Inhibitory phosphorylation of a mitotic cyclin-dependent kinase regulates the morphogenesis, cell size and virulence of the smut fungus *Ustilago maydis*

Cecilia Sgarlata and José Pérez-Martín*

Department of Microbial Biotechnology, Centro Nacional de Biotecnología CSIC, Campus de Cantoblanco-UAM, 28049 Madrid, Spain

*Author for correspondence (e-mail: jperez@cnb.uam.es)

Accepted 13 May 2005
Journal of Cell Science 118, 3607-3622 Published by The Company of Biologists 2005
doi:10.1242/jcs.02499

Summary

The regulation of cyclin-dependent kinase (CDK) activity through inhibitory phosphorylation seems to play an important role in the eukaryotic cell cycle. We have investigated the influence that inhibitory phosphorylation of the catalytic subunit of mitotic CDK has on cell growth and pathogenicity of the corn smut fungus *Ustilago maydis*. This model pathogen is worthy of attention since it is well suited to analyze the relationships between the cell cycle, morphogenesis and pathogenicity. We set out to study these relationships by producing a *cdk1* mutant allele that was refractory to inhibitory phosphorylation. The expression of this mutant in *U. maydis* cells dramatically altered their morphology. Since this kind of mutation makes the CDK catalytic subunit resistant to regulation by Wee1-related kinases in other organisms, we characterized the orthologous Wee1 kinase from *U. maydis*. We found that Wee1 is essential in *U. maydis*. Overexpression of *wee1* produces cell cycle arrest in G2, the target of Wee1 apparently being the Cdk1/Clb2 complex, which is required specifically for the onset of mitosis. Given the connection between the cell cycle control and pathogenesis in *U. maydis*, we also analyzed whether cells with impaired inhibitory phosphorylation of Cdk1 were able to infect plants. We found that inhibitory phosphorylation was required for mating, a prerequisite to initiate pathogenic development. By examining plant-specific expression of the constitutively unphosphorylated *cdk1*^{AF} allele, we also found that appropriate levels of inhibitory phosphorylation were required at stages of infection subsequent to penetration by the fungus. These data reinforces the connections between cell cycle, morphogenesis and virulence in this smut fungus.

Key words: *Ustilago maydis*, cell cycle, inhibitory phosphorylation, Wee1-like kinases, phytopathogenic fungus

Introduction

The correct development of all eukaryotic organisms requires nuclear division and cytokinesis to be strictly coordinated during cell growth. In fungi, the coordination of these two processes raises unique problems. The mechanisms employed must be compatible with simple uninucleate cells in which a single nuclear division is followed by cytokinesis, and more complex multinuclear filamentous fungi in which several rounds of nuclear division must precede septum formation (Harris, 1997; Lew, 2000; Cerutti and Simanis, 2000). A common feature of these processes is the regulation of the cell cycle machinery, including the modulation of the cyclin-dependent kinases (CDKs) via inhibitory phosphorylation of their catalytic subunit (Kellogg, 2003). In fission yeast, the Wee1 and Cdc25 proteins play crucial roles in coordinating cell growth and cell division at the G2/M transition. Wee1 is a protein kinase that phosphorylates and inhibits Cdc2. Phosphorylation of this CDK catalytic subunit on conserved tyrosine and threonine residues prevents entry into mitosis until cells reach a critical size (Russell and Nurse, 1987; Gould and Nurse, 1989; Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1992). Conversely, Cdc25 is a phosphatase that removes the inhibitory phosphate added by Wee1, thereby promoting entry into mitosis (Russell and Nurse, 1986; Gautier et al., 1991; Millar et al., 1991). Loss of Wee1 activity causes cells to enter into mitosis before sufficient growth has occurred and therefore, cytokinesis produces two abnormally small daughter cells. Increasing the gene dosage of *wee1* causes delayed entry into mitosis and an increase in cell size. These responses indicate that the levels of Wee1 activity determine the timing of entry into mitosis (Nurse, 1975; Russell and Nurse, 1987).

In *Saccharomyces cerevisiae*, the budding yeast homolog of Wee1, Swe1, has been linked to a checkpoint that delays cell cycle progression in response to morphogenetic defects (Booher et al., 1993; Sia et al., 1996). Swe1 also modulates pseudohyphal growth in a broad spectrum of conditions (La Valle and Wittenberg, 2001; Martínez-Anaya et al., 2003). A role for Swe1 in the control of cell size was suggested by the finding of a *swe1* mutant allele in a screening for *whi* phenotype (Jorgersen et al., 2002) and more recently it was shown that Swe1 delays entry into mitosis and is required for the control of cell size (Harvey et al., 2003). The common role of the Wee1 and Swe1 kinases may also apply to the filamentous fungus *Aspergillus nidulans*, where the Wee1-like kinase AnkA controls septum formation (De Souza et al., 1999;

Kraus and Harris, 2001). In *A. nidulans* cells, predivisional hyphae cannot septate until a specific cell size is attained, indicating that septum formation is coordinated with cell growth (Wolkow et al., 1996).

