strain *a1/a2 b1/b2*.

Development of *a1/a2 b1/b2* diploids

We observed that the time course of infection by the *a1/a2 b1/b2* diploid was identical to that resulting from co-inoculation of seedlings with *a1 b1* and *a2 b2* haploid strains: no differences were detected between dikaryotic and diploid hyphae with respect to hyphal branching, collapse and growth within host cells (data not shown). Subsequent fragmentation of diploid hyphae and rounding of cells occurred in identical manner to that of dikaryotic hyphae. DAPI staining of the hyphae revealed the presence of a single nucleus in a given hyphal compartment.

Thus, diploid hyphae undergo the same developmental program as dikaryotic hyphae. Our observations thus support Hanna (1929), who reported that teliospores were formed in the same time course whether inoculations were done with paired haploid cells or with solopathogenic diploids.

Development of *a1/a1 b1/b2* diploids

To our surprise, the *a1/a1 b1/b2* diploid developed as vigorous a hyphal network as the *a1/a2 b1/b2* diploid. Hyphae arose from the yeast-like cells, just as they do in the *a1/a2 b1/b2* diploid. The hyphae rapidly branched and spread on the surface of the epidermis (Fig. 9A) and also within host cells, in the same manner as hyphae from the *a1/a2 b1/b2* diploid or the dikaryon (data not shown). These observations indicate that different *a* alleles are not necessary for maintenance of filamentous growth in planta. An intriguing possibility is that the plant produces a signal that substitutes for the pheromones ordinarily coded by the *a* locus (see Discussion).

The time course of subsequent steps in hyphal development into teliospores – hyphal fragmentation, rounding of cells, and wall deposition – were again indistinguishable from the *a1/a2 b1/b2* diploid or the dikaryon (data not shown).

Development of *fuz1*⁻ mutants in planta

fuz1⁻ mutants were isolated as strains defective in filament formation when mated with strains of opposite mating type on solid charcoal agar (Fig. 9B) (Banuett, 1991). Inoculation of plants with *fuz1*⁻ strains carrying different *a* and *b* alleles (*a1 b1 fuz1*⁻ + *a2 b2 fuz1*⁻) results in formation of small tumors which do not produce teliospores (Banuett, 1991). In order to determine whether *fuz1*⁻ strains exhibit filamentous growth in the plant and to determine the step blocked in teliospore formation, we co-inoculated seedlings with *a1 b1 fuz1*⁻ and *a2 b2 fuz1*⁻ strains and followed the time course of infection in parallel with plants inoculated with wild-type *a1 b1* and *a2 b2* strains.

Formation of hyphae by *fuz1*⁻ strains

Co-inoculation of *fuz1*⁻ strains, unexpectedly, led to the formation of hyphae. These hyphae, as in the case of the wild-

