Initiation of Meiotic Recombination in *Ustilago maydis*

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ABSTRACT A central feature of meiosis is the pairing and recombination of homologous chromosomes. *Ustilago maydis*, a biotrophic fungus that parasitizes maize, has long been utilized as an experimental system for studying recombination, but it has not been clear when in the life cycle meiotic recombination initiates. *U. maydis* forms dormant diploid teliospores as the end product of the infection process. Upon germination, teliospores complete meiosis to produce four haploid basidiospores. Here we asked whether the meiotic process begins when teliospores germinate or at an earlier stage in development. When teliospores homozygous for a *cdc45* mutation temperature sensitive for DNA synthesis were germinated at the restrictive temperature, four nuclei became visible. This implies that teliospores have already undergone premeiotic DNA synthesis and suggests that meiotic recombination initiates at a stage of infection before teliospores mature. Determination of homologous recombination in plant tissue infected with *U. maydis* strains heteroallelic for the *nar1* gene revealed that Nar$^+$ recombinants were produced at a stage before teliospore maturation. Teliospores obtained from a *spo11Δ* cross were still able to germinate but the process was highly disturbed and the meiotic products were imbalanced in chromosomal complement. These results show that in *U. maydis*, homologous recombination initiates during the infection process and that meiosis can proceed even in the absence of Spo11, but with loss of genomic integrity.

MEIOSIS has long held fascination for evolutionary biologists, cytologists, and geneticists alike by the puzzle of its evolutionary origin, the chromosome choreography, and its role in generating genetic diversity (Wilkins and Holliday 2009). The distinctive events of meiosis include pairing of homologs along their entire lengths, extensive recombination between homologs, suppression of sister-chromatid separation in the first division, and absence of S phase at the start of the second division. Attention of geneticists has centered primarily on the second step—namely genetic recombination during pairing and its function in maintaining genomic integrity and in generating new linkage relationships among genes. As reciprocal recombination events are essential for proper chromosome segregation at the first meiotic division, this process has profound implications in human development and health (Nagaoka *et al.* 2012). The central question of how homologous chromosomes recognize each other and pair to enable recombination has provoked a great deal of research in a variety of experimental systems. Certain core principles underlie the process, but there is enormous variation in mechanical operations and regulatory mechanisms from one organism to the next and uncertainty in how DNA information is used for pairing and recombination (Pawlowski *et al.* 2007; Schvarzstein *et al.* 2010; Storlazzi *et al.* 2010; Tsai and Mckee 2011; Lake and Hawley 2012). Thus, to geneticists the study of meiosis is irresistibly interesting.

Ustilago maydis is a basidiomycete fungus and notable plant pathogen that was developed several decades ago as an experimental system for studying homologous recombination and DNA repair (Holliday 2004). Indeed the first mutants defective in homologous recombination and meiosis in any eukaryote were obtained in *U. maydis*. Ironically, the intricate biology of the *U. maydis* life cycle has impeded establishment of some of the most basic features of homologous recombination in the meiotic process, so there is a need for greater elucidation in this system.

U. maydis is a biotrophic parasite of maize (Brefort *et al.* 2009). Its life cycle is characterized by three distinct phases

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(Banuett and Herskowitz 1996). In the saprophytic state it propagates as a haploid unicellular yeast-like sporidial form dividing by budding. In the parasitic hyphal state that results from conjugation of two compatible haploids followed by transformation into a proliferating filamentous dikaryon, this mycelial form spreads rapidly in the vicinity of the site of infection and induces galls or tumors in the host plant. Ultimately, the filamentous dikaryon differentiates into a specialized uninucleate diploid cell type, the teliospore, which upon germination completes meiosis to yield the yeast-like haploid form, thus completing the life cycle.

Teliospores are rounded, echinate, thick-walled, melanized cells that are able to tolerate extreme environmental conditions of temperature and desiccation. Placed in nutrient-containing media, teliospores germinate with growth of a tube-like promycelium, or metabasidium, that can reach a length several times the diameter of the teliospore within a day. A single diploid nucleus present in the teliospore moves into the promycelium, then proceeds through both meiotic divisions to yield four nuclei that are distributed into septated compartments along the length of the promycelium (Ramberg and MclLaughlin 1980). Haploid basidiospores bud off the promycelium and continue budding to produce sporidial yeast cells with potential for extensive mitotic division.

Teliospore germination has been investigated by early mycologists using light microscopy, *e.g.*, Hanna 1929, and in more recent years by cell biologists using fluorescence optics and electron microscopy (O'Donnell and McLaughlin 1984; Snetselaar and Mims 1994; Banuett and Herskowitz 1996). While there is consensus that a single diploid nucleus is present in mature teliospores and that meiosis ensues when teliospores germinate, there is scant evidence to establish when or how the onset and progression of homologous recombination, the central genetic process underlying meiosis, is coupled to the morphological events of meiosis that manifest during teliospore germination. It is clear that homologous recombination and the teliospore germination program are connected. When plants are infected with compatible *U. maydis* strains deleted of genes encoding the essential homologous recombination DNA strand exchange factor Rad51 or its primary mediator Brh2, teliospores are formed, but these do not germinate and promycelium formation aborts (Kojic *et al.* 2002). Thus, teliospore formation appears independent of meiotic recombination proficiency, but the developmental processes involved in teliospore germination and growth are intimately connected with execution of the meiotic homologous recombination program.