Here, we set out to investigate the influence of inhibitory phosphorylation of the catalytic subunit of mitotic CDK on cell growth and pathogenicity of the corn smut fungus *Ustilago maydis*. This pathogen is perfectly suited to analyze the relationships between cell cycle, morphogenesis and pathogenicity (Basse and Steinberg, 2004). Haploid cells of this fungus are unicellular and divide by budding. Induction of the pathogenic phase requires the mating of two compatible haploid cells and after cell fusion, the generation of an infective dikaryotic filament that invades the plant (Kahmann et al., 2000). The different morphological changes that the fungal cells undergo during the pathogenic process provide evidence of the tight control of the cell cycle in these transitions. Indeed, we recently showed that manipulation of the transcription of mitotic cyclins affects hyphal proliferation within the plant, resulting in fungal cells unable to produce a successful infection (García-Muse et al., 2004). We also reported that mutations in the Fizzy-related APC activator Cru1 disrupted different stages of plant infection by *U. maydis* (Castillo-Lluva et al., 2004). As a further step toward elucidating the role of cell cycle regulation in the virulence of this fungus, we have analyzed whether impairing the inhibitory phosphorylation of CDK affects the ability of *U. maydis* to infect plants. To achieve this, we took advantage of cells expressing a constitutively unphosphorylated *cdk1* allele to show that deficient inhibitory phosphorylation affects morphogenesis, the coordination of cell growth with the cell cycle, the S phase checkpoint control and virulence. We also characterized the *U. maydis* Wee1 kinase ortholog and found that Wee1 is required to control the G2/M transition in *U. maydis*. The data reported here reinforce the proposed connection between the cell cycle, morphogenesis and pathogenicity in *U. maydis*.

Materials and Methods

Strains and growth conditions

The yeast strains used in this study are listed in Table 1. The *U. maydis* cells were grown in YPD (Sherman et al., 1986), complete medium (CM; 0.25% casaminoacids, 0.1% yeast extract, 0.15% NO_3NH_4, 1% Holliday vitamins solution, 6.25% Holliday salts solution) or minimal medium (MM; 0.3% KNO_3, 6.25% Holliday salts solution) (Holliday, 1974). Controlled expression of genes under the *crg1* and *nar1* promoters, FACS analysis and cell cycle arrest were all carried out as previously described (Brachmann et al., 2001; García-Muse et al., 2003; García-Muse et al., 2004).

DNA, RNA and protein analysis

U. maydis DNA and RNA isolation, the preparation of protein extracts, northern and western blotting, and immunoprecipitations were all performed as described previously (Tsukuda et al., 1988; Garrido and Pérez-Martín, 2003; García-Muse et al., 2004; Garrido et al., 2004). The anti-PSTAIRE (Santa Cruz Biotechnology), anti-myc 9E10, and anti-VSV (Roche Diagnostics Gmb) antibodies were all used at a dilution of 1:10,000 in phosphate-buffered saline + 0.1% Tween + 10% dry milk. The anti-phospho-Cdc2 (Tyr^{15}) antibody (Cell Signaling) was used according to the manufacturer's instructions. The anti-mouse Ig horseradish peroxidase and anti-rabbit Ig horseradish peroxidase (Roche Diagnostics Gmb) secondary antibodies were used at a dilution of 1:10,000. All western blots were visualized by enhanced chemiluminescence (Renaissance®; Perkin Elmer).