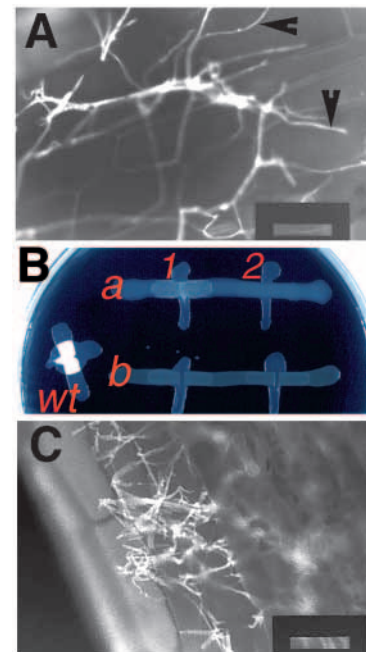


Fig. 9. *a1/a1 b1/b2* diploids and *fuz1*⁻/*fuz1*⁻ dikaryons form hyphae in planta. (A) The diploid *a1/a1 b1/b2* produces a network of filaments (arrowheads) on the leaf surface (compare with Fig. 1D) as seen here with epifluorescence optics. The cell walls of the plant cells can be seen in the background. Scale bar, 50 μ m. (B) *fuz1*⁻ strains result in reduced filament formation on charcoal agar when cross-streaked with a wild-type strain (top horizontal line), and produce no-filaments when cross-streaked against a *fuz1*⁻ strain (bottom horizontal line). Strains used for the reaction in the top horizontal line: a, *a1 b1 fuz1*⁻ versus 1, *a2 b2*, and 2, *a1 b1*. For the reaction in the bottom horizontal line the strains were: b, *a1 b1 fuz1*⁻ versus 1, *a2 b2 fuz1*⁻, and 2, *a1 b1*. wt, *a1 b1* vs *a2 b2*. The plate was sealed with Parafilm and incubated at room temperature for 24 hours before photographing. (C) Network of filaments on epidermal layer produced by inoculation of plants with *a1 b1 fuz1*⁻ + *a2 b2 fuz1*⁻ strains and visualized with epifluorescence optics. Scale: 50 μ m.

type haploid strains, arose from clusters of yeast-like cells. The hyphae spread on the epidermal layer (Fig. 9C), and branch primordia were observed as with co-inoculation of wild-type haploids (data not shown). The hyphae are septate, and the septum separating a branch is Y-shaped (data not shown). These hyphae penetrate host cells, branch extensively (Fig. 10), and traverse from one host cell to another. Collapse of hyphal segments was also evident as hyphal growth progressed (data not shown). Tumors appear at approximately the same time as in co-inoculations with wild-type strains, although the *fuz1*⁻ tumors are smaller. These observations indicate that *fuz1*⁻ mutants form hyphae that are indistinguishable from those of wild-type haploid strains and that these hyphae are competent to form tumors, albeit of reduced size. Thus, the *fuz1* gene, although necessary for filamentous growth in culture, is not necessary for filamentous growth in planta.

Inoculations with a mixture of wild-type haploid strains leads to development of large tumors on the leaf sheath and at the base of the plant and smaller tumors on the leaf blade. In such infections, the leaves in the leaf whorl remain rolled instead of unrolling as occurs in uninfected leaves (data not

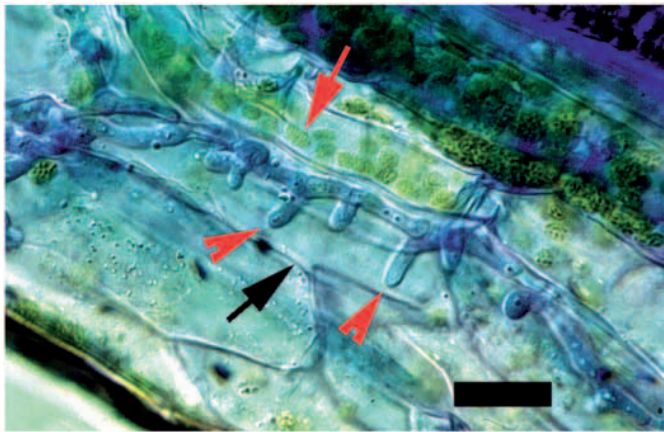


Fig. 10. Branching of *fuz1*⁻ dikaryotic hyphae. The hyphae of *fuz1*⁻ dikaryons exhibit the same type of branching as wild-type dikaryons. This section was obtained from plants 6 days after inoculation and visualized with Nomarski optics. Red arrowheads point to some of the branches. The black arrow points to a plant cell wall; the red arrow points to chloroplasts (green).

shown). In severe infections, water transport is blocked, leading eventually to severe wilting and death of the seedling. In contrast, inoculations with *fuz1*⁻ mutants did not induce such severe symptoms: leaves tend to unroll and the tumors are smaller than those in wild-type inoculations (data not shown). Because *fuz1*⁻ mutants form filaments in planta, we hypothesize that their reduced tumor response may indicate that *fuz1* plays a direct role in tumorigenesis.

