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The cell end marker Tea4 regulates morphogenesis and pathogenicity in the basidiomycete fungus *Ustilago maydis*

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Abstract

Positional cues localized to distinct cell domains are critical for the generation of cell polarity and cell morphogenesis. These cues lead to assembly of protein complexes that organize the cytoskeleton resulting in delivery of vesicles to sites of polarized growth. Tea4, an SH3 domain protein, was first identified in fission yeast, and is a critical determinant of the axis of polarized growth, a role conserved among ascomycete fungi. *Ustilago maydis* is a basidiomycete fungus that exhibits a yeast-like form that is nonpathogenic and a filamentous form that is pathogenic on maize and teozintle. We are interested in understanding how positional cues contribute to generation and maintenance of these two forms, and their role in pathogenicity. We identified a homologue of fission yeast *tea4* in a genetic screen for mutants with altered colony and cell morphology and present here analysis of Tea4 for the first time in a basidiomycete fungus. We demonstrate that Tea4 is an important positional marker for polarized growth and septum location in both forms. We uncover roles for Tea4 in maintenance of cell and neck width, cell separation, and cell wall deposition in the yeast-like form, and in growth rate, formation of retraction septa, growth reversal, and inhibition of budding in the filamentous form. We show that Tea4::GFP localizes to sites of polarized or potential polarized growth in both forms, as observed in ascomycete fungi. We demonstrate an essential role of Tea4 in pathogenicity in the absence of cell fusion. Basidiomycete and ascomycete Tea4 homologues share SH3 and Glc7 domains. Tea4 in basidiomycetes has additional domains, which has led us to hypothesize that Tea4 has novel functions in this group of fungi.

Keywords

Tea4 cell end marker; Polarized growth; Septum positioning; Cell morphogenesis; *Ustilago maydis*; Pathogenicity

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Appendix A. Supplementary material: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fgb.2014.02.010>.

1. Introduction

Cell polarity is a fundamental biological process by which asymmetry of cell components is generated. Cell polarity is crucial for cell morphogenesis, proliferation, and differentiation in all organisms. Positional cues are proposed to be involved in the generation of cell polarity. These cues are interpreted by protein complexes that organize the cytoskeleton for targeted delivery of vesicles to the site of polarized growth. Studies in different eukaryotes have greatly contributed to our understanding of possible mechanisms by which cell polarity is established (reviewed in Bi and Park, 2012; Huisman and Brunner, 2011; Nelson, 2003; Thompson, 2013).

In *Saccharomyces cerevisiae*, the axis of polarity is established before bud emergence, and is marked by cortical landmark proteins (reviewed in Bi and Park, 2012; Herskowitz et al., 1995). Recognition of the landmark results in hierarchical assembly of two GTPase modules: a Ras-like GTPase module (the Bud1/Rsr1 module) for bud site selection, which in turn recruits a Rho-type GTPase module (the Cdc42 module) for polarity establishment (reviewed in Bi and Park, 2012). Absence of the Bud1 GTPase module alters the axis of growth but not polarized growth itself, whereas absence of the Cdc42 GTPase module results in loss of cell polarity. The Cdc42 GTPase module is proposed to polarize the actin cytoskeleton and secretion towards the bud site. This entails activation and localization at the cell tip of the polarisome and the exocyst protein complexes (reviewed in Bi and Park, 2012; He and Guo, 2009).

In fission yeast, the rod-shaped cells grow at their poles, which are activated sequentially in a cell-cycle dependent manner (reviewed in Huisman and Brunner, 2011). The Tea proteins (Tea1, Tea3, Tea4/Wsh3) appear to mark the cell poles as the site of growth: in their absence, cells are bent and establish an ectopic axis of growth forming T-shaped cells, hence the name (Arellano et al., 2002; Martin et al., 2005; Mata and Nurse, 1997; Tatebe et al., 2005). The Tea proteins are thus key determinants of the axis of polarized growth. Tea1, a Kelch domain protein, forms a complex with other proteins, including Tea4/Wsh3 (hereafter referred to as Tea4), an SH3 domain protein, and is anchored at the cell tips by a cell membrane receptor (Mod5) (Martin et al., 2005; Snaith et al., 2005; Tatebe et al., 2005). The microtubule (MT) cytoskeleton and associated proteins (Tea2, a kinesin; Tip1, a CLIP-170 homologue) are required for localization of Tea1 and Tea4 to the poles and to maintain a normal axis of polarity (Busch et al., 2004; Huisman and Brunner, 2011). Tea4 at the cell tips recruits a formin, which is then activated by Cdc42 and Bud6, and nucleates actin cables, resulting in polarization of actin cables towards the cell poles (Martin et al., 2005, 2007). Additional proteins (for example, a Dyrk kinase and a PAK⁶⁵ kinase, and others) are important for marking the cell poles and activation of growth in response to the cell end markers (Bähler and Pringle, 1998; Huisman and Brunner, 2011).

Recent work indicates that *Aspergillus nidulans* homologues of fission yeast Tea1, Tea2, and Tea4 (TeaA, KipA, and TeaC, respectively) are necessary to stabilize the axis of growth. In their absence, hyphae grow in a zig-zag or meandering pattern (Higashitsuji et al., 2009; Konzack et al., 2005; Takeshita et al., 2008). Interactions among the cell end markers and between TeaC and SepA, a formin, suggest that a functionally conserved

module at the cell tip stabilizes the axis of polarized growth and nucleates actin in filamentous fungi (Higashitsuji et al., 2009; Takeshita et al., 2008). Studies in *Magnaporthe oryzae* support a role for Tea4 in stabilization of the axis of polarized growth, and have also uncovered a role for Tea1 and Tea4 in infectious structure development and pathogenicity (Dagdas et al., 2012; Patkar et al., 2010). Loss of MoTea4 leads to a zigzag morphology in the aerial hyphae, drastic reduction in conidiation, and altered pathogenicity (Patkar et al., 2010). Studies in *Colletotrichum lagenarium* have shown that ClaKel2, a Tea1 homolog, is involved in polarized growth. The *clakel2* mutant forms abnormal appressoria on glass slides but not *in planta* (Sakaguchi et al., 2008).

Ustilago maydis is a dimorphic fungus belonging to the Basidiomycota, in contrast to the above fungi, which belong to the Ascomycota. It exhibits a yeast-like non-pathogenic and a filamentous pathogenic form and can switch from one to the other. The switch is controlled by two mating type loci (*a* and *b*) and by nutritional input (Banuett, 1995, 2002; Klosterman et al., 2007; Vollmeister et al., 2012;). Cell fusion of haploid yeast-like cells that carry different *a* and *b* alleles results in formation of the pathogenic filamentous dikaryon (Banuett, 1995, 2002), whose growth in its hosts, maize and teozintle, results in distinct morphologies and the formation of a diploid spore, the teliospore (Banuett and Herskowitz, 1996). Diploid or haploid strains carrying different *a* and *b* alleles bypass cell fusion and are capable of forming a uninucleate filament that is pathogenic (Banuett and Herskowitz, 1989; 1994; Regensfelder et al., 1997; Ruiz-Herrera et al., 1995).

The *U. maydis* yeast-like form is haploid and divides by budding, forming a bud at one of the cell poles, slightly off center (Banuett and Herskowitz, 2002; Jacobs et al., 1994). The bud grows by incorporation of new cell wall material at the tip (Banuett and Herskowitz, 2002; Schuster et al., 2012). Cells can bud at either pole (Banuett and Herskowitz, 2002; Jacobs et al., 1994), and one pole can be used repeatedly (at least three times) before growth switches to the other pole. Daughter cells bud preferentially from the pole opposite their birth site (Valinluck et al., 2010). It is not known how one pole is chosen versus the other, how this switch is triggered, or what stabilizes the axis of growth at one of the cell poles. The *U. maydis* filamentous form is a dikaryon or diploid that grows at the tip cell. It is unbranched in culture, and consists of a long tip cell (>130 μ m) with the nuclei (in a dikaryon) or nucleus (in a diploid) in the cell center. The basal end consists of short septate compartments devoid of cytoplasm (Banuett and Herskowitz, 1994; Day and Anagnostakis, 1971; Steinberg et al., 1998).

We are interested in understating how the spatial organization of these cells contributes to the establishment and maintenance of an axis of polarized growth and the role of this cellular machinery in pathogenicity. We carried out a genetic screen to isolate mutants with altered colony and cell morphology. One of the identified genes has coding capacity for a protein that shares sequence similarity with *S. pombe* Tea4, *A. nidulans* TeaC, and *M. oryzae* MoTea4. Here we describe the characterization of *U. maydis tea4* and show that it is necessary for the axis of polarized growth, for normal septum positioning, and cell wall deposition in the yeast-like cell. We also show that maintenance of unbranched filaments in culture, growth rate of filaments, formation of retraction septa, and integrity of the filamentous tip are dependent on an intact Tea4 protein. In addition, we show that Tea4 is

necessary for pathogenicity of strains that bypass cell fusion to produce the pathogenic filamentous form but is dispensable in strains that undergo cell fusion.

2. Materials and methods

2.1. Strains, media, growth conditions, enzymes, and reagents

U. maydis strains used in these studies were: FB1 (*a1 b1*), FB2 (*a2 b2*), FBD12 (*a1/a2 b1/b2 ade-/ade⁺ met-/met⁺*), (Banuett and Herskowitz, 1989), SG200 (*a1 mfa2 bW1 bE2*) (Regensfelder et al., 1997), FB1 *tea4*, FB2 *tea4*, SG200 *tea4*, SG200 *tea4::gfp*, FBD12 *tea4::gfp/tea4* (this work). Media used were: YEPS (Tsukuda et al., 1988) for routine growth of *U. maydis* strains, UMC broth (*U. maydis* complete; Holliday, 1974) for growth of strains to be tested on charcoal agar (Banuett and Herskowitz, 1989) or for plant inoculations, charcoal agar (Holliday, 1974) and charcoal broth (Banuett and Herskowitz, 1994) for induction of filamentous growth. *U. maydis* strains were grown at 28 °C, unless otherwise stated. Procedures for cell wall, septum, and nuclear staining, as well as source of enzymes and reagents, and bacterial strains used are as described (Banuett and Herskowitz, 2002; Chew et al., 2008; Valinluck et al., 2010).

DNA-mediated transformation of *U. maydis* strains was as described (Chew et al., 2008; Valinluck et al., 2010). Transformants were selected on YEPS 1 M sorbitol supplemented with hygromycin B (200 µg/ml), and purified on the same medium without sorbitol.

2.2. Light microscopy and time-lapse video-microscopy

Cells were observed with Nomarski interference and epifluorescence optics using a BX61 Olympus microscope equipped with an ORCA CCD camera (Hamamatsu). Images were captured using Simple PCI imaging software (Hamamatsu) and processed with Adobe Photoshop CS.