Two studies of teliospore germination, one performed several decades ago and one very recent, raise fundamental questions about the timing of homologous recombination in *U. maydis*. The earlier study focused on the synaptonemal complex (SC). These are characteristic structures of synapsed chromosomes formed in meiotic prophase often in close correspondence with the homologous recombination process. In most organisms it is believed that recombination begins in leptotene with the introduction of DNA-double-strand breaks by Spo11, a topoisomerase-related protein (Zickler and Kleckner 1999). Repair of the DSBs initiates a genome-wide search for homology that drives homolog pairing and alignment, leading to lengthwise synapsis of chromosomes achieved in pachytene through polymerization of the proteinaceous scaffold structure, the synaptonemal complex. Although there are certain notable exceptions, as a general rule it is fair to say that in many organisms, especially fungi, the appearance of SCs can be taken as a temporal landmark signaling onset and procession of homologous recombination. However, a search for SCs in germinating teliospores of *U. maydis* by transmission electron microscopy turned up no evidence for such structures (Fletcher 1981). This finding, although negative, raises the notion that well-defined SCs might not form in *U. maydis* meiosis, as is the case in *Schizosaccharomyces pombe* (Loidl 2006), or alternatively suggests the possibility that SCs (and by inference, onset of homologous recombination) do form, but at a stage prior to teliospore germination. In a more recent study of germinating teliospores using microarray analysis, little evidence for active recombination was found (Zahiri *et al.* 2005). Biomarkers for onset of homologous recombination include the strand exchange proteins Rad51 and the meiosis-specific Dmc1, which are required in large amounts at the strand invasion stage in homologous recombination to promote repair of Spo11-induced DNA double stand breaks (Bishop 1994; Kurzbauer *et al.* 2012). In *U. maydis* only Rad51 is present, the gene encoding Dmc1 apparently having been lost in the course of evolution (Donaldson and Saville 2008; Holloman *et al.* 2008). This being the case, one might even presume that the Rad51 levels could be higher, relatively speaking, to compensate for the lack of Dmc1. However, both microarray analysis and quantitative reverse transcriptase–coupled polymerase chain reaction (qRT–PCR) determination of transcripts in germinating teliospores showed that Rad51 transcript levels actually decrease during germination (Zahiri *et al.* 2005). Thus, it might be construed that the stage at which Rad51 action is required, namely initiation of homologous recombination, occurs prior to teliospore germination.

When might homologous recombination take place in *U. maydis*? The temporal development of teliospore formation in *U. maydis* has been systematically tracked (Banuett and Herskowitz 1996). Teliospores derive from the hyphal heterokaryon form after a series of developmental changes. About 8 days after infection of maize seedlings, visible tumors are formed on leaf surfaces, and within the tumors profuse branching of fungal hyphae is evident. The hyphal tip regions then become lobed and hyphae begin to fragment and round up. Ten days postinfection a single nucleus per cell is observed suggesting that karyogamy has taken place. After 11 days, large masses of rounded uninucleate cells are observed and by day 14, mature teliospores with characteristic echinulate, melanin-rich cell walls appear in

abundance. Given that the meiotic program initiates immediately following karyogamy in a number of fungal model systems (Rossen and Westergaard 1966; Egel and Egel-Mitani 1974; Lu and Jeng 1975; Iyengar *et al.* 1977; Carmi *et al.* 1978; Li *et al.* 1999), it seemed reasonable to us and others (Saville *et al.* 2012) to suppose that a similar process could be in place in *U. maydis* and that homologous recombination becomes activated during the infection stage in plants, after nuclear fusion but before teliospore maturation and not coincident with teliospore germination.

Here we addressed the question of when meiosis initiates during the life cycle of *U. maydis*. Our approach was to examine the dependence of teliospore germination on DNA synthesis, measure homologous recombination during teliospore development, and determine the dependence of teliospore germination and basidiospore formation on initiation of homologous recombination.

Methods and Materials

U. maydis strains and genetic methods

Manipulations with *U. maydis*, media, culture methods, infection of plants, fuzz reaction, meiotic analysis, and mitotic recombination have been described before (see Holliday 1966; Holliday 1974; Kojic *et al.* 2002). The *cdc45^{ts}* mutant used in these studies was originally known as *ts*-92 (Unrau and Holliday 1970) and then subsequently named *tsd2-1* before the gene product was known to be orthologous to Cdc45 (Onel and Holloman 1997). Cdc45 will henceforth replace the name Tsd2. The open reading frame of the mutant allele was amplified by PCR and the sequence was determined. Cdc45 was identified as entry um05770 in the MIPS annotated *U. maydis* database (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/). Multiple sequence alignment of Cdc45 was performed using the T-Coffee alignment tool (http://tcoffee.vital-it.ch/). Crosses were performed by infecting 2-week-old maize seedlings (variety Silver Queen) with UCM266 (*cdc45^{ts} ade1-1 a2 b1*) and UCM737 (*cdc45^{ts} a1 b2*) and maintaining temperature at 22° in an illuminated incubator (14-hr light cycle/10-hr dark cycle). Tumors were harvested after 21 days and teliospores were recovered from macerated plant tissue. Teliospores were spread on YEPS medium (1% yeast extract, 2% peptone, 2% sucrose) at 22° or 32° and allowed to germinate. The Spo11 gene was identified as entry um10420 in the MIPS database. *spo11* null mutants were constructed by standard gene disruption methodology (Kamper 2004). The entire open reading frame on chromosome 12 (MIPS database coordinates chr12:530515–531904) was replaced in haploid strains UCM350 (*pan1-1 nar1-6 a1 b1*) and UCM520 (*met1-2 nar1-1 a2 b2*) by a *nat* resistance cassette (glyceraldehyde 3-phosphate dehydrogenase promoter driving the nourseothricin acetyl transferase gene) to yield UCM738 and UCM762, respectively. *pan*, *met*, *ade*, *nar*, *a*, and *b* indicate auxotrophic requirements for pantothenate, methionine, adenine, inability to utilize nitrate, and mating-type loci, respectively.