Failure of developmental transitions in *fuz1*⁻ mutants

At 9-10 days postinoculation, when the dikaryotic hyphae produced by wild-type strains become embedded in the mucilaginous material, the hyphae produced by *fuz1*⁻ strains appear abnormal and there is little or no production of the mucilaginous material (compare Figs 11A,B with 6B,C). The *fuz1*⁻ hyphae fan out at the junction with the plant cell wall (Fig. 11A,B); in many instances the hyphae appear ruptured (Fig. 11C); other hyphae appear to have cross-striations (Fig. 11D). The ends of broken hyphae bulge out and form cytoplasmic projections (Fig. 11E). We also observed unbroken hyphae with large swellings in the middle that have cytoplasmic projections (Fig. 11F) similar to those seen in ruptured hyphae. The *fuz1*⁻ hyphae form a network of abnormal-looking filaments traversing from one host cell to another (data not shown), but this network is less extensive than that observed in wild-type infections at the same time point.

These observations indicate that *fuz1*⁻ mutants develop normally up to the point at which wild-type hyphae become embedded in the mucilaginous matrix and begin the process of fragmentation. Instead of observing these forms, aberrant hyphae without mucilaginous material are produced.

Twelve to fourteen days postinoculation, the tumors are whitish and have the appearance of a honeycomb under the dissecting microscope (data not shown). In contrast, tumors from wild-type inoculations contain pockets of yellow and yellow-brown coloration which represent sites of teliospore formation (data not shown).

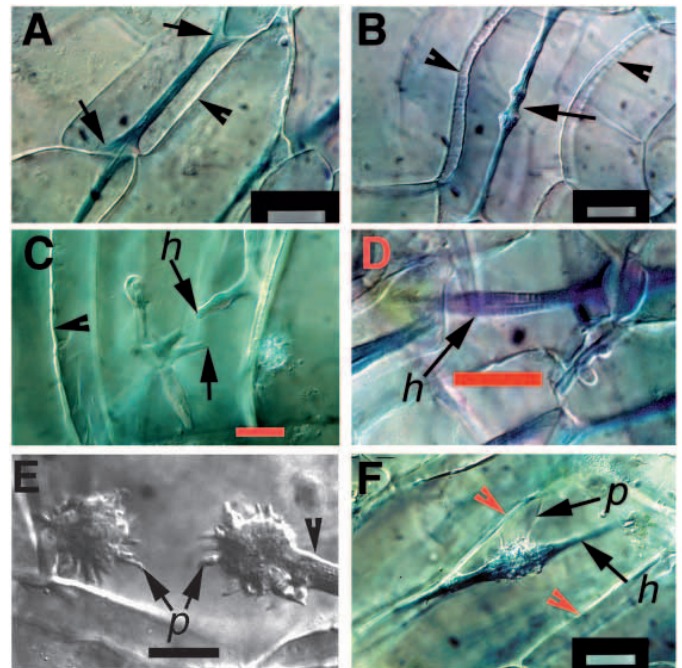


Fig. 11. *fuz1*⁻ dikaryotic hyphae fail to develop further. (A) The *fuz1*⁻ dikaryotic hyphae look abnormal; here it can be seen how they fan out (arrows) at the junction with the host cell wall. The arrowhead points to a plant cell wall. (B) A hypha traverses the length of a single plant cell. Note the swollen parts (arrow) in the middle of the hypha. The arrowheads point to plant cell walls. (C) A hypha appears to have ruptured (arrows). The arrowhead points to the plant cell wall. (D) The hypha (h) shown here exhibits cross-striations, which are not observed in wild-type hyphae. (E) Cytoplasmic projections (p) arise from the swollen ends of a broken hypha. The arrowhead points to the hypha in the vicinity of the swollen end. (F) Cytoplasmic projections (p) are also seen arising from the swollen middle part of a hypha (h). The red arrowheads point to the cell walls of the single plant cell within which we observe this abnormal hypha. Nomarski optics was used in all panels; sections were treated with Fungifluor solution. Scale bars, 20 μ m.

Drastic reduction of teliospore production

At 15 days postinoculation, rare hyphae with lobed tips were observed in a few tumor cells (data not shown). Some of the cells in these hyphae are rounded (Fig. 12A). Most tumor cells appear to be full of debris (Fig. 12B), which may be lysed fungal hyphae. A total of only 7-10 isolated teliospores was observed among the debris after examining many sections from different tumors of the same plant and from tumors from different plants (Fig. 12B). Given that wild-type strains produce an estimated 2.5-6 billion teliospores per cm³ of tumor tissue (Christensen, 1942), production of teliospores by the *fuz1*⁻ mutant is thus estimated to be reduced at least 10⁶-fold.