For analysis of yeast-like morphology using time-lapse video-microscopy, wild type (FB1, FB2, SG200) and mutant strains (FB1 *tea4* and FB2 *tea4*) were grown in 4 mL of UMC broth to an OD₆₀₀ 0.3–0.4. A 3 µl aliquot of the culture was layered onto a pad of 45 µl UMC 1% glucose 0.7% agarose on a 1 × 1 cm well, covered with a glass cover slip, and sealed with parafilm. The slides were incubated at room temperature for 6–8 h before the start of image capture (Valinluck et al., 2010). Images were captured every 30 s, with 2 s light exposure during capture time. Sequences were converted into movie files at a speed of 15 frames/s.

For analysis of the filamentous morphology, wild type SG200 and mutant SG200 *tea4* strains were grown in 4 ml of UMC broth to an OD₆₀₀ 0.4–0.8. An aliquot of the culture was layered as above on a 45 µl charcoal agarose pad. The slides were incubated at room temperature for 6–8 h to allow induction of filamentous growth. Image capture and conversion to movie files were as described above.

2.3. Growth of *tea4* mutant and wild type strains on different media

To test the effect of different chemicals on growth of *tea4* null mutants, FB1 *tea4*, FB2 *tea4*, SG200 *tea4* and the parental strains were grown in YEPS broth to OD₆₀₀ 1.0,

washed once with water, and ten-fold serial dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) of each strain were prepared in a 96-well microtiter plate. A 3 μ l aliquot of each dilution for each strain was spotted on the following media: YEPS agar and YEPS agar containing 1 M sorbitol, 1 M KCl, 1 M NaCl, thiabendazole (TBZ at 0, 2, 5 or 10 μ g/ml), Calcofluor white (40 μ g/ml), or Congo Red (30 μ g/ml). Strains were also streaked on the same media. The plates were incubated at 28 °C. Colony growth and morphology were recorded over a 5 day period and photographed with a Nikon digital camera. At least three independent tests were carried out on three different days.

2.4. Induction of filamentous growth

2.4.1. Charcoal agar—Strains FB1, FB2, FB1 *tea4*, and FB2 *tea4* were grown overnight in UMC broth, and cross-streaked or co-spotted against each other on charcoal agar. Plates were sealed with Parafilm and incubated at room temperature. Strains SG200 and SG200 *tea4* were grown as above, 5-15 μ l aliquots spotted on charcoal agar, and plates incubated as above. Reactions were scored at 16, 24, 48, 72, and 96 h of incubation.

2.4.2. Charcoal broth—To assay ability to form filaments in liquid charcoal medium, strains SG200 and SG200 *tea4* were grown overnight in UMC, a 0.5 ml aliquot transferred to 4 ml of charcoal broth, and grown overnight. The cultures were concentrated 10-fold and 0.3 ml transferred to 10 ml of fresh charcoal broth, and grown at 28 °C in a water bath shaker at 110 rpm (Banuett and Herskowitz, 1994). Aliquots were removed at various times for 16–24 h, beginning 3 h after the start of incubation, stained with Congo Red and DAPI (or Calcofluor/Sytox green), and examined using Nomarski and epifluorescence microscopy.

2.5. Subcellular localization

To determine localization in the yeast-like form, strains FB1 *tea4::gfp*, SG200 *tea4::gfp*, and D12 *tea4::gfp* were grown in UMC broth to early log phase. To determine localization in the filamentous form, strains SG200 *tea4::gfp* and FBD12 *tea4::gfp* were grown in MM charcoal broth (see above) to induce the dimorphic transition. Aliquots were removed at various time intervals for 16–24 h, beginning at 3 h after induction, and examined using epifluorescence optics with a Chroma GFP filter. GFP fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, with a bandwidth of 7.5 nm.

2.6. Deletion of the *tea4* ORF

The entire ORF was deleted following the procedure of Brachmann et al. (2004). Briefly, a 1533 bp and a 1605 bp fragment immediately upstream and downstream of the *tea4* ORF, respectively, were amplified from genomic DNA with primers FBU205 (5'-GGCGCTTAATTAAGCGGCATCAATGTGCAGAG) and FBU206 (5'-GAGGCCATCTAGGCCATGACGCTGACATGATTG), and FBU207 (5'-CATAGTAGGCCTGAGTGGCCATTCTAGCATCGCTCTCCTC) and FBU208 (5'-CGCTTAATTAAGTCACGCACACGGCGTCGAC), respectively. Underline in FBU205 and FBU208 denotes a PacI restriction enzyme site, and in FBU206 and FBU207 a modified SfiI restriction site. The amplified fragments were cloned into pCR2.1 TOPO (LifeTechnologies) to generate plasmids pCR5T4 and pCR3T4, respectively, treated with

SfiI and HindIII, and the 1.6 kb fragment from pCR5T4 and the 5.5 kb fragment from pCR3T4 were ligated together with the SfiI hygromycin resistance cassette from plasmid pMF1-h (Brachmann et al., 2004) to generate pCR2.1-5'T4-*hygB*-3' T4 that lacks the entire ORF (=pCR- *tea4*). The targeting fragment was released by treatment with PacI, then treated as described (Chew et al., 2008; Valinluck et al., 2010), introduced into haploid strains FB1, FB2, and SG200, and transformants selected as described above.

Deletion of the ORF was ascertained by PCR with primers FBU209 (5' - CCAACCACCGTGATACAGC) and FBU114 (5'-CTCGCTGGTAGTTA CCACG) or FBU210 (5'-CTAGATGCTGGCAAGAAGC) and FBU115 (5' - GGAGGAGATGACTACCTCG). Primer FBU209 is upstream of FBU205; FBU210 is downstream of FBU208, and FBU114 and FBU115 are within the *hygB* gene. PCR amplification with the above sets of primers generates a 2.3 kb fragment in the null mutant strains and no fragment in wild type strains (data not shown). This analysis was followed by Southern hybridization as described below.

2.7. Generation of a *tea4::gfp* fusion construct

A 1252 bp fragment from the 3' end of the *tea4* ORF was amplified with primers FBU 524 (5'-GGGATCGTCGAACAAGTTCCGCTA), which contains an endogenous XmnI site (underline), and FBU525 (5' -TAATATGGCCAAGAACCAACGAATGACGTC), which introduces a MscI site (underline). The amplified fragment, lacking the stop codon, was cloned into pCR2.1 TOPO, and the resulting plasmid digested with KpnI and MscI to obtain a 1296 bp fragment, which was ligated into KpnI and MscI-digested pMF3-h (Brachmann et al., 2004). This generates an in frame fusion of truncated *tea4* ORF to *gfp* (p'*tea4*ORF: :gfp-*hygB*). A 967 bp fragment from the 3' region immediately downstream of the *tea4* ORF was amplified with primers FBU522 (5'-ATTGGCCTGAGTGGCCTTCCGATATCTTAGC; underline = SfiI site) and FBU523(5'-TCGGCGCGAATTAATTCTTCCCCAAAAGTGCAC; underline = XmnI site), and cloned into pCR2.1 TOPO (pCR2.1-*tea4*G3). Digestion of this plasmid and of plasmid p '*tea4*ORF: :gfp-*hygB* with KpnI and SfiI generates a 4840 bp and a 5023 bp fragment, respectively, that were ligated to generate pCR2.1-*tea4*ORF: :gfp-*hygB*-3'*tea4* (=p*tea4*: :gfp). The targeting fragment was released with XmnI, treated as above, and introduced into strains FB1, SG200, and FBD12, with selection for hygromycin resistant transformants. All PCR amplified fragments used in generation of the *tea4*:*gfp* fusion construct were sequenced. Gene replacement was confirmed using Southern hybridization analysis (see below).

2.8. Isolation of genomic DNA, Southern hybridization analysis, and labeling of probes

Genomic DNA from putative FB1 *tea4*, FB2 *tea4*, and SG200 *tea4* transformants and the parental wild type strains (FB1, FB2, and SG200) was treated with restriction endonuclease AatII, fractionated on a 0.7% agarose gel, and transferred to an Immobilon membrane (Millipore) as described (Chew et al., 2008; Valinluck et al., 2010). The membrane was probed with a 521 bp DIG-labeled PCR fragment from the 3' region of *tea4* (see below), stripped, and re-probed with a 872 bp DIG-labeled PCR fragment from the *hygB* gene in pMF1-h (Brachmann et al., 2004).

For Southern hybridization analysis of putative strains FB1 *tea4::gfp*, SG200 *tea4::gfp*, and FBD12 *tea4::gfp*, and the parental strains FB1, SG200, and FBD12, genomic DNA was treated with *NarI* restriction endonuclease, fractionated as above, and the membrane probed with a 533 bp DIG-labeled PCR fragment from the *tea4* ORF (see below), stripped and reprobed as above with a 872 bp DIG-labeled PCR fragment from the *hygB* gene in pMF1-h.

The following probes were labeled with the PCR DIG-labeling kit (Roche): a 522 bp fragment from the *tea4* 3' region with primers FBU252 (5' -ACCCTCAATCTCTCCTCG) and FBU253 (5' -TGGTGCTGTTACGCCATC); a 872 bp fragment from the *hygB* gene with primers FBU79 (5' -GCGAGTACTTCTACACAG) and FBU80 (5' -GCTTTCAGCTT CGATGTAGG); a 533 bp fragment from the *tea4* ORF with primers FBU347 (5' -CGTCTGGTGCCGACAGCC) and FBU348 (5' -CATCCCCGTCGCTTGCG). Conditions for PCR and purification of probes were as described (Chew et al., 2008; Valinluck et al., 2010).

2.9. Plant inoculations

Teozintle (*Zea mays subsp. mexicana* [Durango] or *subsp. parviglumis* [Guerrero]) and maize cultivar W23, grown in a greenhouse, were inoculated with a mixture of (a) FB1 + FB2, (b) FB1 + FB2 *tea4*, (c) FB2 + FB1 *tea4*, (d) FB1 *tea4* + FB2 *tea4*, or with pure cultures of (e) SG200 or (f) SG200 *tea4*. Seedlings were inoculated 7 days post-germination and symptom development monitored on a daily basis for 3 weeks and recorded photographically. The inoculations were repeated on three different occasions.