Table 1. *U. maydis* strains used in this study

Strain	Relevant genotype	Reference
FB1	*a1 b1*	Banuett and Herskowitz, 1989
FB2	*a2 b2*	Banuett and Herskowitz, 1989
FBD11	*a1a2 b1b2*	Banuett and Herskowitz, 1989
UMC37	*a1a2 b1b2 wee1/Δwee1*	This work
TAU17	*a1 b1 cdk1-1*	García-Muse et al., 2004
UMC5	*a1 b1* P_{tef1}*:cdk1-myc*	This work
UMC6	*a1 b1* P_{tef1}*:cdk1*^{AF}*-myc*	This work
UMC8	*a2 b2* P_{tef1}*:cdk1-myc*	This work
UMC9	*a2 b2* P_{tef1}*:cdk1*^{AF}*-myc*	This work
UMC38	*a1 b1 wee1-myc*	This work
UMC33	*a1 b1* P_{crg}*:wee1-myc*	This work
UMC41	*a1 b1* P_{crg}*:wee1-myc tub1-GFP*	This work
UMC23	*a1 b1 wee1*^{nar}	This work
TAU42	*a1 b1 clb2*^{nar}	García-Muse et al., 2004
UMP40	*a1 b1 clb2*^{nar} *wee1*^{nar}	This work
UMC45	*a1 b1 clb2*^{hsp70} P_{crg}*:wee1-myc*	This work
SONU58	*a1 b1* P_{crg}*:cln1-myc*	Castillo-Lluva, this laboratory
UMP19	*a1 b1 clb1-vsv*	García-Muse et al., 2004
UMP27	*a1 b1 clb2-myc*	García-Muse et al., 2004
UMC44	*a1 b1 wee1*^{scp}	This work
UMP9	*a1a2 Δcru1*	Castillo-Lluva et al., 2004
UMC10	*a1 b1* P_{nar}*:cdk1-myc*	This work
UMC11	*a1 b1* P_{nar}*:cdk1*^{AF}*-myc*	This work
UMC9	*a2 b2* P_{nar}*:cdk1-myc*	This work
UMC12	*a2 b2* P_{nar}*:cdk1*^{AF}*-myc*	This work
SG200	*a1 mfa2 bW2 bE1*	Bolker et al., 1995b
UMC14	*a1 mfa2 bW2 bE1* P_{nar}*:cdk1-myc*	This work
UMC13	*a1 mfa2 bW2 bE1*P_{nar}*:cdk1*^{AF}*-myc*	This work
UMP50	*a1 b1* P_{mig1}*:cdk1-myc*	This work
UMP51	*a1 b1* P_{mig1}*:cdk1*^{AF}*-myc*	This work

Plasmids and constructs

Plasmid pGEM-T easy (Promega) was used for cloning, subcloning and sequencing of genomic and PCR fragments. The plasmids pRU11, pRU2, pCU2 and pCU3 were used to express genes under the control of P*crg1*, P*nar1*, P*scp* and P*tef1* promoters, respectively, and the plasmids pBS-MYC-HYG and pGNB-myc to produce C-terminal myc-tagged protein fusions as already described (Brachmann et al., 2001; Brachmann, 2001; García-Muse et al., 2004; Garrido et al., 2004). The oligonucleotides used for PCR amplification are shown in Table 2 and the PCR fragments generated were analyzed using an automated sequencer (ABI 373A) and standard bioinformatic tools. To construct the different *U. maydis* strains, protoplasts were transformed with the constructs indicated using the protocol described by Tsukuda et al. (Tsukuda et al., 1988). Integration of the plasmids into the corresponding loci was verified by diagnostic PCR and Southern blot analysis in all cases.

The mutant *cdk1*^{AF} allele was constructed by assembling two PCR fragments that carried the T14A and Y15F mutations, generated with the primer pairs CDK1/CDK2-b and CDK3-b/CDK4. The wild-type *cdk1* allele was amplified using the CDK1 and CDK4 primers. In both cases, the resulting 1.1 kb fragment was inserted into pGNB-myc to obtain the myc-epitope-tagged protein. The tagged alleles were then inserted into pCU3 (P*tef1*-dependent expression) or pRU2 (P*nar1*-dependent expression) to construct the pCU3-Cdk1, pCU3-Cdk1AF, pRU2-Cdk1 and pRU2-Cdk1AF plasmids. These plasmids were linearized and integrated into the *cbx1* locus by homologous recombination, as described by Brachmann et al. (Brachmann et al., 2001). To express the *cdk1* and *cdk1*^{AF} alleles under the control of the

Table 2. Oligonucleotides used in this study

Primer	Sequence
CDK1	5′GGACTAGTCATATGGACAAGTATCAAAGGATCGAA3′
CDK2-b	5′GTATACGACACCGTATGTTCCTGTAGAGGC3′
CDK3-b	5′GCCTCTACAGGAACATACGGTGTCGTATAC3′
CDK4	5′CGGAATTCTGTGAGGAGCCTCCTGAAGTACGG3′
MIG1-1	5′ATGGGCCCAACTCGGAGTCGAGACACACA3′
MIG1-2	5′CATATGGATCTGGAGGAAGAGAATGGA3′
WEE1-7	5′GGCTTAAGTCCGCCGCTTTGCATCGCCCTCT3′
WEE1-8	5′CCGCTCGAGGTGTTTCACGATAGGATAGC3′
WEE1-9	5′CGGGAATTCATCGGAGACTTTGGCATGGCG3′
WEE1-10	5′GGGGTACCTCGTCGATCGTAGCGCGTTGCTC3′
WEE1-27	5′GGGGTACCCGCAACTCCAATGTCATCA3′
WEE1-31	5′CAATTGACCATCCAGATCCATCGAGTA3′
WEE1-32	5′AATTCCATATGCATCAGAACGACGACG3′
WEE1-35	5′GGGGTACCGAATAATATTGATAATAAATC3′
WEE1-36	5′CGGAATTCAAACAGGATGGATGGGAGGCA3′

plant-specific promoter P*mig1*, a 2 kb fragment carrying the P*mig1* promoter was inserted into pCU3-Cdk1 and pCU3-Cdk1AF to replace a 0.25 kb fragment carrying the P*tef1* promoter. This fragment was obtained by PCR amplification with primers MIG1-1 and MIG1-2, using *U. maydis* genomic DNA as the template. The resulting plasmids, pMIG1-Cdk1 and pMIG1-Cdk1AF were integrated by homologous recombination into the *mig1* locus.