DISCUSSION

In the present work we have analyzed the time course of fungal development in the plant, specifically, the morphological changes in the hyphae and the pathway of teliospore formation using coinfection with haploid strains having different *a* and *b*

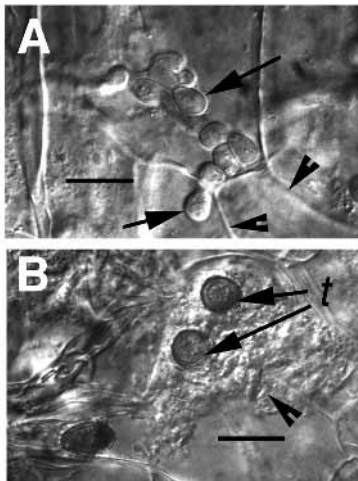


Fig. 12. Drastic reduction in teliospore formation by *fuz1*⁻ mutants. (A) Rare *fuz1*⁻ hyphae in a few tumor cells are seen undergoing cell rounding (arrows). (B) Rare teliospores (arrows) produced by the *fuz1*⁻ mutant. The arrowhead points to debris that is present in most tumor cells induced by *fuz1*⁻ mutants. Nomarski optics was used for both panels. Scale bars, 20 μ m.

alleles or infection with diploid strains heterozygous at both *a* and *b*. The existence of a pathway of teliospore development is supported by analysis of mutants defective in the *fuz1* gene, which appear to exhibit a specific defect in this pathway. The role of the *a* locus in development has been ascertained by comparison of *a1/a1 b1/b2* and *a1/a2 b1/b2* diploid strains. These studies show that hyphal development in planta does not require different alleles at the *a* locus.

The pathway of teliospore development

Several aspects of teliospore formation, for example, fragmentation and morphological changes, have been reported earlier (Knowles, 1898; Lutman, 1910; Rawitscher, 1912; 1922; Christensen, 1931; Sleumer, 1932; Mills and Kotzé, 1981; Snetselaar and Mims, 1994), though complete documentation was not provided. Earlier studies also reported the change in the consistency of the hyphae in the tumors, which was referred to as 'gelatinization'. For example, Christensen (1931) says: "...at the time of spore formation the hyphae gelatinize so completely that they appear as short, angular, and irregular segments lying in a clear matrix...". He also described the appearance of 'grape-like clusters' in the hyphal tips, which we show is due to increased hyphal branching and subsequent formation of a lobed hyphal tip (Fig. 6). Our studies extensively document the steps involved in teliospore formation, which are summarized next.

Upon co-inoculation with *a1 b1* and *a2 b2* haploids or with pathogenic diploids (*a1/a2 b1/b2* and *a1/a1 b1/b2*), rapid proliferation of the fungus ensues. This proliferation involves formation of dikaryotic (or diploid) hyphae, extensive hyphal branching on the leaf surface and invasion of host cells (days 1-2; Fig. 13A). By days 3-5, hyphae are observed to branch inside host cells, traverse from cell to cell, and exhibit collapse of hyphal compartments (Fig. 13B,C). Tumors arise by days 5-6. Within the tumors, the hyphae undergo a number of discrete developmental steps: first, hyphal branching occurs at closer intervals, particularly near the tips (days 7-8; Fig. 13D),