3. Results

3.1. Umtea4 codes for a protein with similarity to the *Schizosaccharomyces pombe* cell end marker Tea4

The *U. maydis tea4* gene was identified in a genetic screen for mutants with altered colony and cell morphology (Banuett, unpublished). The *tea4* open reading frame (ORF) spans 5052 nucleotides, has no introns, and a coding capacity for a protein of 1684 amino acids (Fig. 1) with a predicted MW 181 kDa and a pI of 5.6 (MUMDB um01012; <http://mips.gsf.de/genre/proj/ustilago/>; GenBank, Accession No. KF896301). The derived amino acid sequence of the putative Tea4 protein was compared with proteins in the NCBI database using Blast analysis (<http://www.ncbi.nlm.nih.gov/blast>). The *Ustilago* protein has similarity to the well-characterized *S. pombe* Tea4 and *S. cerevisiae* Bud14 proteins, and to the more recently identified *A. nidulans* TeaC and *M. oryzae* MoTea4 proteins (Martin et al., 2005; Tatebe et al., 2005; Knaus et al., 2005; Higashitsuji et al., 2009; Patkar et al., 2010). The putative *Ustilago* Tea4 protein contains several domains (Fig. 1): Src homology (SH3; amino acids 202–263), phosphatase type I (Glc7) binding domain (amino acids 338–342), coiled-coil (CC; 422–438; not shown); putative nuclear localization signal (NLS; amino acids 651–665), Ras associating (RA; amino acids 783–917), conserved domain of unknown function (amino acids 979–1155), and Ala-rich tract (amino acids 1453–1465; not shown) (<http://prosite.expasy.org>; <http://pfam.sanger.ac.uk>; <http://smart.embl-heidelberg.de>; http://www.ch.embnet.org/software/COILS_form.html). Multiple sequence alignment of UmTea4 with other Tea4 homologues in the Basidiomycota and Ascomycota using ClustalW

(Thompson et al., 2002) and MultAlin (Corpet, 1988) showed that all Tea4 homologues in these phyla contain the SH3 and Glc7 binding domains, whereas the RA domain the NLS, and other conserved domains of unknown function appear to be specific to Tea4 homologues in the Basidiomycota (Fig. 1). The presence of a conserved RA domain and other domains exclusively in Tea4 homologues in the Basidiomycota suggests that Tea4 may have novel functions in this group of fungi. It is possible that these new domains are present in Tea4-interacting partners in the Ascomycota.

3.2. Tea4 mutants exhibit altered cell morphology, septum positioning, and cell wall deposition in the yeast-like form

To determine the *tea4* null phenotype in several cell processes, we generated a *tea4* null mutation in haploid strains FB1, FB2, and SG200 by a one-step gene replacement procedure (see Materials and Methods). This mutation removes the entire ORF and replaces it with a 2722 bp hygromycin resistance cassette (Fig. S1A). Gene replacement was confirmed by Southern hybridization analysis (Fig. S1B and C).

Wild type strains form smooth colonies on YEPS agar (Fig. S1D), whereas *tea4* mutants form colonies with multiple ridges and rough edges, giving a lacy appearance (Fig. S1E). Because altered colony morphology may reflect altered cell morphology, we examined cells of the *tea4* null mutants in asynchronously growing cultures in YEPS broth with Nomarski optics during early, mid, and late logarithmic phase of growth.

Wild type cells form a bud at one of the cell poles, slightly off center, once per cell cycle (Fig. 2) (Banuett and Herskowitz, 2002; Jacobs et al., 1994) and continue forming buds from the same cell pole for at least three cell cycles before switching to the opposite pole (Fig. 2A–D). Daughter cells bud preferentially from the distal end (the end farthest from their birth site) at least for the first three cycles of budding (Fig. 2C and D) (Valinluck et al., 2010). The doubling time for wild type cells grown on agarose pads averages 1 h 42.5 min with a growth rate of 7.1 $\mu\text{m}/\text{h}$, which corresponds roughly to the doubling time of cells grown in YEPS medium in shaking culture (Banuett and Herskowitz, 2002; Valinluck et al., 2010). Wild type cells maintain their characteristic cigar shape with an average cell length of $15.7 \pm 1.1 \mu\text{m}$ and an average width of $3.1 \pm 0.2 \mu\text{m}$. The width of the mother-bud neck remains constant at $0.81 \pm 0.03 \mu\text{m}$ throughout the budding process (see also Valinluck et al., 2010). This budding pattern is consistent among different wild type haploid and diploid strains grown in rich medium, with slight strain-dependent variations in cell length and width (Valinluck and Banuett, unpublished). Even after long periods of growth in slab culture, wild type cells maintain their normal elongated shape and budding pattern.

In contrast, the *tea4* mutants form buds at one or both cell poles (Fig. 3A and B). In early log phase, it is common to observe cells with bifurcate buds (Fig. 3A; buds 2a and 2b; buds 3a and 3b) or a fan of buds (Fig. 3B; buds 1–5). These buds grow at an angle approximately 45–90° with respect to another bud (Fig. 3A, arrowhead). These observations indicate that *tea4* is necessary for the axis of polarized growth in early and mid-log phase cells. In late log phase, mutant cells increase in cell width (Fig. 3C) and a septum (Fig. 3C, arrowhead) forms in the cell middle, partitioning the cell into two smaller ones (Fig. 3C, M1 and M2). In wild type cells, septa do not form in the cell middle. These observations indicate that Tea4 is

necessary for maintenance of normal cell width and septum positioning. Saturated cultures contain mostly clusters of spherical cells with long appendages that remain attached to the mother cell (not shown), forming large cell clumps.

To further investigate septum positioning and cell wall deposition, we examined cells from asynchronously exponentially growing cultures after staining with Congo Red or Calcofluor White. Wild type cells contain one, two, or no septa in the neck region depending on the cell cycle stage (Fig. 4G–I). Cells ready to separate (e.g., large-budded cells) have two closely apposed septa (Fig. 4I) (Banuett and Herskowitz, 2002; O'Donnell and McLaughlin, 1984; Valinluck et al., 2010; Weinzierl et al., 2002). Wild type cells exhibit uniform faint cell wall staining, with more intensely staining bud scars and septa (Fig. 4G–I). In contrast, *tea4* mutant cells exhibit abnormal septum positioning and aberrant cell wall deposition. Wide and elongated cells or clusters of spherical cells contain a septum in the cell mid region (Fig. 4A, C; asterisks) and exhibit abnormal cell wall deposits in the form of striations or bands on the cell surface (Fig. 4C; cws; 4E, acw). These abnormal deposits of cell wall material are not observed in wild type cells. Taken together, these observations indicate that Tea4 is necessary for normal positioning of the septum and for normal cell wall deposition. Nuclear staining of *tea4* mutant cells and cell clusters reveals a single nucleus per cell. We did not observe multinucleated cells (Fig. S2).

Additional observations indicate that the mutants have altered cell wall properties or composition: increased sensitivity to the presence of compounds that interfere with cell wall biosynthesis (e.g. CongoRed, Calcofluor White; Roncero and Durán, 1985) and to high salt in the medium (Fig. S3B–D). In addition to altered cell wall properties, the *tea4* mutants are more sensitive to 1 M sorbitol (Fig. S3E), indicating that they have a defect in the stress response to high osmolarity. In *S. pombe*, *tea4* mutants were shown to be defective in the stress response (Tatebe et al., 2005). Lastly, *tea4* mutants are slightly more sensitive than wild type strains to the microtubule depolymerizing drug thiabendazole, suggesting that the microtubule cytoskeleton is slightly compromised (Fig. S3F).

3.3. Analysis of cell morphology of *tea4* mutant yeast-like cells using time-lapse video-microscopy

To gain a better understanding of the temporal sequence of events leading to the aberrant morphology of *tea4* mutants, we followed cell growth on UMC agarose pads for at least 11 h using time-lapse video-microscopy (Fig. 5; $t = 0$ to $t = 11$ h; see Movie 1). These events were compared to those observed during growth of wild type strains under the same conditions (see Fig. 2).

Tea4 mutant cells exhibit several morphological alterations, which increase in severity as the cells become older. They initially grow at a rate similar to that of wild type cells (7.1 $\mu\text{m}/\text{h}$), but growth rate decreases as a function of time, and cells become aberrantly shaped and form abnormal buds (Fig. 5; $t = 0$ h to $t = 11$ h). Newly formed *tea4* cells have similar dimensions to wild type cells, with an average width of $2.9 \pm 0.2 \mu\text{m}$ and an average length of $12.4 \pm 0.3 \mu\text{m}$ compared to $3.1 \pm 0.2 \mu\text{m}$ and $15.7 \pm 1.1 \mu\text{m}$, for wild type cells. The width of *tea4* mutant cells increases gradually to $4.3 \pm 0.3 \mu\text{m}$, and even to $5.63 \pm 0.31 \mu\text{m}$, approximately 1.5–2 $\times$ wider than wild type cells. The neck region broadens as the bud

grows ($2.32 \pm 0.11 \mu\text{m}$ compared to $0.81 \pm 0.03 \mu\text{m}$ in wild type cells) and in some cases its width equals that of the mother and daughter cells (Fig. 5, $t = 3 \text{ h}$ to $t = 11 \text{ h}$). Increase in cell width and reduced elongation results in rounded cells in older cultures. Altered septum placement occurs early in the growth of cells (Fig. 5, $t = 0$ to $t = 4 \text{ h}$) and leads to formation of clusters of enlarged attached cells (Fig. 5, $t = 2 \text{ h}$; cells #1–4). The outermost cells in this cluster give rise to buds (Fig. 5, $t = 3 \text{ h}$), which can be wild type-like (Fig. 5, $t = 4$ to $t = 9 \text{ h}$; [buds b3–1, b3–2, b3–3]), or aberrant (bent, wider, and with constrictions near their subapical region) (Fig. 5, $t = 7 \text{ h}$ to $t = 10 \text{ h}$ [buds b4a-1 and b4b-1]). These buds in turn can form lateral buds at right angles to the axis of the cell (Fig. 5, $t = 10 \text{ h}$; arrow) (see also Fig. 5, $t = 4 \text{ h}$ to $t = 9 \text{ h}$). Older rounded cells in a cluster form appendages that grow to an average length of $28.1 \pm 0.3 \mu\text{m}$ (Fig. 5, $t = 9 \text{ h}$ to $t = 11 \text{ h}$; ap). These appendages bud in two different ways: by constriction in the subapical region (Fig. 5, $t = 9 \text{ h}$ [b5–1, arrowheads]) or by extension of a bud from the sides (Fig. 5, $t = 11 \text{ h}$; [b, arrow]).

The altered axis of polarized growth, gradual increase in cell and neck width combined with centrally misplaced septa and reduced elongation rate as well as a defect in cell separation result in clusters of aberrantly shaped cells characteristic of *tea4* null mutants. These observations clearly demonstrate that Tea4 is necessary not only for the axis of polarized growth and septum positioning but also for several other processes that contribute to generation of the normal cell shape of *U. maydis* yeast-like cells.

3.4. Tea4 mutants exhibit altered filament formation on charcoal agar

We determined ability to form filaments on charcoal agar by cross-streaking strains FB1, FB2, FB1 *tea4*, and FB2 *tea4* against each other (Fig. S4A–C). A mixture of two wild type strains results in a characteristic white fuzzy reaction after overnight incubation (16–24 h) due to cell fusion followed by filamentous growth of the dikaryon. A mixture of a wild type and a *tea4* strain results in a reaction that is approximately 60% that between wild type strains at 24 h (Fig. S4A, B and D), and a mixture of two *tea4* strains results in a reaction that is approximately 25% that between wild type strains at 24 h (Fig. S4A, B, and E) but becomes indistinguishable from the wild type reaction after 48 h (data not shown). Thus, the *tea4* mutation appears to delay but not abolish filament formation upon cell fusion of two *tea4* strains (see below).