To produce a C-terminal tagged version of Wee1, a 4.5 kb fragment encompassing the *wee1* ORF without the stop codon was amplified by PCR from *U. maydis* genomic DNA with the primers WEE1-32 and WEE1-31. This fragment was cloned into pGEM-T easy to produce the pGEMT-Wee1 plasmid. A 2.6 kb fragment from pGEMT-Wee1 was inserted into pBS-MYC-HYG to produce the endogenous myc-tagged *wee1*, and the resulting pBS-Wee1-myc plasmid was integrated into the *wee1* locus by homologous recombination. To overexpress *wee1*, a C-terminal myc-epitope tag was first introduced by inserting the 4.5 kb pGEMT-Wee1 fragment into pGNB-myc. From the resulting pGNB-wee1-myc plasmid, a 4.7 kb fragment carrying the tagged allele was inserted into the pRU11 vector (P*crg1*-dependent expression). The resulting pRU11-Wee1-myc plasmid was linearized and integrated into the *cbx1* locus by homologous recombination as described by Brachmann et al. (Brachmann et al., 2001).

To delete the *wee1* gene, we used the pKOWee1 plasmid generated by ligating two DNA fragments flanking the *wee1* ORF into pNEB-HYG (+), a *U. maydis* integration vector containing a hygromycin resistance cassette (Brachmann et al., 2001). The 5′ fragment was produced by PCR with the primers WEE1-7 and WEE1-8 using *U. maydis* genomic DNA as the template, and the 3′ fragment using the primers WEE1-9 and WEE1-10. After linearization, the pKOWee1 plasmid was integrated into the *wee1* locus by homologous recombination.

To produce a conditional $wee1^{nar}$ allele we inserted two PCR fragments into pRU2. The 5′ fragment was produced using the primers WEE1-35 and WEE1-36 and spanned from nucleotide –872 to nucleotide –120 (considering the adenine in the ATG as nucleotide +1). The 3′ fragment was obtained with the primers WEE1-32 and WEE1-27 and spans from nucleotide +1 to nucleotide +1524. The resulting plasmid pWEE1nar was linearized and integrated into the *wee1* locus by homologous recombination.

To produce the $wee1^{scp}$ allele, a 2.28 kb fragment from pWEE1nar was inserted into pCU2 (P*scp*-dependent expression). The resulting plasmid pWEE1scp was linearized and integrated into the *wee1* locus by homologous recombination.

Microscopy

Microscopy was carried out using a Leica DMLB microscope with phase contrast optics. Standard FITC and DAPI filter sets were used for epifluorescence analysis of nuclear staining with DAPI (see García-Muse et al., 2003) and WGA staining, performed as described by Castillo-Lluva et al. (Castillo-Lluva et al., 2004). Photomicrographs were obtained with a Leika 100 camera and the images were processed with Photoshop (Adobe).

Mating and plant infection

To test for mating, compatible strains were co-spotted on charcoal-containing PD plates that were sealed with parafilm and incubated at 21°C for 48 hours (Holliday, 1974). Plant infections were performed with the maize cultivar Early Golden Bantam as described previously (Old Seeds, Madison, WI, USA) (Gillissen et al., 1992). Filaments inside the plant tissue were stained with Chlorazole Black E as described by Brachmann et al. (Brachmann et al., 2003).

Sequence analyses

Protein sequences of fungal Wee1-like kinases were downloaded from PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Alignments and phylogenetic dendrograms were constructed using ClustalW and NJPlot programs (Thompson et al., 1997).

Results

Expression of a *cdk1* allele insensitive to inhibitory phosphorylation dramatically alters the morphogenesis of *U. maydis* cells

The catalytic subunit of the mitotic CDK from *U. maydis*, encoded by the *cdk1* gene, contains the conserved amino terminal Thr^{14} and Tyr^{15} residues. These residues are the targets of inhibitory phosphorylation by Wee1-related kinases in other systems (Fleig and Gould, 1991; García-Muse et al., 2004). A specific antibody raised against the phosphorylated human Cdc2-Y^{15}P peptide recognized one major band in total protein extracts of *U. maydis*. Moreover, this phosphorylated protein co-migrated with a protein recognized by the anti-PSTAIRE antibody (Fig. 1A). To determine whether this band corresponded to the Cdk1 subunit, we introduced a *cdk1-1* allele that produces Cdk1 carrying a C-terminal copy of the FLAG epitope into fungal cells by homologous recombination (García-Muse et al., 2004). In protein extracts from these strains the phospho-specific antibody recognized a single band with a slightly lower electrophoretic mobility to wild-type Cdk. This retardation was due to the increase in size provoked by adding the FLAG tag, indicating that the protein recognized by the phospho-specific antibody in both wild-type and recombinant strains was Cdk1.