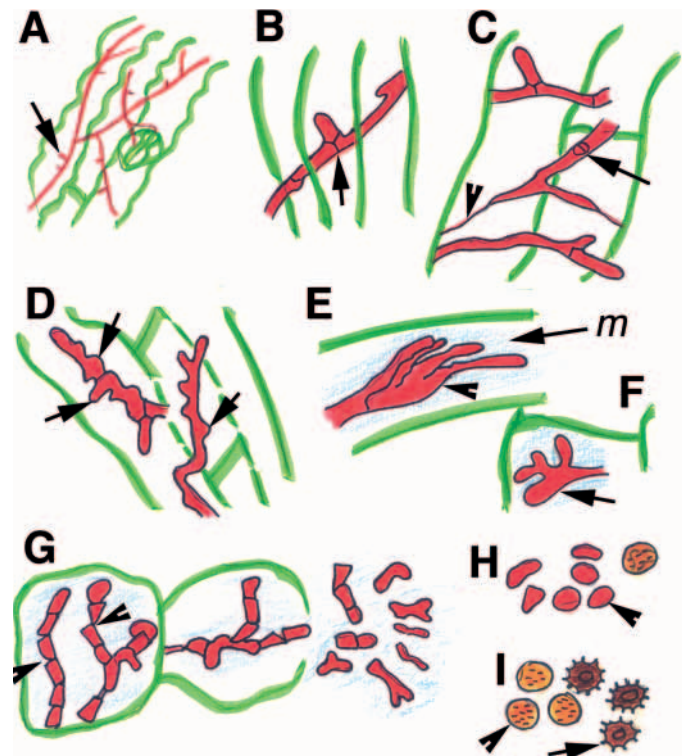


Fig. 13. Schematic diagram of hyphal development and teliospore formation within the plant. The developmental steps that occur after infection of maize plants with *U. maydis* are shown in the different panels. (A) Branching (arrow) and spreading of hyphae on the leaf surface and penetration through the stomata (days 1-2); (B) branching (arrow) and proliferation within plant cells (days 3-4); (C) hyphal collapse (arrowhead)(days 3-5). The arrow points to a branch arising towards the plane of the paper. Tumors arise at days 5-6. (D) Increased branching (arrows), particularly near the hyphal tip (days 7-8); (E) Appearance of mucilaginous matrix (m) causing the hyphae to look bloated and indistinct (arrowhead) (days 8-9); (F) formation of lobed tips (arrow) (day 9). G, hyphae become fragmented (arrowheads point to line of fragmentation) and produce segments of two to several cells (days 9-12); (H) the released cells (arrowhead) undergo morphological changes leading to rounding (days 10-15), and deposition of the pigmented and sculpted wall (arrow) characteristic of the teliospore (I) (days 12-15).

second, the hyphae become embedded within a mucilaginous matrix (days 8-9; Fig. 13E) and lobed hyphal tips are observed (day 9; Fig. 13F); third, hyphae become fragmented and release cells (days 9-12; Fig. 13G); fourth, the released cells undergo morphological changes leading to rounding (days 10-15; Fig. 13H); fifth, a pigmented and sculpted cell wall is deposited (days 12-15; Fig. 13I). This progression is disrupted by the *fuz1*⁻ mutation at the stage where production of the mucilaginous matrix takes place and fragmentation of hyphae occurs (Figs. 13E-G). The *fuz1*⁻ mutants instead form abnormal hyphae (Fig. 14A-C) and do not produce the mucilaginous matrix.

This fungal developmental process consists of an ordered sequence of events, but the process is not completely synchronous – different parts of a tumor or different tumors contain a heterogenous population undergoing the different morphological transitions and transformations just described. We next comment on different steps in this pathway of teliospore formation.

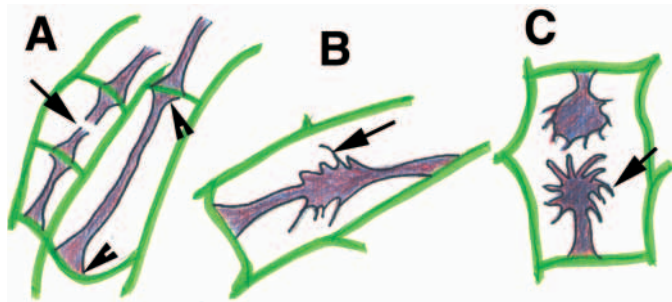


Fig. 14. Diagram of abnormal hyphae produced by *fuz1*⁻ mutants. *fuz1*⁻ mutants disrupt the progression of teliospore formation at the stage where production of the mucilaginous matrix takes place and fragmentation of hyphae occurs (Fig. 13E-G). The *fuz1*⁻ mutants instead form abnormal hyphae which fan out at the junction with a plant cell wall (A, arrowheads). The hyphae also appear ruptured (arrow), and cytoplasmic projections arise from swollen parts of the hypha (arrow in B,C).