The above results suggest that the *tea4* mutation does not impair cell fusion or filament formation, albeit it delays the response. To further examine the role of *tea4* in filament formation, we analyzed a strain that bypasses cell fusion for filament formation (SG200) and its isogenic derivative (SG200 *tea4*) on charcoal agar. Strain SG200 forms filaments after overnight incubation (16–24 h) whereas SG200 *tea4* does not, even after prolonged incubation (72 h or longer; (Fig. S4F, compare a and b). These results are rather surprising and indicate that Tea4 is necessary for filament formation on charcoal agar in strains that bypass cell fusion but is dispensable in strains that undergo cell fusion to form a filament. Alternatively, the SG200 *tea4* strain produces aberrant filaments but these do not result in the characteristic white fuzziness.

3.5. The *tea4* mutation alters septum positioning and nuclear division in the filamentous form

To help us understand the above observations, we examined the morphology of filaments using an independent assay in which filaments are induced in charcoal broth under nitrogen-limiting conditions (Banuett and Herskowitz, 1994). Strains were grown as described in Materials and Methods and aliquots analyzed over a period of 16–24 h beginning 3 h post-induction. SG200 (or FBD12, another solopathogenic strain) initiates filament formation from one of the cell poles of a yeast-like cell after 3 h incubation (Banuett and Herskowitz, 1994). The filament consists of a long tip cell ($>130\ \mu\text{m}$) (Figs. 6A and 7A) that grows at the apical end. The basal end consists of empty compartments separated by septa (Figs. 6A and 7B). The tip cell of these filaments contains a single nucleus (Fig. 6B, arrows), located approximately in the cell middle. This nucleus is arrested in the G2 phase of the cell cycle (García-Muse et al., 2003). Growth of SG200 *tea4* under the same conditions results in formation of filament-like structures, which arise from clusters of spherical cells (Fig. 6D). These filament-like structures are partitioned by septa into compartments (Fig. 6D, arrowheads) that contain cytoplasm and form lateral buds or branches (Fig. 6D, arrows). In contrast, wild type filaments do not form lateral buds or branches. The *tea4* filament-like structures contain multiple nuclei, with one nucleus per compartment (Fig. 6E). Taken together, these results indicate that *tea4* is necessary for septum positioning, maintenance of G2 nuclear arrest, and inhibition of lateral budding in the filamentous form. Furthermore, these studies indicate that SG200 *tea4* mutants are able to form filaments in liquid medium, albeit abnormal ones, but these abnormal filaments for unknown reasons do not produce the white fuzziness on charcoal plates (see Fig. S4).

3.6. Analysis of filamentous growth of wild type and *tea4* mutants using time-lapse video-microscopy

To gain a better understanding of the temporal sequence of events that lead to the *tea4* filamentous morphology, we used time-lapse video microscopy and compared the sequence of events in *tea4* mutants to those of wild type strains (Figs. 8–10; see Movies 2, 3, and 4). Strains SG200 and SG200 *tea4* were grown on agarose charcoal pads and their growth followed for at least 9 h. Image capture and processing are described in Materials and Methods.

3.6.1. Wild type strains—Wild type filaments have a consistent width of $2.50 \pm 0.02\ \mu\text{m}$ ($n = 186$) and extend to greater than $130\ \mu\text{m}$ in length (Fig. 8, $t = 0$ to $t = 3$ h; see Movie 2). The filament grows by apical extension (Fig. 8, $t = 0$ h, arrows) in a straight manner and can turn at sharp angles when encountering a physical barrier. The basal end forms vacuoles that coalesce to leave a compartment devoid of cytoplasm (Fig. 8, $t = 0$ h (b; arrowhead) to 1 h [v], inset c1). This compartment is separated from the tip cell by a septum (Fig. 8, $t = 30$ min [s; arrows]; $t = 1$ h [s; arrowheads]; inset c1; arrow). Continued tip growth and vacuolization at the basal end result in the filamentous morphology characteristic of *Ustilago* (Fig. 8, $t = 0$ h to $t = 3$ h). The growth rate is $75\ \mu\text{m/h}$, at least $10\times$ faster than that of yeast-like cells ($7.1\ \mu\text{m/h}$). This growth resembles, in many respects, an amoeboid-like movement: one end of the cell elongates and moves forward while cytoplasm at the other end retracts towards the growing end (See Movie 2).

Amazingly, wild type filaments are capable of reversing their growth direction, that is, the basal end now becomes the fast growing end (the apical end) and the old apical end becomes the new basal end (Fig. 9, $t = 1$ h 30 min to $t = 8$ h 30 min; see Movie 3). This can be readily observed in filament f2 (Fig. 9, $t = 2$ h 30 min to $t = 5$ h 30 min; f2). Filament f1 reverses growth direction twice during the duration of this sequence (Fig. 9, $t = 1$ h 30 min to $t = 5$ h 30 min; f1). It forms a new axis of growth at the basal end, while the apical end becomes the new basal end (Fig. 9, $t = 5$ h 30 min to $t = 6$ h 30 min; f1). The process is repeated once more (Fig. 9, $t = 6$ h 30 min to $t = 8$ h 30 min; f1). Growth reversal is commonly observed in wild type filaments. Physical barriers do not appear to be the inducing signal since many of the filaments are able to grow over other filaments or parallel to them (see Fig. 8) without induction of growth reversal. The factors involved in generation of this switch are unknown. We have observed increased activity of microtubules at the basal end prior to reversal (data not shown).

3.6.2. *tea4* mutants—The events occurring during growth of *tea4* filaments are in stark contrast to those of wild type filaments (Fig. 10, $t = 0$ to 6 h; see Movie 4; compare to Fig. 8 and Movie 2). The *tea4* cells initially form wild type-like filaments that exhibit amoeboid-like movement except that they grow in a less straight pattern compared to wild type filaments (Fig. 10, $t = 0$ h; filaments f1, f2, f3). The average width of the filaments is $2.70 \pm 0.05 \mu\text{m}$ ($n = 190$), slightly wider than wild type filaments, and the average length of the tip cell is $122 \mu\text{m}$. Even though the filaments exhibit wild type-like morphology, their rate of growth is slower than that of wild type filaments ($45 \mu\text{m/h}$ versus $75 \mu\text{m/h}$, respectively), and gradually decreases to $7 \mu\text{m/h}$. As growth rate decreases, the amoeboid-like growth also decreases, resulting in fewer instances of vacuolization and septation at the basal end of the filament. Diminished growth rate is accompanied by formation of apical and lateral buds or long appendages. In some instances, narrowing of the tip of the filament precedes apical budding (Fig. 10; $t = 1$ h; f1 arrowhead; $t = 2$ h; f1, arrowhead; inset; $t = 3$ h, filament f1 and cell #1), whereas in other cases, apical budding occurs by constriction of the subapical region to delimit a bud (Fig. 10; $t = 2$ h to $t = 3$ h, filament f2 and cell 2; arrow). Formation of lateral buds or long appendages increases as time progresses (Fig. 10; $t = 3$ h to $t = 4$ h; filament f3 and cell 3, arrow; filament f4 and cell 4, arrow; and inset in $t = 3$ h). These lateral buds reach a length of $30 \mu\text{m}$, longer than wild type buds, and initially extend at a rate of $24 \mu\text{m/h}$, greater than that of a wild type bud ($7.1 \mu\text{m/h}$) but slower than that of wild type filaments ($75 \mu\text{m/h}$) (see Movie 4). The lateral buds or appendages in turn bud from the tip (Fig. 10, $t = 6$ h; cells 4 and 4–1) or laterally. The region from which lateral buds arise is broad (Fig. 10, $t = 3$ h; inset), and has little resemblance to the characteristic neck region that separates mother from bud in wild type yeast-like cells. The *tea4* filamentous cells are unable to maintain filamentous growth: aberrant yeast-like growth becomes the dominant mode of growth (Fig. 10; see Movie 4). Thus, Tea4 is necessary for maintenance of growth rate of the filamentous form. Reduced growth rate may trigger a signal to switch to budding, which occurs in an aberrant manner.

3.7. Sub cellular localization of Tea4::GFP

To gain a better understanding of the function of Tea4 in *U. maydis*, we determined subcellular localization using Tea4::GFP in which GFP was fused in frame to the carboxy-

terminus of Tea4 (see Materials and Methods). Haploid strains containing a gene replacement with *tea4::gfp* expressed from the endogenous *tea4* promoter, and providing the sole source of Tea4, exhibited normal cell morphology, implying that the fusion protein is functional. In the filamentous form, Tea4::GFP localizes to the tip of the filament as a compact dot (Fig. 11A, arrow), cap (Fig. 11B), or multiple discrete dots (Fig. 11D, arrowheads). In some filaments, discrete faint dots of Tea4::GFP can be seen along the length of the filament, with a higher concentration in the subapical region close to the tip (Fig. 11A, C, E; arrowheads). These dots move towards the apical end and also to the basal end. A weak signal of Tea4::GFP can be detected at the basal end of the filaments (not shown). In the yeast-like form, Tea4::GFP localizes to one or both cell poles in unbudded cells (Fig. 11F, G; arrows) and to the bud tip in budded cells (not shown), but it was not possible to determine if this localization consisted of discrete spots or a cap. In some of the yeast-like cells, discrete dots of Tea4::GFP can also be observed throughout the cell. The Tea4::GFP signal in yeast-like cells is weaker than that in filaments. Localization to the neck region was faint and close to the limit of resolution (not shown). Localization of Tea4 to the basal end of the filament supports a role for Tea4 in growth reversal, or formation of retraction septa or both. In summary, Tea4::GFP localizes to sites of active or potential polarized growth.

3.8. Role of *tea4* in pathogenicity

To determine the role of *tea4* in pathogenicity, we inoculated maize and teozintle seedlings using two sets of strains (Fig. 12). In Set I, we inoculated with a mixture of wild type (*a1 b1* × *a2 b2*) or *tea4* mutant strains (*a1 b1 tea4* × *a2 b2 tea4*) (Fig. 12A and B). In Set II, we inoculated with pure cultures of a wild type solopathogenic strain (SG200) or its isogenic derivative (SG200 *tea4*) (Fig. 12C and D). The results were strikingly different. In Set I, inoculations with wild type haploid strains (*a1 b1* × *a2 b2*) or *tea4* mutant haploid strains (*a1 b1 tea4* × *a2 b2 tea4*) led to similar reactions (Figs. 12A, B and 5SB-D). Tumor induction was 80% in the former (Fig. 12A), and 60% in the latter (Fig. 12B). These tumors occurred on all aerial plant parts (leaf blade, ligule, leaf sheath) (Fig. 55B–D). Anthocyanin induction was very strong in teozintle plants (Fig. S5). Teratomas were commonly observed in infections of teozintle plants with mixtures of wild type strains but not with the wild type solopathogenic strain or mixtures of *tea4* mutant strains (Fig. S5D [ter; arrow]). In Set II, inoculations with the wild type strain (SG200) led to 70% tumor induction (Fig. 11C and S5A) on all aerial plant parts. In contrast, inoculations with SG200 *tea4* resulted in no tumors (Figs. 12D and S5E and F). The majority of plants (70%) did not show symptoms; about 10% showed chlorosis only, and about 20% showed anthocyanin pigmentation (Fig. 12D), which occurred at the edges of the leaf blade, along the midrib, or in a mottled appearance on the leaf blade (Fig. S5E and F). These results indicate that Tea4 is essential for tumor induction in strains that bypass cell fusion (e.g., the solopathogenic strains) but is dispensable when cell fusion occurs (See Discussion).