To address what role this phosphorylation might play in *U. maydis* cells, we constructed a mutant allele, $cdk1^{AF}$, in which these putative inhibitory phosphorylation sites in Cdk1 were replaced with residues that could not be phosphorylated (Thr^{14} to Ala and Tyr^{15} to Phe, respectively). However, we were unable to replace the endogenous *cdk1* locus with this $cdk1^{AF}$ allele (not shown) and therefore, we overexpressed this mutant allele and the wild-type allele under the control of the *tef1* promoter that produces high levels of transcription (Spellig et al., 1996). We used 3xMyc-tagged versions of both the wild-type and the constitutively unphosphorylated Cdk1 to discriminate between the levels of protein produced by the endogenous locus and the ectopically expressed alleles (Fig. 1B). Overexpression of a wild-type allele had no discernible effect on cell growth or the morphology of the *U. maydis* cells

(Fig. 1C, left column). Cells grew by budding and newly formed buds emerged at the pole of the elongated mother cell. Furthermore, when the cell wall of these cells were stained with FITC-labeled wheat germ agglutinin (WGA), a lectin that binds to oligomeric chitin, the bud scar as well as newly formed septa at the stage of cell separation were labeled. In contrast, typical budding pattern was lost in the cells expressing the constitutively unphosphorylated allele and the morphology of these cells was dramatically altered (Fig. 1C, right column). Large aggregates of these cells formed, composed of long chains of rounded cells that divided by septation but which remained attached to each other. Each cell compartment in these chains of cells contained a single nucleus, and WGA reacted strongly with septa and the lateral cell wall. These data indicate that the inability to phosphorylate Cdk1 at its amino terminus interferes with normal growth in *U. maydis*.

S phase checkpoint control in *U. maydis* could be mediated through inhibitory phosphorylation of Cdk1

Inhibitory phosphorylation of the catalytic subunit of CDK is part of the S phase checkpoint control in response to damage or incompletely replicated DNA in several organisms. For instance, *S. pombe*, *A. nidulans* and human cells are dependent of this regulation. In contrast, *S. cerevisiae* cells do not require inhibitory phosphorylation to arrest cell cycle after activation of a S phase checkpoint (Lew and Kornbluth, 1996). To address whether in *U. maydis* the inhibitory phosphorylation is involved in the response to S phase checkpoint, we firstly examined the levels of Tyr^{15} phosphorylation in response to an arrest in S phase caused by addition of 1 mg/ml hydroxyurea (García-Muse et al., 2004). We found that the level of tyrosine-phosphorylated Cdk1 increased upon addition of hydroxyurea (HU) (Fig. 2A), suggesting an involvement of this negative regulation in the S phase checkpoint as in other organisms. To reinforce this conclusion, we tested cells expressing high levels of the unphosphorylated $cdk1^{AF}$ allele for sensitivity to HU at sub-lethal concentrations. As control we used wild-type cells and cells expressing high levels of wild-type *cdk1* alelle. Consistently, we found that cells with impaired inhibitory phosphorylation showed a high degree of HU sensitivity (Fig. 2B).

U. maydis contains a single Wee1-related protein

The dramatic phenotype observed by overexpression of the constitutively unphosphorylated Cdk1 protein indicates