Optical methods

DNA content was measured by flow cytometry (Garcia-Muse *et al.* 2003). Cells (10^7) were harvested, washed twice in cold water, fixed in 70% ethanol overnight, and resuspended in 50 mM sodium citrate, pH 7.6. Cellular RNA was destroyed by incubation with RNaseA (0.25 mg/ml) at 50° for 1 hour. Then proteinase K (1 mg/ml) was added and cells were incubated for another hour at 50°. Cells were stained with propidium iodide (10 μg/ml) and analyzed with a Coulter Epics XL-MLC. For each acquisition, 10^4 events were measured at a flow rate of 60–100 events per second in both DNA content (FL3) and cell size (FSC). In the DNA histograms, relative fluorescence intensities are given on the horizontal axis and cell numbers in the vertical axis. Microscopy was carried out using a Nikon CF600 microscope. A standard diamidino-2-phenylindole (DAPI) filter set was used for epifluorescence analysis of nuclear staining with DAPI (Garrido and Perez-Martin 2003). Photomicrographs were obtained with an ORCA G digital camera (Hamamatsu) and the images were processed with Metamorph software.

Recombination assays

For tracking recombination *in situ* during the course of *U. maydis* infection, heteroallelic recombination at the *nar1* locus was measured by determining Nar$^+$ prototrophy (Kojic *et al.* 2002). Plant seedlings were wounded at multiple points with a 26-gauge needle and inoculated with a mixed cell suspension ($\sim 10^6$) of strains UCM350 and UCM520. Plants were maintained over a 2-week period under illumination at 28° for a 14-hr light cycle and in the dark at 22° for a 10-hr dark cycle. At 2-day intervals, tumor tissue (1–2 g) from three to four plants per sampling was excised with a razor, ground with a mortar and pestle, and suspended by mixing with 1 ml water. Plant debris was removed by filtration through gauze, and aliquots of the aqueous fraction were plated on minimal medium containing ammonium to determine the frequency of diploids and on minimal medium containing nitrate to determine frequency of Nar+ recombinants.

Meiotic allelic recombination at *nar1* was measured by random products analysis (Holliday 1974) in crosses of strains UCM350 × UCM520. Teliospores ($\sim 10^6$) were germinated on YEPS agar at 28°. After 40 hr, microcolonies were washed from the plate and the suspension was vortexed to disperse cells, which were then plated on nitrate minimal medium supplemented with pantothenate and methionine to enable growth of the haploid meiotic progeny. Nar$^+$ prototrophs were counted after 5 days. Mitotic allelic recombination at *nar1* was measured by fluctuation analysis in diploids produced from mating strains UCM350 × UCM520 on charcoal-containing medium (Kojic *et al.* 2002). In three independent experiments cells from

seven to nine cultures were spread on nitrate minimal medium to select for Nar^+ recombinants. The median values were used to calculate the recombination frequency.

Results

Teliospore germination during inhibition of DNA replication

In meiosis a single round of DNA replication is followed by two rounds of cell division. In *U. maydis* the tacit understanding among investigators has been that the diploid nucleus present in teliospores is competent to undergo meiosis. However, it has never been clear at what stage the teliospore nucleus is arrested because the opacity of the teliospore's heavily melaninized, extremely tough cell wall has precluded intracellular microscopic observation and accurate biochemical measurement of DNA content. In view of the "black box"-like nature of the teliospore, it has not been possible to discern whether the nucleus of the dormant teliospore is arrested prior to entry into the meiotic round of DNA replication or in meiotic prophase following replication. We reasoned, however, that by considering the consequences of DNA synthesis inhibition during teliospore germination, it should be possible to deduce the nuclear arrest point. In the event that arrest occurs at the premeiotic S-phase stage, teliospore germination should herald the onset of the meiotic DNA replication round. If this scenario were the case, then inhibition of DNA synthesis during teliospore germination should block chromosome duplication and preclude nuclear division, with the result that the growing promycelium should arrest in development with only a single nucleus. On the other hand, if the diploid nucleus present in teliospores were arrested in meiotic prophase after having already passed through meiotic S phase, then reductional and equational divisions should ensue and four compartmentalized nuclei should become evident in the germinated promycelium in spite of the block to DNA replication.