4. Discussion

We have characterized the homologue of the *S. pombe* cell end marker Tea4 in the dimorphic basidiomycete fungus *Ustilago maydis*. To our knowledge, this is the first time

this important cell end marker is characterized in a member of the Basidiomycota. Our work reveals that Tea4 is an important positional marker for polarized growth and septum location, functions conserved with Tea4 homologues in ascomycete fungi. In addition, our studies uncover Tea4 roles in maintenance of cell and neck width, cell separation, and cell wall deposition in the yeast-like form, and in growth rate, formation of basal compartments, and inhibition of budding in the filamentous form, and possibly a role in growth reversal. Our studies show that Tea4 localizes to sites of active or potential growth in the yeast-like and filamentous forms, which supports observations in ascomycete fungi (Higashitsuji et al., 2009; Martin et al., 2005; Patkar et al., 2010; Tatebe et al., 2005). We also demonstrate an essential role of Tea4 in pathogenicity in the absence of cell fusion.

4.1. Tea4 has acquired new domains in the Basidiomycota

Our analysis shows that the Tea4 protein in *U. maydis* contains domains not present in Tea4 homologues in the ascomycete fungi. In particular, the presence of a RA-domain suggests a possible link with small GTP-binding proteins in the control of cell morphogenesis. Potential candidates include Rho1 and Rac1, both of which have been shown to regulate cell morphogenesis in *U. maydis* (Mahlert et al., 2006; Pham et al., 2009). Rho1 as the catalytic subunit of β -glucan synthase could provide a critical link to the cell wall integrity pathway. We are currently exploring the role of the RA domain in Tea4 function and identifying potential binding proteins.

The UmTea4 protein has the signature Glc7 binding site (R/K-X-V/I-X-F). This domain is important for localization of a type I phosphatase to the bud cortex in budding yeast or the cell tips in fission yeast (Alvarez-Tabarés et al., 2007; Knaus et al., 2005). In budding yeast, Bud14 (Tea4 homologue) and the type I phosphatase Glc7 physically interact. The complex is thought to stabilize microtubule interactions at sites of polarized growth. The role of the Glc7 domain in filamentous fungi remains to be explored.

4.2. Tea4 is necessary for the axis of polarized growth

Our work demonstrates that Tea4 is an important determinant of the axis of polarized growth in *U. maydis*. Its localization to active and potential sites of polarized growth supports this contention. The *U. maydis* genome lacks sequences that code for most of the landmark proteins that direct axial or bipolar budding in *S. cerevisiae* but does contain the genes coding for components of the *S. cerevisiae* Bud1/Rsr1 and Cdc42 modules (Banuett et al., 2008; Kämper et al., 2006). The Cdc42 module plays a role in cytokinesis, not polarized growth. It is the Rac homologue, absent from the genomes of *S. cerevisiae* and *S. pombe*, which plays a role in polarized growth (Böhmer et al., 2007; Mahlert et al., 2006; Weinzierl et al., 2002). Our observations suggest that the strategy used by *U. maydis* for budding is not a replica of that used by *S. cerevisiae* and may involve novel combinations of components of the machinery used by *S. pombe* and *S. cerevisiae* in determining polarized growth.

In *U. maydis* yeast-like cells, absence of Tea4 results in mother cells that bud simultaneously at both cell poles, that is, both cell poles become active. The buds have an altered axis of growth. In *S. pombe*, deletion of *tea4* results in activation of an ectopic axis of growth leading to T-shaped or bent cells. These mutants fail to activate bipolar growth

during NETO, thus the cells exhibit monopolar growth (Martin et al., 2005; Tatebe et al., 2005). In *U. maydis*, Tea4 appears to be necessary for activation of one of the cell poles and to maintain the other one quiescent. The fact that Tea4 localizes to both cell poles of the yeast-like cell, as observed in *S. pombe* (Martin et al., 2005; Tatebe et al., 2005), suggests that additional polarity factors are involved in activation or suppression of one of the cell poles. As in fission yeast, polarity factors likely form distinct clusters at the cell cortex. It has been hypothesized that segregation of different polarity factors to distinct clusters that locate in different cortical subdomains may allow temporal and spatial regulation of cell polarity (Dodgson et al., 2013).

In *U. maydis* filaments, absence of Tea4 results in a less straight pattern of growth compared to that of wild type filaments, and in formation of lateral buds (secondary axes of growth), which are not observed in wild type filaments. Thus, Tea4 plays a role in growth direction of filaments and in inhibition of secondary axes of growth as demonstrated in *A. nidulans* and *M. oryzae* (Higashitsuji et al., 2009; Patkar et al., 2010).

4.3. Tea4 is necessary for septum positioning, cell wall deposition, and G2 arrest

In *U. maydis* wild type yeast-like cells, the site of cytokinesis is determined by the site of bud emergence, as in budding yeast, and occurs in the narrow neck region that separates the bud from the mother cell. (Banuett and Herskowitz, 2002; O'Donnell and McLaughlin, 1984; Weinzierl et al., 2002). A role for Tea4 in septum positioning is supported by the fact that *tea4* mutants form a septum in the cell middle, and when these cells form buds, only a single septum is present in the neck region in most buds. Mutants lacking components of the *U. maydis* Cdc42 GTPase module and the diaphanous-like formin Drf1 form a single septum in the neck region and fail to separate, but these mutants retain their normal cigar shape and do not form a septum in the cell middle (Böhmer et al., 2007; Freitag et al., 2011; Weinzierl et al., 2002). Rho1 depleted mutants form aberrant cell clusters with multiple buds. These buds contain a single septum in the neck region and fail to separate (Pham et al., 2009). Taken together, these observations indicate that the presence of two septa in the neck region is necessary for cell separation. As suggested above, Tea4 may control morphogenetic processes by interaction with Rho1.

Our observations indicate that the Tea4 mutant has altered cell wall composition or structure. Increased cell wall deposition was reported in *Motea4* mutants (Patkar et al., 2010). *MoTea4* is necessary for organization of the actin cytoskeleton suggesting that integrity of the actin cytoskeleton is important for delivery of cell wall components to the site of growth in this fungus (Patkar et al., 2010). Tea4 in *U. maydis* may act similarly. Precedent for a role of Tea4 in actin organization was first demonstrated in *S. pombe*, where Tea4 at the cell poles interacts with and recruits a formin, which nucleates actin filaments (Martin et al., 2005). A Tea4-formin interaction appears to be conserved in *A. nidulans* but remains to be demonstrated in other fungi (Higashitsuji et al., 2009).

In wild type *U. maydis* filaments, septum formation is restricted to the basal end, where vacuoles coalesce to form empty compartments separated by retraction septa. In contrast, *tea4* mutants form multiseptate filament-like structures, where each compartment contains cytoplasm and a single nucleus per compartment. This indicates that Tea4 controls

positioning of the septum at the basal end. Formation of basal compartments in *U. maydis* has been shown to be dependent on a kinesin, the diaphanous-like formin Drf1, and the RNA binding protein Rrm4 (Becht et al., 2006; Freitag et al., 2011; Steinberg et al., 1998). *U. maydis drf1* mutants also form multiseptate filaments, suggesting that Tea4 and Drf1 may share a function in formation of the retraction (basal) septa. Conceivably, Tea4 at the basal end could recruit Drf1 during active formation of retraction septa. It remains to be determined if Tea4 interacts with Drf1 or Srf1 (the other formin encoded by the *U. maydis* genome Banuett et al., 2008; Freitag et al., 2011; Kämper et al., 2006) to control septation.

Studies in *S. pombe*, *A. nidulans*, and *M. oryzae* support a role for Tea4 in septation. In *S. pombe*, Tea4 inhibits formation of septa at inappropriate sites, and in *A. nidulans*, TeaC inhibits formation of excess septa, whereas in *M. oryzae* Tea4 is necessary for the formation of two septa in the conidium and to inhibit formation of multiseptate germ tubes (Higashitsuji et al., 2009; Huang et al., 2007; Patkar et al., 2010).

U. maydis filaments in culture contain a single nucleus in a diploid or haploid strain or two nuclei in a dikaryon. The nucleus (or nuclei) appears to be arrested in G2 (García-Muse et al., 2003). The presence of multinucleate filament-like structures in *tea4* mutants suggests that Tea4 controls, directly or indirectly, nuclear division to ensure the presence of a single nucleus in each of the compartments. Although Tea4 contains a putative NLS, we did not detect Tea4 in the nucleus. This could be due to a weak signal from Tea4: :GFP or, alternatively, to a nonfunctional NLS.

4.4. Tea4 is necessary for cell and neck width

The increase in cell width in *tea4* mutant cells is likely due to increased delivery of membrane and cell wall remodeling components to the lateral cell surface. Interestingly, *S. pombe* Rga4 (GAP for Cdc42) mutants have increased cell width (Das et al., 2007; Kelly and Nurse, 2011). Rga4 is excluded from cell tips by the action of the DYRK kinase Pom1, and this appears to be important for activation of Cdc42 at the tips (Tatebe et al., 2008). The Tea1–Tea4 complex recruits Pom1 to sites of growth (Behrens and Nurse, 2002). Future work will determine if the Rga4 homologue in *U. maydis* acts in concert with Tea4 to control cell width.

U. maydis tea4 mutants exhibit a broad neck, and in some cases the boundary between mother and daughter cell is completely lost. The mechanism by which neck width is controlled in *U. maydis*, in general, and by *tea4*, in particular, remains to be determined, but may involve septins. *U. maydis* has four septins, deletion of either one results in lemon-shaped cells with wider necks (Alvarez-Tabarés and Pérez-Martín, 2010), however, the increase in neck width is not as dramatic as that described for *tea4* mutants. In *M. oryzae*, septins, F-actin, and Tea1 colocalize as a ring at the appressorium pore. It was hypothesized that septins scaffold F-actin via Tea1 at the appressorium pore during formation of the infection peg (Dagdas et al., 2012). This scaffolding is thought to provide rigidity to the emerging infection peg. In *U. maydis*, septins (Alvarez-Tabarés and Pérez-Martín, 2010; Böhmer et al., 2009) as well as Tea4 (this work) and Tea1 (a cell end marker) (Woraratnadharm and Banuett, unpublished) localize to the neck region in the yeast-like cell. It is possible that UmTea1 may have a similar role at the neck as Tea1 in the *M. oryzae*

appressorium pore. Absence of Tea4 may disrupt the proper localization of Tea1 at the neck or its ability to recruit proteins important for neck integrity.