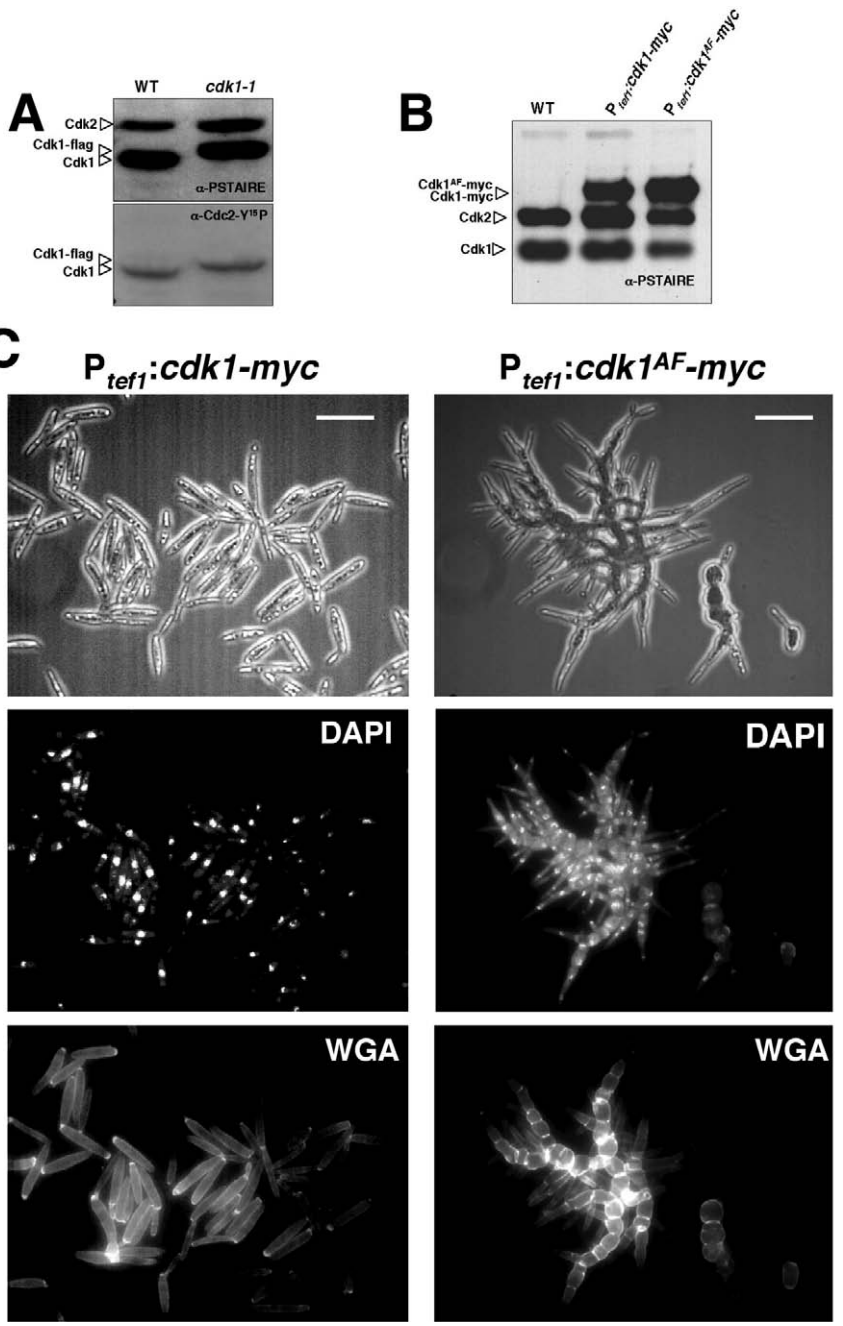


Fig. 1. The absence of inhibitory Cdk1 tyrosine phosphorylation causes severe morphological defects in *U. maydis*. (A) Cdk1 is phosphorylated at Tyr^{15}. Protein extracts from wild-type and TAU17 cells expressing a FLAG-tagged version of Cdk1 (*cdk1-1*) were subjected to immunoblotting with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies. Cdk2 is the *U. maydis* homolog of *S. cerevisiae* Pho85, which is also recognized by the anti-PSTAIRE antibody. (B) Expression of the ectopic myc-tagged Cdk1. Protein extracts from wild-type, and UMC5 and UMC6 cells expressing myc-tagged Cdk1 (P_{tef1}:*cdk1-myc*) and the constitutively unphosphorylated Cdk1 allele (P_{tef1}:$cdk1^{AF}$-*myc*), respectively, were immunoblotted with anti-PSTAIRE antibodies. (C) UMC5 and UMC6 cells were grown to mid-exponential phase in rich medium (YPD) and then stained with DAPI and FITC-WGA, to visualize nuclei and cell walls, respectively. Scale bars: 20 μm.

that the inhibition induced by Wee1-related kinases may be critical for the correct growth of *U. maydis*. Therefore, we searched the available *U. maydis* genomic sequence for potential homologues of Wee1-related kinases (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/index.html). We found a single gene, *wee1*, encoding a putative protein of 1525 amino acids (accession no. AY995171). Sequence analysis indicated that Wee1 contained a kinase domain near the carboxyl terminus which shared 43-28% sequence similarity with that in other fungal Wee1-related proteins (Fig. 3A). In addition, Wee1 contained the Glu-Gly-Asp triplet motif characteristic of kinase subdomain VIII in the Wee1 protein kinase family (Fig. 3C) (Booher et al., 1993). A dendrogram analysis (Fig. 3B) indicated that the Wee1-related proteins from fungi fall in two different branches, *U. maydis* Wee1 lies in a group with *S. pombe* Wee1 and *A. nidulans* AnkA.