This idea was tested using teliospores homozygous for a conditional mutation in a gene essential for DNA synthesis. The approach was to germinate teliospores under conditions restrictive for the gene's function and to visualize the nucleus/nuclei in the promycelium at a time corresponding to a point after the second meiotic division. The teliospores used were derived from strains that contained a temperature-sensitive (ts) allele of *cdc45* (Figure 1A), a gene required for initiation of DNA synthesis and for replication fork progression (Onel and Holloman 1997). Cdc45 couples the Mcm2–7 complex with the GINS complex enabling DNA helicase activity required for replication origin unwinding and fork progression (O'donnell *et al.* 2013). Mutation of the gene in the ts allele is due to a single-amino-acid change (G303D) at a conserved but not invariant residue in a tyrosine-rich region of the protein. The mutant grows robustly at 22° but is severely crippled at 32° (Figure 1B). In an early study of this mutant it was determined that over a period of 6 hr after shifting cell cultures to 32°, the relative increase in DNA synthesis in the mutant was only about 10% compared to wild type (Unrau and Holliday 1970). In addition, approximately half the cells in the population were no longer viable after 8 hr. Therefore, at the restrictive temperature the mutant has a limited, exponentially diminishing capacity for DNA synthesis. If DNA replication were to accompany teliospore germination, then the process would take at least 10 times longer to be completed in the mutant. Meiotic S phase in every organism in which it has been investigated is several times longer than S phase in mitotically growing cells, presumably because chromosomes are being prepared for subsequent events. Measured values range from about three times longer in *Saccharomyces cerevisiae* (Cha *et al.* 2000) to five times longer in the newt *Triturus vulgaris* (Callan 1973) to six times longer in *Lilium longiforum* (Holm 1977). Given that mitotic S phase in wild-type cells of *U. maydis* is about 1 hr (Holliday 1965), it might be reasonable to suppose meiotic S phase would take 3 hr to complete and, by extrapolation, at least 30 hr for the $cdc45^{ts}$ mutant at restrictive temperature. Since our planned experiment was designed for observing germinating teliospores after 16 hr (see below), it seems unlikely that the $cdc45^{ts}$ mutant would be able to complete DNA synthesis within this time frame under the restrictive condition.

We germinated both wild-type and $cdc45^{ts}$ teliospores at 32° and, 16 hr later, after promycelia developed, stained the preparations with DAPI to enable visualization of nuclei by fluorescence microscopy (Figure 1C). In the control wild-type strain, four primary nuclei were observed to be distributed in different compartments in the mature promycelium obtained at 22° and 32°. Onset of basidiospore budding from the promycelia was evident at both temperatures. In the case of $cdc45^{ts}$, promycelium formation was efficient, but was aberrant in that there appeared to be a high frequency of secondary and tertiary promycelia (Figure 1D). Nevertheless, it was evident that four nuclei were produced in promycelia formed at 32° regardless of promycelium distribution, suggesting that four genome complements were present in the nucleus of the ungerminated teliospore. These findings strongly support the notion that the single nucleus in dormant teliospores is arrested in meiotic prophase at a presumptive checkpoint after meiotic S phase but before the first meiotic division.

Teliospore developmental stages and homologous recombination

In light of the conclusion that the teliospore nucleus is arrested in meiotic prophase and assuming that the stage of arrest is late prophase given the reported absence of any vestige of SC, it seemed reasonable to suppose that meiosis and associated meiotic recombination would necessarily have initiated prior to teliospore formation. This would place the onset of the meiotic process at a stage during biotrophic growth within the plant tissue. From *in situ* analysis of teliospore development in infected plant tissue (see