Our observations are for the most part in agreement with observations reported by previous workers. However, differences in timing of hyphal branching and location of teliospore formation exist. We observed branching as early as 1 day after inoculation in agreement with Mills and Kotzé (1981). In contrast, Snetselaar and Mims (1994) report that hyphae are unbranched in the first 2 days postinoculation. These differences may have resulted from use of different *U. maydis* strains, maize varieties, or plant growth conditions. Our observations on hyphal fragmentation and subsequent steps contrast somewhat with those by Snetselaar and Mims (1994) and are discussed in Results.

The branch primordium and its associated Y-shaped septum in *U. maydis* appear similar to the clamp connection of other Basidiomycetes such as *Schizophyllum commune* and *Coprinus cinereus*. The clamp connection, which participates in maintaining the dikaryotic state in these fungi, is a projection of the apical cell that fuses with the subapical cell to deliver a duplicated nucleus to the subapical cell (Casselton, 1978; Novotny et al., 1991). The clamp connection becomes separated from the apical cell by a septum formed at an angle. Although the *U. maydis* branch primordia exhibit structural similarity to clamp connections, branch primordia were not seen fusing with the adjacent cell, and thus we do not consider them to be true clamps, in agreement with the observations and conclusions of Sleumer (1932). These clamp-like structures of *U. maydis* have been observed by several other workers (Hanna, 1929; Christensen 1931; Sleumer, 1932; Snetselaar and Mims, 1994). Even though the branch primordia of *U. maydis* do not appear to serve the same function as clamp connections in other Basidiomycetes, it is plausible that the morphogenetic changes in the branch primordia and in the clamp connection may result from similar regulatory processes.

Several possible roles can be proposed for the mucilaginous material. During hyphal fragmentation, cells become separated from each other at their septum, a process that is likely to involve cell wall degrading enzymes. In addition, the fragmented cells undergo dramatic changes in shape, which require that the cell wall become loosened and remodeled. Such changes in cell wall rigidity could have serious consequences for maintenance of turgor pressure. The mucilaginous material

may contain enzymes involved in cell wall degradation or may act as an osmotic stabilizer for the cells changing in shape.

The requirement for the *fuz1* gene for late stages of teliospore formation and possibly for tumor induction

We have observed that development of *fuz1*⁻ mutants proceeds normally until the time in wild-type infections when hyphae become embedded in the mucilaginous matrix and begin to fragment. Inactivation of *fuz1* blocks teliospore production because of a block in hyphal fragmentation. Associated with this defect is the lack of mucilaginous material: as noted above, this material may be required for or result from fragmentation. The *fuz1* gene could control production of the mucilaginous matrix by the fungus or by the plant. The defect in fragmentation of the *fuz1*⁻ hyphae may be related to the defect in cell separation exhibited by *fuz1*⁻ yeast cells during budding in culture (F. Banuett, unpublished).

Inoculations with *fuz1*⁻ strains have an overall reduced effect in the tumor response. First, the developing leaves tend to unroll completely, whereas in wild-type inoculations leaves tend to remain rolled. Secondly, the smaller *fuz1*⁻ tumors do not block water transport as severely as wild-type tumors. These differences in the tumor response indicate that *fuz1* may play a direct role in tumorigenesis.

Role of putative plant signals in fungal differentiation

Several observations argue for the involvement of plant signals in hyphal development: (1) branching and clamp-like structures occur only in planta and not in culture; (2) different *a* alleles are necessary for filamentous growth in culture but not in planta; (3) nuclear fusion in the dikaryon occurs only in planta not in culture; (4) *fuz1*⁻ mutants exhibit filamentous growth in planta but not in culture; (5) teliospores form only in planta, not in culture. We next discuss these observations.

Induction of hyphal branching

Because branching is not observed in culture, we hypothesize that either contact with the plant surface or a signal produced by the plant causes induction of branching. One possibility is that a plant hormone, for example, the gas ethylene, is the inductive signal. Flaishman and Kolattukudy (1994) recently demonstrated that ethylene induces germination of conidia, branching of the germ tubes, and formation of penetration structures (appressoria) in *Colletotrichum* species that attack ripening fruits. Because ethylene production increases dramatically 1 day after inoculation of maize seedlings with *U. maydis* (Andrews et al., 1981), it is plausible that ethylene triggers branching of *U. maydis* hyphae. Contact with plant surface materials may also trigger branching (see Podila et al., 1993).