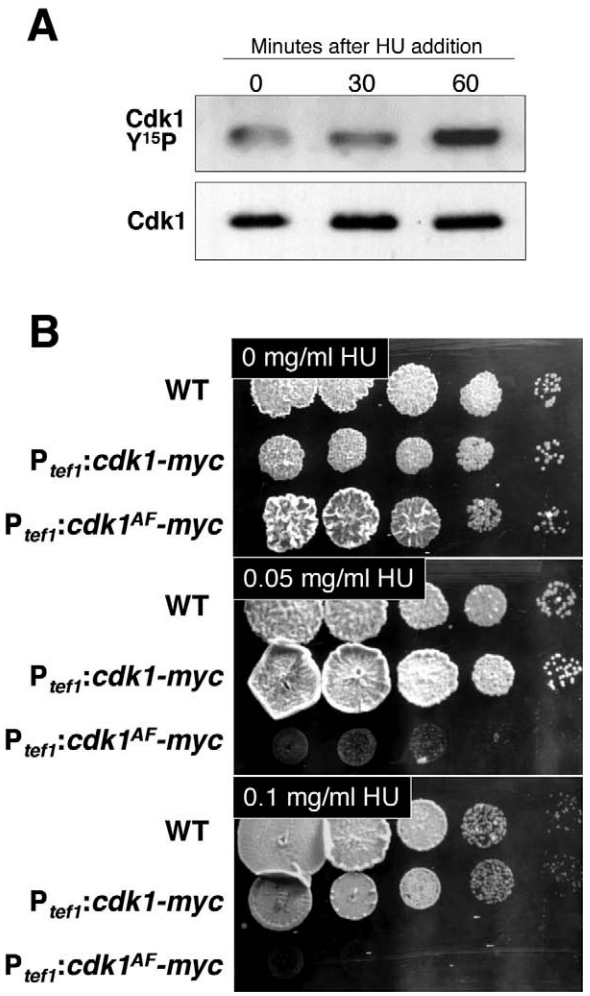


Fig. 2. Cdk1 phosphorylation and S phase checkpoint. (A) Effects of inhibition of DNA synthesis by hydroxyurea on Cdk1 phosphorylation. HU (1 mg/ml) was added to an early log phase culture of FB1 cells and samples were taken at 30 and 60 minutes after HU addition. (B) Impaired tyrosine phosphorylation of Cdk1 causes sensitivity to HU. Wild-type (FB1), and UMC5 and UMC6 cells expressing myc-tagged Cdk1 (P_{tef1}*:cdk1-myc*) and the constitutively unphosphorylated Cdk1 allele (P_{tef1}*:cdk1*^{AF}*-myc*), respectively, were spotted as serial dilutions onto YPD plates containing a series of HU concentrations and the plates were incubated at 28°C for 3 days.

We created a fungal strain in which the genomic copy of *wee1* was replaced with a C-terminal myc-tagged Wee1 protein. Cell growth and morphology was not noticeably affected by this replacement (not shown). Using this strain, we investigated whether the levels of Wee1 fluctuated in different phases of the cell cycle. Because no reproducible synchronization method is so far available for *U. maydis,* we performed western blotting on protein extracts isolated from cultures of wild-type cells enriched in G1 phase, or cells arrested in S phase by the presence of hydroxyurea, or M phase with benomyl. We detected Wee1 under all conditions, but it appeared to undergo a mild down-regulation that coincided with M phase arrest (Fig. 3D), as described for Swe1 in *S. cerevisiae* (Harvey and Kellogg, 2003) and Wee1 in *S. pombe* (Aligue et al., 1997). We also analyzed the mRNA levels and found that *wee1* mRNA is preferentially expressed at G1 phase with the lowest levels again being found in M phase-arrested cells (Fig. 3E), as described for *SWE1* in *S. cerevisiae* (Rey et al., 1996).

High levels of *wee1* expression causes cell cycle arrest at G2 phase

Overexpression of Wee1-related kinases delays entry into mitosis in several systems, resulting in G2 arrest as a consequence of accumulation of phosphorylated inactive CDK complexes (Russell and Nurse, 1987; Booher et al., 1993; McGowan and Russell, 1995). To determine whether overexpression of *wee1* has similar inhibitory activity in *U. maydis*, an extra copy of *wee1* was introduced into wild-type *U. maydis* cells. This additional *wee1* allele was under the control of the *crg1* promoter which can be induced by arabinose and repressed by glucose (Bottin et al., 1996). These cells were unable to grow in solid medium containing arabinose (Fig. 4A) indicating that the overexpression of *wee1* was deleterious to the cells.

We evaluated this response in more detail, characterizing the morphology of the cells as well as their DNA content in liquid cultures. After 6 hours in arabinose-containing medium, virtually all *wee1*-overexpressing cells ceased dividing and remained as single, uninuclear cells with a bud-like protrusion that continued elongating over time (Fig. 4B). These cells contained long microtubules that reached the tip of the growing pole, thereby supporting active polar growth (Fig. 4C) a hallmark of G2 phase in *U. maydis* cells (Steinberg et al., 2001; Banuett and Herskowitz, 2002). Flow cytometry showed these cells to have a 2C DNA content (Fig. 4D), all of which was compatible with G2 arrest. To determine whether the Wee1-induced cell cycle arrest correlated with the increased tyrosine phosphorylation of Cdk1, we examined the phosphotyrosine content of Cdk1 during the growth of the wild-type and *wee1*-overexpressing cells (Fig. 4E). We consistently found an increase in the levels of Tyr^{15}P-Cdk1 when *wee1* overexpression was induced with respect to the wild-type cells.