4.5. Tea4 is necessary for pathogenicity of strains that bypass cell fusion

We demonstrate that Tea4 is necessary for pathogenicity of strains that bypass cell fusion to form the pathogenic form. In inoculations with mixtures of wild type haploid strains that differ at the *a* and *b* mating type loci, cell fusion results in formation of a dikaryotic filament that is pathogenic. Stable diploid strains have been generated in the laboratory by mating two haploid strains carrying different *a* and *b* alleles and complementing auxotrophic markers and selection for prototrophs (Banuett and Herskowitz, 1994). These diploids contain a single nucleus and the same genetic information as the dikaryon, however, differential gene expression is observed between the dikaryon and the diploid (Babu et al., 2005). The diploid strains can grow as yeast-like or filaments by simple manipulation of the medium. When inoculated into plants as pure culture, they grow as filaments and induce formation of tumors; the strains are said to be solopathogenic (Banuett and Herskowitz, 1994; Ruiz-Herrera et al., 1995). Haploid solopathogenic strains have been generated by genetic engineering. These strains induce tumors (Regensfelder et al., 1997). We observed that Tea4 is dispensable for pathogenicity when cell fusion is involved in formation of the filamentous pathogenic dikaryon (e.g., *a1 b1 dtea4* × *a2 b2 dtea4*). In contrast, Tea4 is essential for pathogenicity of solopathogenic strains, that is, strains that bypass cell fusion.

We propose that there are two functionally redundant pathways for pathogenicity: a cell fusion-dependent pathway (not necessarily *a* locus-dependent) that activates an unknown protein (designated X) and a Tea4-dependent one (downstream of the *b* locus). If cell fusion occurs, Tea4 becomes dispensable because X is active. If cell fusion does not occur, as in solopathogenic strains, X is inactive and Tea4 becomes essential for pathogenicity. Other explanations are also plausible.

The abnormal morphology of *tea4* mutant filaments may hinder penetration of the host or proliferation within host cells. The fact that 20% of the plants inoculated with the solopathogenic *tea4* mutant develop anthocyanin pigmentation suggests that there is penetration, albeit quite inefficiently. Studies in *M. oryzae* have shown that *Motea4* mutants are defective in pathogenicity and this appears to be the result of a defect in development of infection structures (Patkar et al., 2010). The studies in *M. oryzae* and *U. maydis* provide evidence for the important role of the Tea4 cell end marker in fungal pathogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Alvarez-Tabarés I, Pérez-Martín J. Septins from the phytopathogenic fungus *Ustilago maydis* are required for proper morphogenesis but dispensable for virulence. *PLoS One*. 2010; 5:e12933. [PubMed: 20885997]
- Alvarez-Tabarés I, Grallert A, Ortiz JM, Hagan IM. *Schizosaccharomyces pombe* protein phosphatase 1 in mitosis, endocytosis and a partnership with Wsh3/Tea4 to control polarized growth. *J Cell Sci*. 2007; 120:3589–3601. [PubMed: 17895368]
- Arellano M, Niccoli T, Nurse P. Tea3p is a cell end marker activating polarized growth in *Schizosaccharomyces pombe*. *Curr Biol*. 2002; 12:751–756. [PubMed: 12007420]
- Babu MR, Choffe K, Saville BJ. Differential gene expression in filamentous cells of *Ustilago maydis*. *Curr Genet*. 2005; 47:316–333. [PubMed: 15809875]
- Bähler J, Pringle JR. Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis. *Gene Dev*. 1998; 12:1356–1370. [PubMed: 9573052]
- Banuett F. Pathogenic development in *Ustilago maydis*: a progression of morphological transitions that results in tumor formation and teliospore production. In: Osiewacz, HD., editor. *Molecular Biology of Fungal Development*. Marcel Dekker; New York, Basel: 2002. p. 349-398.
- Banuett F. Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. *Ann Rev Gen*. 1995; 29:179–208.
- Banuett F, Herskowitz I. Bud morphogenesis and the actin and microtubule cytoskeleton during budding in the corn smut fungus, *Ustilago maydis*. *Fungal Genet Biol*. 2002; 37:149–170. [PubMed: 12409100]
- Banuett F, Herskowitz I. Discrete developmental stages during teliospore formation in the corn smut fungus *Ustilago maydis*. *Development*. 1996; 122:2965–2976. [PubMed: 8898211]
- Banuett F, Herskowitz I. Morphological transitions in the life cycle of *Ustilago maydis* and their genetic control by the *a* and *b* loci. *Exp Mycol*. 1994; 18:247–266.
- Banuett F, Herskowitz I. Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. *Proc Natl Acad Sci USA*. 1989; 86:5878–5882. [PubMed: 16594058]
- Banuett F, Quintanilla RH Jr, Reynaga-Peña CG. The machinery for cell polarity, cell morphogenesis, and the cytoskeleton in the basidiomycete fungus *Ustilago maydis*: a survey of the genome sequence. *Fungal Genet Biol*. 2008; (Suppl. 1):S3–S14. [PubMed: 18582586]
- Behrens R, Nurse P. Roles of fission yeast tea1p in the localization of polarity factors and in organizing the microtubular cytoskeleton. *J Cell Biol*. 2002; 157:783–793. [PubMed: 12034771]
- Becht P, König J, Feldbrügge M. The RNA-binding protein Rrm4 is essential for polarity in *Ustilago maydis* and shuttles along microtubules. *J Cell Sci*. 2006; 119:4964–4973. [PubMed: 17105762]
- Bi E, Park HO. Cell polarization and cytokinesis in budding yeast. *Genetics*. 2012; 191:347–387. [PubMed: 22701052]
- Böhmer C, Ripp C, Bölker M. The germinal centre kinase Don3 triggers the dynamic rearrangement of higher-order septin structures during cytokinesis in *Ustilago maydis*. *Mol Microbiol*. 2009; 74:1484–1496. [PubMed: 19906182]
- Böhmer C, Böhmer M, Bölker M, Sandrock B. Cdc42 and the Ste20-like kinase Don3 act independently in triggering cytokinesis in *Ustilago maydis*. *J Cell Sci*. 2007; 121:143–148. [PubMed: 18089648]
- Brachmann A, König J, Julius C, Fedbrügge M. A reverse genetic approach for generating gene replacement mutants in *Ustilago maydis*. *Mol Gen Genom*. 2004; 272:216–226.
- Busch KE, Hayles J, Nurse P, Brunner D. Tea2p kinesin is involved in spatial microtubule organization by transporting Tip1p on microtubules. *Dev Cell*. 2004; 6:831–843. [PubMed: 15177031]
- Chew E, Aweiss Y, Lu Cy, Banuett F. Fuz1, a MYND domain protein, is necessary for cell morphogenesis in *Ustilago maydis*. *Mycologia*. 2008; 100:31–46. [PubMed: 18488351]
- Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucl Acids Res*. 1988; 16:10881–10890. [PubMed: 2849754]

- Dagdas YF, Yoshino K, Dagdas G, Ryder LS, Bielska E, Steinberg G, Talbot NJ. Septin-mediated plant cell invasion by the rice blast fungus, *Magnaporthe oryzae*. *Science*. 2012; 336:1590–1595. [PubMed: 22723425]
- Das M, Wiley DJ, Medina S, Vincent HA, Larrea M, Oriolo A, Verde F. Regulation of cell diameter, For3p localization, and cell symmetry by fission yeast Rho-GAP Rga4p. *Mol Biol Cell*. 2007; 18:2090–2101. [PubMed: 17377067]
- Day PR, Anagnostakis AK. Corn smut dikaryon in culture. *Nat New Biol*. 1971; 231:19–20. [PubMed: 4932892]
- Dodgson J, Chessel A, Yamamoto M, Vaggi F, Cox S, Rosten E, Albrecht D, Geymonat M, Csikasz-Nagy A, Sato M, Carazo-Salas RE. Spatial segregation of polarity factors into distinct cortical clusters is required for cell polarity control. *Nat Commun*. 2013; 4:1834. [PubMed: 23673619]
- Freitag J, Lanver D, Böhmer C, Schink KO, Bölker M, Sandrock B. Septation of infectious hyphae is critical for appressoria formation and virulence in the smut fungus *Ustilago maydis*. *PLoS Pathog*. 2011; 7:e1002044. [PubMed: 21625538]
- García-Muse T, Steinberg G, Pérez-Martín J. Pheromone-induced G2 arrest in the phytopathogenic fungus *Ustilago maydis*. *Euk Cell*. 2003; 2:494–500.
- He B, Guo W. The exocyst complex in polarized exocytosis. *Curr Opin Cell Biol*. 2009; 21:537–542. [PubMed: 19473826]
- Herskowitz I, Park HO, Sanders S, Valtz N, Peter M. Programming of cell polarity in budding yeast by endogenous and exogenous signals. *Cold Spring Harb Symp Quant Biol*. 1995; 60:717–727. [PubMed: 8824447]
- Higashitsuji Y, Herrero S, Takeshita N, Fischer R. The cell end marker protein TeaC is involved in growth directionality and septation in *Aspergillus nidulans*. *Euk Cell*. 2009; 8:957–967.
- Holliday, R. *Ustilago maydis*. In: King, RC., editor. *Handbook of Genetics*. Vol. 1. Plenum; New York: 1974. p. 575-595.
- Huang Y, Chew TG, Wanzhong GW, Balasubramanian MK. Polarity determinants Tea1p, Tea4p, and Pom1p inhibit division-septum assembly at cell ends in fission yeast. *Dev Cell*. 2007; 12:987–996. [PubMed: 17543869]
- Huisman SM, Brunner D. Cell polarity in fission yeast: a matter of confining, positioning, and switching. *Semin Cell Dev Biol*. 2011; 22:799–805. [PubMed: 21803169]
- Jacobs CW, Mattichak SJ, Knowles JF. Budding patterns during the cell cycle of the maize smut pathogen *Ustilago maydis*. *Can J Bot*. 1994; 72:1675–1680.
- Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ, Banuett F, Kronstad JW, Gold SE, Muller O, Perlin MH, Wosten HA, de Vries R, Ruiz-Herrera J, Reynaga-Pena CG, Snetselaar K, McCann M, Perez-Martin J, Feldbrugge M, Basse CW, Steinberg G, Ibeas JI, Holloman W, Guzman P, Farman M, Stajich JE, Sentandreu R, Gonzalez-Prieto JM, Kennell JC, Molina L, Schirawski J, Mendoza-Mendoza A, Greilinger D, Munch K, Rossel N, Scherer M, Vranes M, Ladendorf O, Vincon V, Fuchs U, Sandrock B, Meng S, Ho EC, Cahill MJ, Boyce KJ, Klose J, Klosterman SJ, Deelstra HJ, Ortiz-Castellanos L, Li W, Sanchez-Alonso P, Schreier PH, Hauser-Hahn I, Vaupel M, Koopmann E, Friedrich G, Voss H, Schluter T, Margolis J, Platt D, Swimmer C, Gnirke A, Chen F, Vysotskaia V, Mannhaupt G, Guldener U, Munsterkötter M, Haase D, Oesterheld M, Mewes HW, Mauceli EW, DeCaprio D, Wade CM, Butler J, Young S, Jaffe DB, Calvo S, Nusbaum C, Galagan J, Birren BW. Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature*. 2006; 444:97–101. [PubMed: 17080091]
- Kelly FD, Nurse P. Spatial control of Cdc42 activation determines cell width in fission yeast. *Mol Biol Cell*. 2011; 22:3801–3811. [PubMed: 21849474]
- Klosterman SJ, Perlin MH, Garcia-Pedrajas M, Covert SF, Gold SE. Genetics of morphogenesis and pathogenic development of *Ustilago maydis*. *Adv Genet*. 2007; 57:1–47. [PubMed: 17352901]
- Knaus M, Camerini E, Pedruzzi I, Tatchell K, De Virgilio C, Peter M. The Bud14p–Glc7p complex functions as a cortical regulator of dynein in budding yeast. *EMBO J*. 2005; 24:3000–3011. [PubMed: 16107882]
- Konzack S, Rischitor PE, Enke C, Fischer R. The role of the kinesin motor KipA in microtubule organization and polarized growth of *Aspergillus nidulans*. *Mol Biol Cell*. 2005; 16:497–506. [PubMed: 15563609]