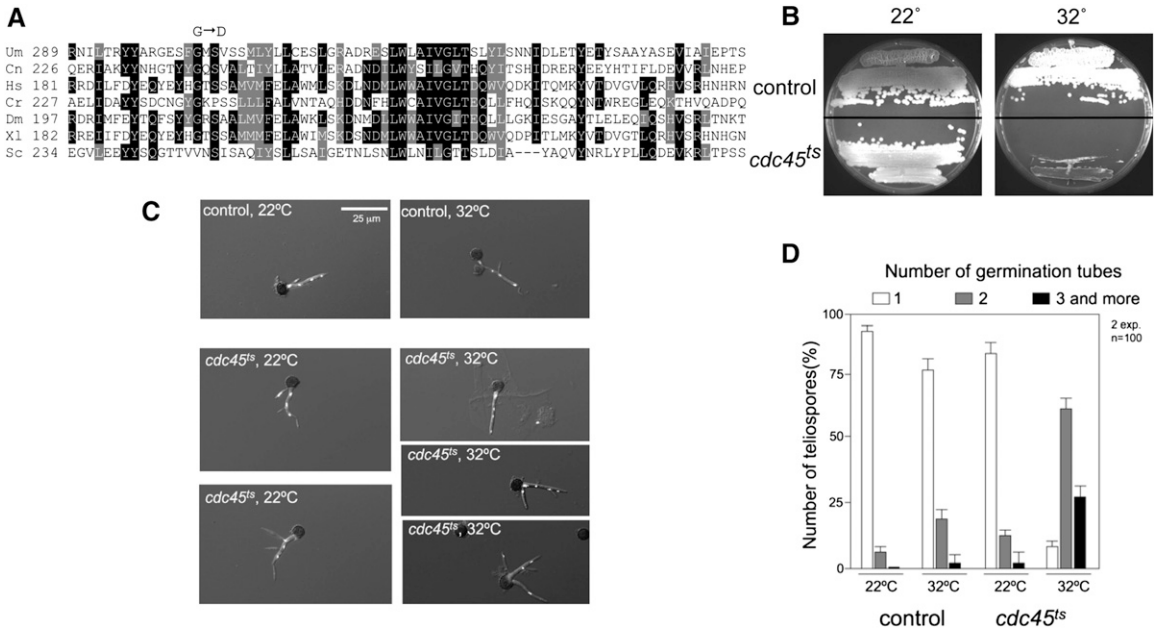

Figure 1 Cdc45 function is not required for teliospore germination. (A) The *cdc45^{ts}* allele of *U. maydis* is due to a mutation at residue 303, which lies within a conserved region in the aligned Cdc45 orthologs (GenBank accession numbers indicated). Um (*Ustilago maydis* XP_761917); Cn (*Cryptococcus neoformans* AFR95427); Hs (*Homo sapiens* AAC67521); Cr (*Chlamydamonas reinhardtii* XP_001696501); Dm (*Drosophila melanogaster* AAD09003); Xl (*Xenopus laevis* AAC67520); Sc (*Saccharomyces cerevisiae* NP_013204). (B) *cdc45^{ts}* mutant strain UCM266 and wild-type control plated at permissive and restrictive temperatures. (C) Teliospores obtained from a wild-type control or *cdc45^{ts}* × *cdc45^{ts}* cross were germinated at 22° for 24 hr or 32° for 16 hr and then stained with DAPI. (D) Promycelia, or germ tubes, emerging during teliospore germination were tallied.

Figure 2A), it was previously reported that dikaryotic nuclei undergo karyogamy concomitant with hyphal fragmentation and rounding 8–10 days postinfection (Banuett and Herskowitz 1996). Since it has been long known in *U. maydis* that yeast-form, mitotically growing diploid cells could be recovered from gall tissue well before the appearance of teliospores (Holliday 1961), we considered the possibility that such diploids might derive from cells that initiated meiosis but somehow exited the program before committing to the first meiotic division and returned to mitotic growth. If so, it might be predicted that homologous recombination would have commenced in these cells.

To test this notion, we recovered diploids from infected plant tissue and then determined the frequency of recombination at a reporter gene locus. For this test the *U. maydis* strains used to infect the plants were compatible haploids each carrying (i) a specific biosynthetic deficiency to enable selection of prototrophic diploids (*pan1-1* or *met1-2*, requirement for pantothenic acid or methionine, respectively) and (ii) an allele of the *nar1* (nitrate reductase) recombination reporter gene. Diploids could be selected on minimal medium by complementation of the biosynthetic deficiencies, and the subset, having undergone heteroallelic recombination by gene conversion at the *nar1* locus to restore a functional *nar1* gene, could then be detected by growth on nitrate-containing minimal medium. As gene conversion is not reciprocal, recombination events could be easily detected in diploids without need for analysis of haploid meiotic progeny.

At intervals several days after infection, plant tumor tissue was excised and ground in a mortar with water to yield an aqueous broken-cell extract. After removal of debris, aliquots were plated on ammonium minimal medium for selection of diploids and on nitrate minimal medium for selection of Nar$^+$ recombinants (Figure 2B). Small galls were apparent on plant leaf surfaces 6 days postinfection (dpi) and diploids could be detected then. Nar$^+$ recombinants were detectable at day 8 and reached a peak level at day 10, several days before the appearance of teliospores. The frequency of *nar1* recombination observed was 4.5×10^{-3}. This is certainly in the range of meiotic allelic recombination at *nar1* ($1.6 \pm 0.6 \times 10^{-3}$) that we determined by the traditional random products method of scoring haploid meiotic progeny and also comparable to the induced levels of recombination reported ($\sim 5 \times 10^{-3}$) in *nar1* heteroallelic diploids after irradiation with high doses of gamma rays (Holliday 1971). However, this frequency is well beyond the level of spontaneous recombination ($2.4 \pm 0.8 \times 10^{-6}$) we observed in mitotically growing diploid cells. These findings show that homologous recombination is actively underway *in planta* before teliospores are formed and are consistent with the notion that in *U. maydis*, diploid cells likely enter the meiotic program shortly after karyogamy.