Independence of hyphal growth in planta from different *a* alleles

Studies of *U. maydis* in culture demonstrate that different *a* alleles are necessary for maintenance of filamentous growth but not for tumor formation (Banuett and Herskowitz, 1989). In other words, an *a1/a1 b1/b2* strain is Fil⁻ Tum⁺, in apparent violation of the correlation between filamentous growth and tumor induction. We have shown here that this Fil⁻ Tum⁺ diploid strain produces

hyphae in planta that develop exactly like those produced by co-inoculation of haploid strains carrying different *a* and *b* alleles or by single inoculation of a diploid heterozygous at *a* and *b*. The correlation between filamentous growth and tumor induction thus is maintained when filamentous growth is assessed in the plant. The failure of *a1/a1 b1/b2* strains to exhibit filamentous growth in culture is due to lack of autocrine induction by the pheromones encoded by the *a* locus (Banuett and Herskowitz, 1989; Bölker et al., 1992; Spellig et al., 1994), which appear to function in a MAP kinase pathway (Banuett and Herskowitz, 1994a). It would be interesting if infected plant cells produce a peptide pheromone such as *a*-factor or another signal that activates this MAPK pathway.

Nuclear fusion during hyphal development

As noted earlier, nuclear fusion occurs only rarely in dikaryotic hyphae grown in culture. In contrast, nuclear fusion is a programmed event during fungal development in planta. Several independent observations suggest that karyogamy occurs around the time of tumor formation (Christensen, 1931; Sleumer, 1932; Ehrlich, 1958). Our observations, though incomplete, suggest that by the time the hyphae (dikaryotic) are embedded in the mucilaginous matrix, each hyphal cell contains a single nucleus. Electron microscopic evidence indicates that nuclear fusion involves the close apposition of spindle pole bodies (Snetselaar and Mims, 1994). The role of the plant in karyogamy remains to be elucidated. It is possible that the plant produces a peptide that induces karyogamy and thus may play a similar role to the pheromones of *Saccharomyces cerevisiae* in regulating karyogamy (Rose et al., 1986).

Requirement of *fuz1* for hyphal growth

The *fuz1* gene is necessary for filamentous growth in culture (Banuett, 1991) but not in planta: *fuz1*⁻ mutants form hyphae in the plant that branch in the same manner as those from wild-type inoculations. Barring residual activity due to leakiness of the *fuz1*⁻ mutation, these observations are consistent with our view that different pathways of filamentous growth may be activated by different environmental conditions (Banuett and Herskowitz, 1994a), as has also been suggested for *Candida albicans* (Liu et al., 1994). Further support for this proposal comes from observations of the behavior of other *Fuz*⁻ mutants in planta. Inactivation of three other genes, *fuz7* (a MEK homolog), *fuz2* (unknown function), and *rtf1* (an inhibitor of tumor formation) abolishes formation of filaments in culture (Banuett, 1991; Banuett and Herskowitz, 1994a) but, surprisingly, not in planta (F. Banuett, unpublished).

The studies described here have established the time course of events in fungal development that occur during infection of maize with *U. maydis*. The behavior of *fuz1*⁻ mutants provides support for the existence of an ordered developmental pathway leading to teliospore formation, although the precise role of *fuz1* remains to be elucidated. The different morphological forms exhibited by the fungus provide a means of following events during the time course of infection and to ascertain the effect of mutations in different genes on this process. We expect to identify other genes responsible for the program of teliospore formation by looking for fungal mutants defective in tumor formation and teliospore production.

We would like to thank Jim DeVay for his kind gift of maize seed, Betsy O'Neill and Frank Sprenger for help with Photoshop, and John Kaloyeros, without whose prompt maintenance and repair of the Conviron chamber this work would not have been possible. This work was supported by NIH grant AI 18738.

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(Accepted 12 July 1996)