- Mahlert M, Leveleki L, Hlubek A, Sandrock B, Bölker M. Rac1 and Cdc42 regulate hyphal growth and cytokinesis in the dimorphic fungus *Ustilago maydis*. *Mol Microbiol*. 2006; 59:567–578. [PubMed: 16390450]
- Martin SG, Rincón SA, Basu R, Pérez P, Chang F. Regulation of the formin for3p by cdc42p and bud6p. *Mol Biol Cell*. 2007; 18:4155–4167. [PubMed: 17699595]
- Martin SG, McDonald WH, Yates JR 3rd, Chang F. Tea4p links microtubule plus ends with the formin for3p in the establishment of cell polarity. *Dev Cell*. 2005; 8:479–491. [PubMed: 15809031]
- Mata J, Nurse P. *Tea1* and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell. *Cell*. 1997; 89:939–949. [PubMed: 9200612]
- Nelson WJ. Adaptation of core mechanisms to generate cell polarity. *Nature*. 2003; 422:766–774. [PubMed: 12700771]
- O'Donnell KL, McLaughlin DJ. Postmeiotic mitosis, basidiospore development, and septation in *Ustilago maydis*. *Mycologia*. 1984; 76:486–502.
- Patkar RN, Suresh A, Naqvi NI. MoTea4-mediated polarized growth is essential for proper asexual development and pathogenesis in *Magnaporthe oryzae*. *Euk Cell*. 2010; 9:1029–1038.
- Pham CD, Yu Z, Sandrock B, Bölker M, Gold SE, Perlin MH. *Ustilago maydis* Rho1 and 14-3-3 homologues participate in pathways controlling cell separation and cell polarity. *Euk Cell*. 2009; 8:977–989.
- Regensfelder E, Spellig T, Hartmann A, Lauenstein S, Bölker M, Kahmann R. G proteins in *Ustilago maydis*: transmission of multiple signals? *EMBO J*. 1997; 16:1934–1942. [PubMed: 9155019]
- Roncero C, Durán A. Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: *in vivo* activation of chitin polymerization. *J Bacteriol*. 1985; 162:1180–1185. [PubMed: 2987185]
- Ruiz-Herrera J, Leon CG, Guevara-Olvera L, Carabez-Trejo A. Yeastmycelial dimorphism of haploid and diploid strains of *Ustilago maydis* in liquid culture. *Microbiology*. 1995; 141:695–703.
- Sakaguchi A, Miyaji T, Tsuji G, Kubo Y. Kelch-repeat protein Clakel2p and calcium signaling control appressorium development in *Colletotrichum lagenarium*. *Euk Cell*. 2008; 7:102–111.
- Schuster M, Treitschke S, Kilaru S, Molloy J, Harmer NJ, Steinberg G. Myosin-5, kinesin-1, and myosin-17 cooperate in secretion of fungal chitin synthase. *Embo J*. 2012; 31:214–227. [PubMed: 22027862]
- Snaith HA, Samejima I, Sawin KE. Multistep and multimode cortical anchoring of tea1p at cell tips in fission yeast. *Embo J*. 2005; 24:3690–3699. [PubMed: 16222337]
- Steinberg G, Schliwa M, Lehmler C, Bölker M, Kahmann R, McIntosh JR. Kinesin from the plant pathogenic fungus *Ustilago maydis* is involved in vacuole formation and cytoplasmic migration. *J Cell Sci*. 1998; 111:2235–2246. [PubMed: 9664045]
- Takeshita N, Higashitsuji Y, Konzack S, Fischer R. Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus *Aspergillus nidulans*. *Mol Biol Cell*. 2008; 19:339–351. [PubMed: 18003978]
- Tatebe H, Nakano K, Maximo R, Shiozaki K. Pom1 DYRK regulates localization of the Rga4 GAP to ensure bipolar activation of Cdc42 in fission yeast. *Curr Biol*. 2008; 18:322–330. [PubMed: 18328707]
- Tatebe H, Shimada K, Uzawa S, Morigasaki S, Shiozaki K. Wsh3/Tea4 is a novel cell-end factor essential for bipolar distribution of Tea1 and protects cell polarity under environmental stress in *S. pombe*. *Curr Biol*. 2005; 15:1006–1015. [PubMed: 15936270]
- Thompson BJ. Cell polarity: models and mechanisms from yeast, worms, and flies. *Development*. 2013; 140:13–21. [PubMed: 23222437]
- Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics*. 2002 Unit 2.3 (Chapter 2).
- Tsukuda T, Carleton S, Fotheringham S, Holloman WK. Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. *Mol Cell Biol*. 1988; 8:3703–3709. [PubMed: 2851726]
- Valinluck M, Ahlgren S, Sawada M, Locken K, Banuett F. Role of the nuclear migration protein Lis1 in cell morphogenesis in *Ustilago maydis*. *Mycologia*. 2010; 102:493–512. [PubMed: 20524583]

- Vollmeister E, Schipper K, Baumann S, Haag C, Pohlmann T, Stock J, Feldbrügge M. Fungal development of the plant pathogen *Ustilago maydis*. FEMS Microbiol Rev. 2012; 36:59–77. [PubMed: 21729109]
- Weinzierl G, Leveleki L, Hassel A, Kost G, Wanner G, Bölker M. Regulation of cell separation in the dimorphic fungus *Ustilago maydis*. Mol Microbiol. 2002; 45:219–231. [PubMed: 12100561]

Fig. 1.

Domain organization of *U. maydis* Tea4 and Tea4 homologues in the Basidiomycota and Ascomycota. Length of the predicted Tea4 protein in *U. maydis* is indicated as well as the range in length of the predicted Tea4 protein in the Basidiomycota and Ascomycota. The length of most Tea4 homologues in the Basidiomycota is approximately twice that in the Ascomycota. Sequences of Tea4 homologues were retrieved from fungal databases at Mycocosm (<http://genome.jgi.doe.gov/programs/fungi/index.jsf>) and the Broad Institute (<http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genome-initiative>) and their domain organization was determined using various protein databases (<http://prosite.expasy.org>; <http://pfam.sanger.ac.uk>; <http://smart.embl-heidelberg.de>). Sequence comparisons among Tea4 homologues in the Ustilaginomycotina, Pucciniomycotina, and Agaricomycotina reveal similar domain organization to that of UmTea4. Some Tea4 homologues in the Agaricomycotina contain additional domains (e.g., GPCR; not shown). Sequence comparisons among Tea4 homologues in different subphyla of the Ascomycota reveal that they all have a SH3 and Glc7 binding domain but lack the RA domain and other domains present in the Basidiomycota. Yellow rectangle = Src homology (SH3) domain; dark orange rectangle = Glc7 (phosphatase type 1) binding domain; light blue rectangle = putative nuclear localization signal (NLS); red rectangle = Ras-associating domain; green rectangle = other conserved domains of unknown function; coiled coil domain (CC; not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Cellular morphology and budding pattern of wild type strains. The sequence of photomicrographs documents morphology of wild type cells through multiple cycles of budding (Fig. 2A–D). Mother cell (M1) forms 3 buds (D1, D2, D3) from the same pole ($t = 1$ h to $t = 4$ h) and none from the opposite pole during this sequence. Daughter cell (D1) forms a bud ($t = 3$ h; D1–1) from the pole distal to its birth site. Mother cell (M2) forms two additional buds, B2 and B3, from the opposite pole from which B1 emerged ($t = 2$ h to $t = 3$ h; arrow). Time in hours is shown on the upper right-hand corner of each panel. Scale bar = 5 μ m in all panels.

Fig. 3.

Cell morphology of *tea4* null mutants. (A) Aberrant cell with bifurcate buds at both cell poles, for example, bud 2a and 2b (formed laterally on bud 1; arrowhead), and 3a and 3b. (B) Cell with a fan of buds (buds 1,2,3,4,5) at one cell pole. Each bud appears to arise laterally from the preceding bud. (C) Older cell partitioned into two cells (M1 and M2) by a septum in the cell middle (arrowhead). M1 and M2 form several aberrant buds (D1, D2, D3a and D3b, and B1 and B2, respectively). Scale bar = 5 μ m in all panels.

Fig. 4. Septum positioning and cell wall deposition in wild type and *tea4* mutant cells. (A, C, E) Staining of septa, bud scars, and cell wall in *tea4* mutants with Congo Red. (B, D, F) Nomarski view of cells in A, C, E, respectively. (G–I) Wild type cells stained with Congo Red. (A) Abnormal placement of septum (asterisk) in cell middle divides the cell into two (M1 and M2). M1 and M2 have abnormal cell wall deposition (arrowhead, acw) and their entire cell surface stains more intensely than that in the buds (arrows). Bud scars (bs, arrowhead) exhibit heavy cell wall deposition not observed in wild type cells (compare with G–I). (C) Clusters of rounded or abnormal cells with cell wall striations (arrowhead, cws) and more intense cell wall staining over the entire cell surface compared to that in the buds (for example, buds b1 and b2). These clusters of large cells are separated by a single septum (asterisks). (E) Elongated, wide cell (M) with multiple buds at one of the cell poles (arrow). Bud scars (bs, arrow) stain intensely. Arrowhead points to a band of intense cell wall

staining (acw). (G) Unbudded cell with bud scars (arrowhead). (H) Cell with large bud and a single septum (arrow, s) in the neck region. (I) Large budded cell with two septa (arrow, s). Scale bar = 5 μ m in all panels.

Fig. 5.