Homologous recombination-deficient meiosis

As a control to show that the observed Nar$^+$ prototrophs arose as a consequence of activation of the meiotic homologous recombination program, we wished to analyze

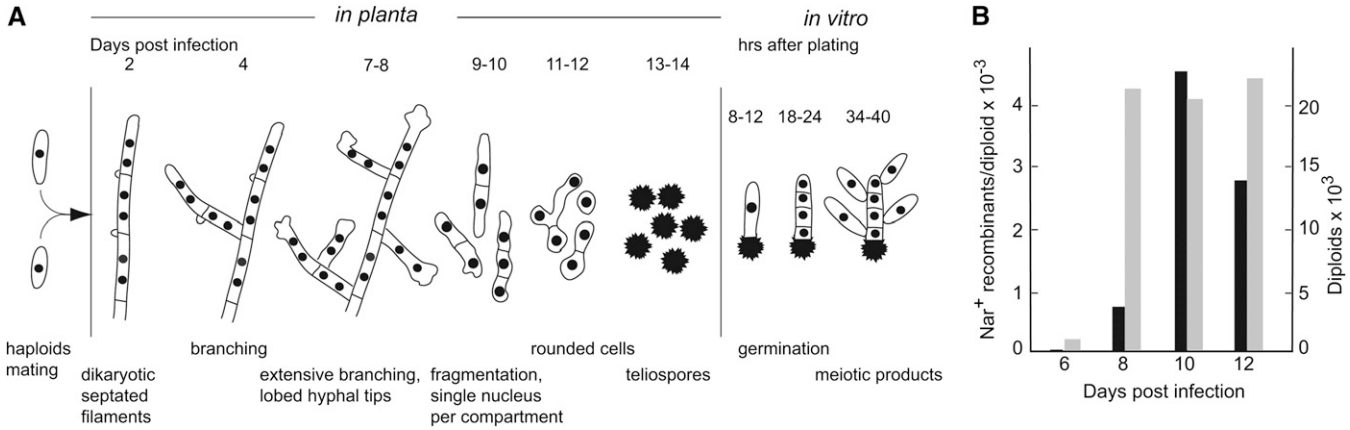

Figure 2 Formation of Nar+ recombinants during the course of infection. (A) The *U. maydis* life cycle is depicted schematically (adapted from Banuett and Herskowitz 1996) starting with mating of compatible haploids on the plant surface, proceeding in the plant through the switch to the dikaryon hyphal form, karyogamy, and the development and germination of teliospores. Forty 7-day-old seedlings were inoculated with freshly grown cultures of UCM350 and UCM520 and cultured under a daily light/dark cycle. At 2-day intervals, three to four plants were harvested, tumor tissue was excised and macerated, and aliquots of the suspension were plated on minimal media for selection of diploids and Nar+ recombinants as described in the *Materials and Methods*. (B) The bar graph indicates the frequency of Nar+ recombinant formation (solid bars) and the level of diploids (shaded bars) during the course of infection.

diploids from plant tissue obtained after infection with *spo11*Δ compatible strains since the DNA double-strand breaks initiating homologous recombination would not be formed in such cells. For this purpose we constructed strains deleted of the entire *spo11* open reading frame and will present a detailed characterization of the *spo11* gene and a description of the phenotype of mutant variants elsewhere. For this study, however, our expectation was that Nar^+ prototrophs from a *spo11*Δ cross would be rare, since homologous recombination would not initiate in the absence of Spo11. Indeed, we recovered no Nar^+ prototrophs among *spo11*Δ diploids obtained from plant tissue ($<1 \times 10^{-4}$). However, the accuracy of this finding in comparison with the results obtained from the wild-type cross must be considered tentative because diploid formation itself was attenuated and was delayed in appearance relative to wild type. Simply put, we were unable to recover an equivalent number of diploids from the *spo11*Δ cross. In general, plants infected with the *spo11*Δ mutants exhibited a markedly reduced frequency of the hallmark signs of pathology associated with *U. maydis* infection. Tumor formation was delayed by 5–7 days relative to wild type. Tumors that did form were generally small, they sometimes resorbed, and they often remained undarkened, indicating an absence of mature teliopsores. Nonetheless, there was an occasional darkened tumor (in ~1 in 25 plants infected with *spo11*Δ) with mature teliospores that could germinate to produce sporidial meiotic progeny. Germination of these teliospores, however, was highly disturbed (Figure 3). Often, basidiospore formation from the emergent promycelia was stunted. Growth of progeny sporidial cells was extremely variable, as was evident by the scraggly, uneven colony formation and the range of morphologies. On charcoal-containing mating medium, there was frequent appearance of white fuzzy colonies, a sign of heterozygosity (*i.e.*, disomy) at the *b* mating type locus (Schulz *et al.* 1990). On minimal medium lacking supplements the frequency of colony formation was ~80%, which is much higher than expected (25%) for two independently segregating biosynthetic markers, suggesting diploidy or aneuploidy. Heteroallelic recombination at *nar1* in the meiotic products was $<10^{-7}$, far below that observed in a normal meiosis (1.6×10^{-3}). FACS analysis of randomly sampled individual meiotic progeny revealed each one to have a genome content greater than a haploid control. Together, these findings suggest that a meiosis-like developmental program can be completed by *spo11*Δ mutants, but the process is lacking in homologous recombination and the distribution of chromosomes among the progeny is highly disturbed.

Discussion

Three conclusions can be drawn from these studies. First, the nucleus of a dormant *U. maydis* teliospore is diploid with four genome complements and is arrested at a stage in meiosis after initiation of homologous recombination, but prior to the first meiotic division. Second, homologous recombination in *U. maydis* initiates during the infection stage in plants before teliospores mature. Third, if homologous recombination does not initiate due to absence of Spo11, teliospore maturation can still proceed, although at much reduced efficiency, and teliospores can still germinate to produce viable progeny. The meiotic process, however, is deranged due to chromosome missegregation and the progeny exhibit extreme variation in growth due presumably to chromosome imbalance.

It was established years ago that *U. maydis* teliospores contain a single diploid nucleus and are competent to

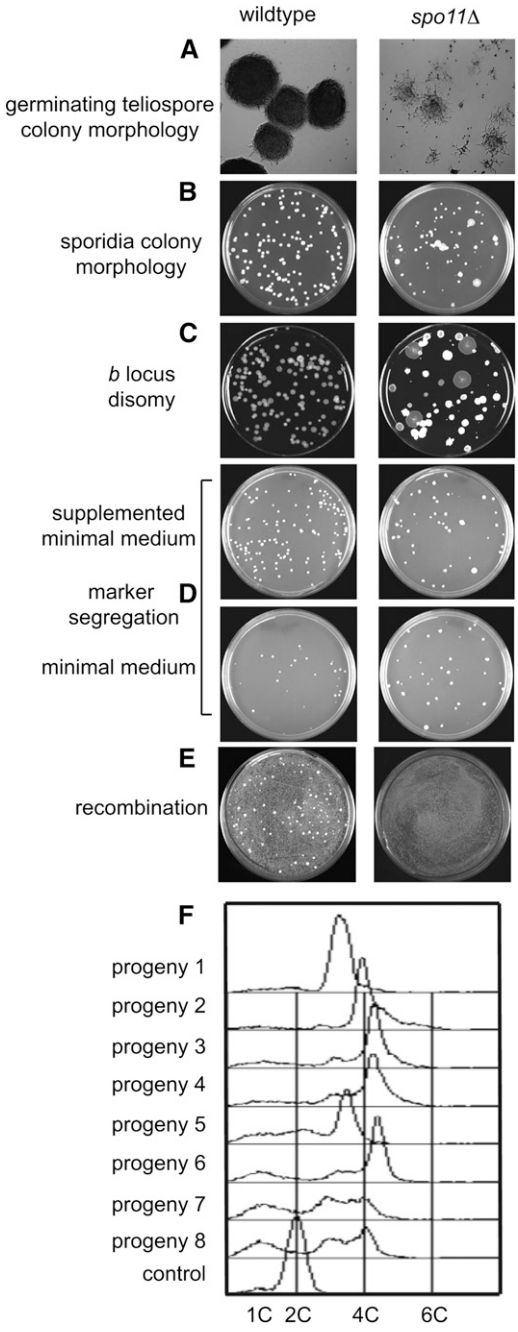

Figure 3 *spo11Δ* aberrant meiosis. Teliospores issuing from crosses between wild type (UCM350 × UCM520) or *spo11Δ* (UCM738 × UCM762) were (A) germinated on YEPS medium for 54 hr. Colonies were washed off plates, resuspended, and vortexed in water to disperse cells. Aliquots (~100 cells) were plated on (B) YEPS medium to display colony morphology of meiotic products; (C) charcoal-containing mating medium to reveal the fuz reaction indicating disomy at the b locus; (D) ammonium minimal medium with or without pantothenate and methionine to show non-Mendelian distribution of biosynthetic markers. (E) Cells (10^5 wild type or 10^7 *spo11Δ*) were spread on nitrate minimal with pantothenate and methionine to measure allelic recombination at *nar1* as Nar^+ prototrophs. (F) DNA content of individual isolated of *spo11Δ* meiotic progeny. Histograms indicate DNA content (*x*-axis), with 2C corresponding to G2 content of wildtype haploid control, and cell number (*y*-axis).

complete meiosis upon germination. However, it has not been clear whether the dormant teliospore diploid nucleus is present in a premeiotic S-phase state or arrested in meiotic prophase before the first meiotic division. The opaque nature of the dormant teliospore has precluded direct microscopic observation, so conclusions drawn have been based on inference from studies of teliospore development and germination. Part of the argument for premeiotic S-phase status of teliospores came from the early finding that diploids of *U. maydis* could be obtained from infected plant tissue. This suggested that free-living diploid cells with capacity for proliferative division could be part of the normal *U. maydis* life cycle and might undergo differentiation into teliospores but would arrest at a stage prior to entry into the meiotic program. On the other hand, accumulating evidence from other systems would argue that the teliospore nucleus is arrested after the onset of meiosis—after homologous recombination has started, but before the first meiotic division.

In a number of fungal systems it is commonly found that premeiotic DNA synthesis takes place before nuclear fusion of heterokaryons. This has been observed in both ascomycetes and basidiomycetes (Rossen and Westergaard 1966; Lu and Jeng 1975; Iyengar *et al.* 1977; Carmi *et al.* 1978). In these systems once karyogamy has occurred, the diploid nucleus immediately enters the meiotic program and proceeds with chromosome condensation, homolog alignment, synapsis, SC formation, SC disassembly, and then transitions to metaphase. It would seem reasonable that this same paradigm would apply in *U. maydis* in which case meiosis would be predicted to commence at the stage of infection in plant tissue when the dikaryotic hyphal cells become fragmented and rounded and a single nucleus becomes apparent. As teliospore development proceeds to maturity within the plant, the single nucleus present would then be predicted to arrest in meiotic prophase I after the major chromosome dynamic events of homologous recombination have taken place.