Time-lapse analysis of cell morphology of *tea4* yeast-like cells. Changes in cell morphology during growth of *tea4* (FB1 *tea4*) mutants on UMC agarose pads for at least 11 h ($t = 0$ to $t = 11$ h; see Movie 1). Image capture and processing were as described in Materials and Methods. A mother cell is divided into cells M1 and M2 by a septum in the cell middle ($t = 0$ h; white arrow). A bud forms at the pole of M2 and a bifurcate bud (arrowheads) at the pole of M1. Neck regions (black arrows) are ill-defined. New septa (arrows) partition M1 and M2 into a total of 4 cells ($t = 2$ h, cells 1, 2, 3, 4; arrowheads point to buds emerging from cells 3 and 4; arrows point to septa). Cell 3 forms wild type-like buds in sequential manner from the same cell pole and these buds in turn bud from their distal pole ($t = 3$ h to $t = 9$ h; buds b3-1, b3-2, b3-3, and b3-4). At the opposite end of the 4-celled cluster, the bifurcate bud has a different fate: 4a gives rise to bud b4a-1, and 4b to bud b4b-1 ($t = 3$ h to $t = 6$ h). Bud b4a-1 increases in length and width ($t = 3$ h to $t = 9$ h), and forms a bud at its tip ($t = 6$ h; white arrowhead points to broad neck region). The neck region between b4a-1 and its bud widens and the boundary between these two cells becomes blurred ($t = 7$ h; arrowhead points to neck region; arrow points to site of septum formation). Bud b4a-1 also forms a lateral bud near the septum ($t = 10$ h; arrow). Cell 4b gives rise to a wild type-like apical bud, b4b-1 ($t = 6$ h; neck region, black arrowhead). Growth of this bud becomes abnormal, with a constriction near the apical region ($t = 9$ h; arrowheads) and formation of a region devoid of cytoplasm ($t = 10$ h; arrowhead), eventually resulting in a wild type-like bud (b4b-1-1) ($t = 11$ h). Bud b4b-1 also forms a lateral bud, b4b-1-2, near the septum separating b4b-1-1 from b4b-1 ($t = 11$ h). Long appendages arise from the central cluster of rounded cells ($t = 10$ h to $t = 11$ h; ap) and form apical and lateral buds ($t = 11$ h; b, arrow). Time in hours is shown near the upper left hand corner of each panel. Scale bar = 5 μ m in all panels.

Fig. 6.

Septum and nuclear positioning in wild type and *tea4* filaments. Strains SG200 and SG200 *tea4* were grown in charcoal broth. Aliquots were stained with DAPI or Congo Red at various time intervals and examined microscopically using Nomarski and epifluorescence optics (see Materials and Methods). (A–C) Wild type filaments. (D–F) Filament-like structures formed by *tea4* mutants. (A, D) Strains treated with Congo Red to stain septa. (B, E) Strains treated with DAPI to stain nuclei. (A) Wild type filaments consist of a long tip cell and empty compartments at the basal end separated by septa (arrowheads). Arrow points to the yeast-like cell from which the filament emerged during induction of the dimorphic transition. (B) Filaments f1 and f2 have a single nucleus (n1 and n2, respectively) located approximately in the cell middle. (C) Nomarski view of filaments f1 and f2 in B. Arrowheads point to yeast-like cells that gave rise to the filaments. (D) Multiple filament-like structures formed by *tea4* strains upon induction of the dimorphic switch. Septa (arrowheads) separate compartments, which contain cytoplasm (not all septa are marked). Lateral buds or branches (arrows) arise from these compartments. (E) DAPI-stained *tea4* filament-like structures containing multiple nuclei, with a single nucleus per compartment in each of the filaments. For example, filament f1 contains nuclei n1–1 and n1–2 (arrows), and filament f2 contains nuclei n2–1, n2–2, n2–3, and n2–4 (arrows). (F) Nomarski view of filament-like structures in E. Black specks are pieces of charcoal in the medium used for induction of the dimorphic switch. Scale bar = 5 μ m in all panels.

Fig. 7.

Filamentous morphology of wild type *U. maydis* strains. Strains SG200 and FBD12 were grown in charcoal broth and examined microscopically using Nomarski optics. (A) Wild type *U. maydis* filaments consist of a long apical cell ($>130\ \mu\text{m}$) that exhibits hyperpolarized growth at the tip (the apical end). (B) The basal end consists of empty compartments formed by the coalescence of vacuoles (arrows) and retraction of the cytoplasm at the basal end as the filament extends at the tip. The empty compartments are separated by septa (arrowheads). Scale bar = $5\ \mu\text{m}$ in both panels.

Fig. 8.

Time-lapse analysis of filamentous growth of *U. maydis* wild type strains. Time-lapse sequence documenting the filamentous morphology of wild type strains grown on MM charcoal agarose pads for at least 9 h (see Movie 2). Image capture and processing are described in Materials and Methods. Several filaments (f2–f5) exhibit apical growth ($t = 0$ h; arrows; inset a1 shows magnification of a tip). The basal end of filament f6 is clearly visible ($t = 0$ h; b, arrowhead). As the filament grows at the apical end, the cytoplasm at the basal end retracts towards the growing tip in an amoeboid-like movement. Active coalescence of vacuoles (v) at the basal (b) end and generation of empty compartments separated by septa can be observed in several filaments (f6; $t = 30$ min to $t = 1$ h; s, arrows in Panel B and arrowheads in Panel C; f2 and f3; v, arrows; inset c1 shows magnification of vacuoles in the basal region of filament f2. Arrow, septum; arrowhead, vacuoles). At $t = 1$ h, filament f6 has moved out of the field of view, leaving a track of empty compartments (arrowheads). Apical growth of filament f1 is reactivated and the basal end becomes visible ($t = 3$ h; arrowhead). The apical end of other filaments (f7–f10) appears in the field of view ($t = 1$ to $t = 3$ h). Filamentous growth continues in the same fashion for many hours without budding or lateral branch formation. Growth rate of filaments is $75 \mu\text{m/h}$ (see Movie 2). The yeast-like cells in the upper right hand corner in Panel D are cells that did not undergo the dimorphic transition. Black specks in the background are pieces of charcoal in the medium. Time in hours is shown on the bottom right hand corner of each panel. Scale bar = $5 \mu\text{m}$ in all panels.

Fig. 9.

Reversal of tip growth in *U. maydis* wild type filaments. Time-lapse sequence documenting growth reversal in wild type filaments (see Movie 3). The sequence demonstrates that *U. maydis* filaments can form a new axis of growth at the basal end and that the apical end becomes the new basal end. Filament f1 grows at the apical end (arrow) and forms empty compartments separated by septa (arrowheads) at the basal end ($t = 1$ h 30 min) as described in Fig. 8. It extends from left to right. Filament f2 appears in the field of view and grows over an empty compartment of f1 ($t = 2$ h 30 min to $t = 3$ h 30 min). It grows from top to bottom (long arrow). Filament f2 reverses growth direction from bottom to top ($t = 5$ h 30; long arrow). Filament f1 forms a new axis of growth ($t = 6$ h 30 min; long arrow) at the old basal end (arrowhead) and now grows from right to left (long arrow) (compare $t = 1$ h 30 min with $t = 6$ h 30 min). The asterisk (Panel E) marks the new basal end (formerly the apical end). Growth is reversed once again, from left to right ($t = 8$ h 30 min), and a new apical end forms (asterisk) at the previous basal end. The former apical end ($t = 6$ h 30 min) now becomes the new basal end ($t = 8$ h 30 min; short arrow). Black specks are pieces of charcoal in the medium. Time in hours is shown in the upper right hand corner of each panel. Scale bar = 5 μ m in all panels.

Fig. 10.

Time-lapse analysis of growth of *U. maydis tea4* filaments. Time-lapse sequence documenting changes in morphology of *tea4* filaments during growth on MM charcoal agarose pads for at least 9 h (see Movie 4). Filaments (f1, f2, f3) exhibit wild type-like growth early in the sequence ($t = 0$ to $t = 1$ h), albeit their growth rate is slower than that of wild type filaments ($45 \mu\text{m/h}$ vs $75 \mu\text{m/h}$, respectively) and they tend to grow in a less straight pattern compared to wild type filaments (see Fig. 8 for comparison). Growth rate continues to decrease and reaches $7 \mu\text{m/h}$ as the number of yeast-like cells increases and other changes occur. Filament f1 forms a wild type-like apical bud ($t = 1$ h to $t = 2$ h; arrowhead). In this process, the tip becomes narrower ($t = 2$ h; inset) followed by tip elongation and expansion to form a bud ($t = 3$ h; cell #1). This bud (cell #1) continues to grow and eventually separates from the tip ($t = 4$ h). Likewise, filament f2 forms a bud from its tip ($t = 2$ h to $t = 6$ h; cell #2). New buds arise from the tip of f1 and f2 ($t = 4$ h to $t = 6$ h; cells #1–2 and #2–2, respectively). The process of tip budding is observed in all *tea4* filaments but never in wild type filaments. Filaments f3 and f4 form lateral buds, which lack a clearly defined neck region ($t = 3$ h; cells #3 and #4, arrows; inset shows lateral bud #3 from filament f3). Lateral buds elongate ($t = 3$ h to $t = 6$ h) and form new buds ($t = 6$ h; for example, #4–1). Vacuole coalescence and formation of empty compartments at the basal end decreases ($t = 2$ h; b) and is no longer observed after 3 to 4 h of growth. Black specks are pieces of charcoal in the medium. Time in hours is shown on the upper left hand corner of each panel. Scale bar = $5 \mu\text{m}$ in all panels.

Fig. 11.

Subcellular localization of Tea4::GFP. Strain SG200 *tea4::gfp* was grown in minimal medium charcoal broth to induce filamentous growth (Materials and Methods). Strain FB1 *tea4::gfp* was grown in UMC broth to early log phase. Cells were examined using epifluorescence optics. (A) Tea4::GFP localizes to the apical end of filaments as a compact dot (arrow). Arrowheads point to discrete faint spots of Tea4::GFP in the subapical region. (B) Tea4::GFP localizes as a cap at the tip of the filament. (C) Enlarged view of the tip in A showing discrete spots of Tea4::GFP (arrowheads) in the subapical region. (D) Tea4::GFP localizes as multiple discrete dots (arrowhead) at the tip of the filament. (E) Discrete spots of Tea4::GFP (arrowheads) are seen along the length of the filament, with a higher concentration in the subapical region. The presence of Tea4::GFP at the tip is blocked by a piece of charcoal (arrow). (F) Tea4::GFP cap (arrow) at one of the cell poles in a yeast-like cell. (G) Tea4::GFP at both cell poles (arrows). Arrowheads point to discrete spots of Tea4::GFP in the cell. The intensity of the Tea4::GFP signal is stronger in the filamentous form than in yeast-like cells. Scale bar = 1 μ m in all panels.

Fig. 12.

Quantification of symptoms induced by wild type and *tea4* null strains. Seven day-old teozintle seedlings were inoculated with wild type or *tea4* mutants as indicated below (see Fig. 5S for symptoms). In Set I, infection is with a mixture of haploid strains requiring cell fusion to form the infectious pathogenic dikaryotic filament (A and B). In Set II, infection is with pure cultures of solopathogenic strains; no cell fusion is required to induce the infectious filamentous form (C and D). Symptom development was followed for 3 weeks and occurs as follows (color in graph indicated in parenthesis): chlorosis (light yellow), anthocyanin pigmentation (dark yellow), small tumors on leaves, leaf sheath, ligule (orange), large tumors on aerial plant parts and near the root system (dark brown), and teliospore development (not shown). Green indicates no symptom development. The Y axis shows the percentage of plants exhibiting a particular symptom. (A) Infection with two mating compatible wild type haploid strains (*a1 b1* + *a2 b2*). (B) Infection with two mating compatible *tea4* null mutants (*a1 b1 tea4* + *a2 b2 tea4*). (C) Infection with a wild type solopathogenic strain (SG200). (D) Infection with a *tea4* null derivative strain (SG200 *tea4*). At least 80 plants were inoculated in each set. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)