Ultrastructure studies on teliospores from several species of the basidiomycete rust fungi support the notion that the nuclei are developmentally arrested in prophase I (Boehm *et al.* 1992; Mims *et al.* 1996). In particular, teliospores of the red morning glory rust *Coleosprorium ipomoeae* have cell walls thin enough to enable staining and visualization of nuclei (Mims and Richardson 2005). SCs were apparent in dormant teliospores indicative of arrest in pachytene. Upon hydration of the teliospores and germination, the SCs rapidly disappeared within 2 hr as the nuclei entered first meiotic division. Similarly, in other rust fungi, SCs were observed in the nuclei of maturing spores shortly after karyogamy but disappeared as the teliospores entered dormancy, implying arrest at late prophase I.

The results of our study provide support for the above model in which the meiotic program of *U. maydis* initiates *in planta* immediately following karyogamy and arrests in late prophase I with teliospore maturation. The production of

four nuclei during germination of *cdc45^{ts}* teliospores at temperature restrictive for DNA replication strongly implies that dormant teliospores already contain four complete chromosome complements. Further, our demonstration that diploids could be recovered from infected plant tissue with heteroallelic recombination at the *nar1* locus comparable to that observed following meiosis places the timing of meiotic homologous recombination at a stage in the life cycle shortly after that for karyogamy, well before teliospore maturation. Therefore, germination of teliospores can be taken as a visual landmark for meiotic checkpoint release and entrance into the first meiotic division.

Diploid strains of *U. maydis* can arise through failure of reduction during meiosis. These can be recovered at low frequency as prototrophs after germinating teliospores issuing from crosses of strains with complementing nutritional markers (Holliday 1961). But in addition, diploids with unlimited growth potential can also be recovered from tissue of infected plants well before the appearance of teliospores (Holliday 1961). This procedure, which again relies on the presence of complementing nutritional markers in the parental strains to enable selection of prototrophs, was useful for strain construction in early genetic studies on mitotic recombination in diploids. Our finding that meiotic levels of allelic recombination (gene conversion) have occurred in such diploids recovered from infected plants speaks to their origin and nature. The high level of allelic recombination implies that these diploids must have entered the meiotic recombination program initiated by introduction of Spo11-mediated DSBs. However, the recovery of mitotically growing diploid recombinants after extracting plant tissue and plating on selective medium *in vitro* implies that these cells entered the meiotic program, but then exited back into the mitotic cell cycle before complete commitment to meiosis. Given that the teliospore nucleus is arrested in late meiotic prophase, the return to growth of these pre-teliospore diploids must occur prior to the teliospore meiotic arrest point. This is analogous to the return to mitotic growth observed in the case of diploids of budding yeast induced to enter the meiotic program by starving for nitrogen and eliminating glucose. When challenged by a change in nutritional status, yeast cells can return to mitotic growth until a certain temporal event at which a point of commitment to meiosis is reached (Simchen 2009; Dayani *et al.* 2011). In yeast this event appears to involve separation of the duplicated spindle pole body in preparation for formation of the meiosis I spindle, and possibly a similar event limits the ability to recover *U. maydis* diploids from the infection stage. If it is true that *U. maydis* diploids enter the meiotic program immediately after fusion of the heterokaryon nuclei, and that those that can be recovered are done so under the artificial *in vitro* condition of selecting for prototrophy on minimal medium, then it seems unlikely that in the natural course of plant infection, diploids would exist in a free living state.

An unexpected finding was the attenuation of virulence in crosses with *spo11*Δ. When plants were infected with *spo11*Δ strains they exhibited mild symptoms of disease and only rarely developed mature teliospores. Since Spo11 action is required to introduce DSBs in DNA (Keeney 2008) to initiate homologous recombination in diploid cells formed well into the infection process, it is not clear why or how the pathogenesis program would be compromised by a gene function that would seem relatively late acting relative to the other major events occurring during hyphal growth and development. Perhaps this means that there is an interdependent signaling between the homologous recombination program and the differentiation program underlying the pathogen–host relationship that is in place at early steps in the course of infection. Disease symptoms, tumors, and teliospores do form readily in crosses with mutants defective in homologous recombination such as *brh2*Δ and *rad51*Δ, but these gene functions act downstream of Spo11 and therefore might be past a crucial point of pathway interplay.

It was interesting to note that teliospores obtained from the *spo11*Δ cross were capable of germinating and producing sporidial progeny, in contrast to *brh2*Δ or *rad51*Δ teliospores, which failed altogether to germinate (Kojic *et al.* 2002). This may not be surprising given that unrepaired DSBs would be expected to cause greater lethality than an imbalance in chromosomes. The *spo11*Δ meiosis, however, was highly disturbed and characterized by lack of recombination and severe chromosome missegregation among the surviving progeny. In the absence of the primary system for ensuring accurate pairing and segregation of homologs, it is all the more remarkable that viable progeny were still capable of being produced. Given that the chromosome number in *U. maydis* is 23 (Kamper *et al.* 2006), it would be extremely unlikely that viable progeny could arise by random segregation of a perfect complement of chromosomes as happens at a modest frequency in organisms with low chromosome number, such as *S. pombe* (Davis and Smith 2003). This attests to an extreme plasticity in *U. maydis* in its ability to tolerate severe chromosomal imbalances.

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