In summary, high levels of Wee1 induce G2 arrest, correlated with a high level of Tyr^{15} phosphorylation of Cdk1 in *U. maydis*. On these grounds, we consider *U. maydis* Wee1 to be a Wee1-related protein kinase.

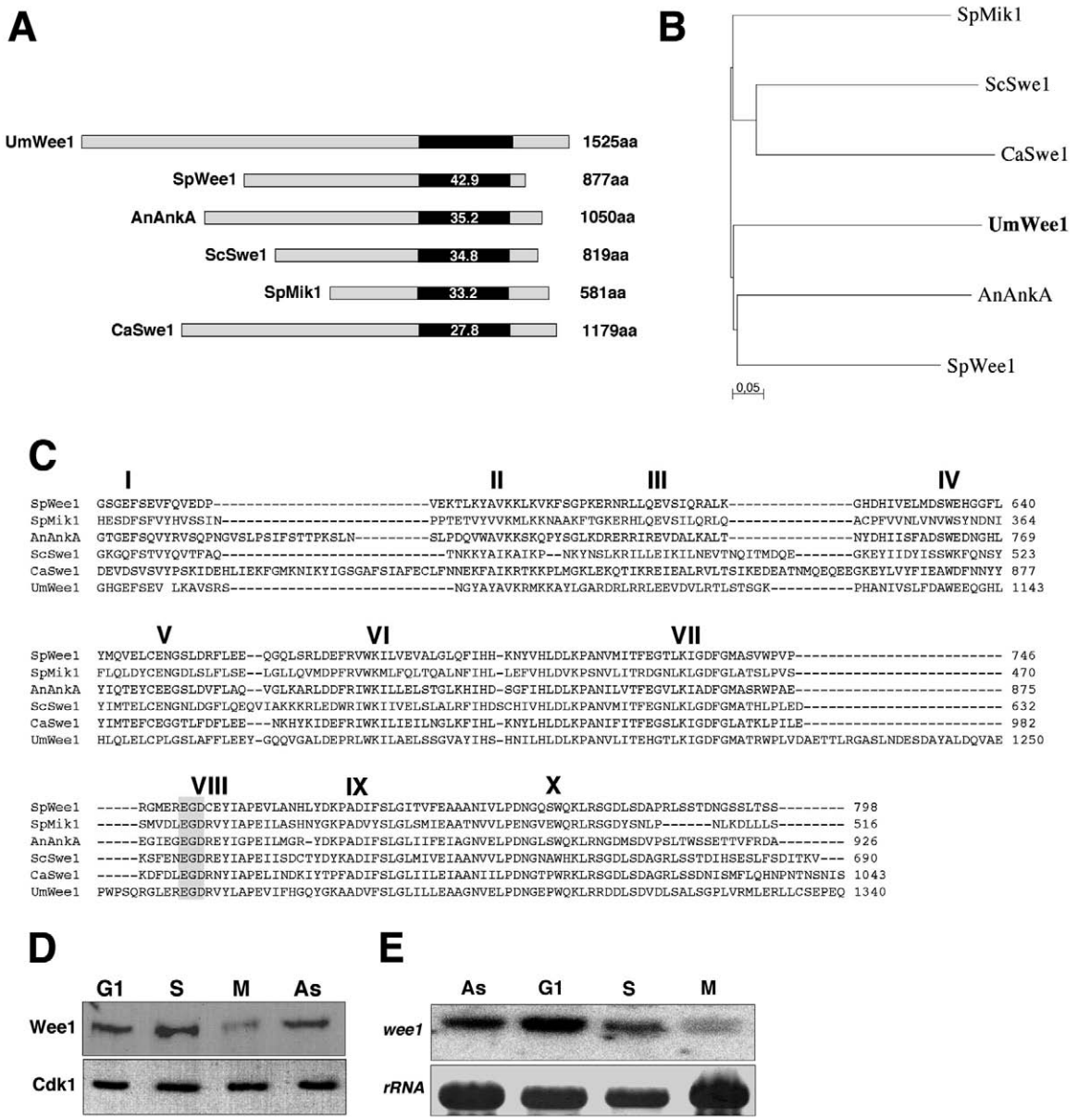


Fig. 3. Sequence analysis of a *U. maydis* Wee1-like protein. (A) Schematic representation of the Wee1 protein in relation to other fungal Wee1-like proteins. The catalytic domains are shown in black and were identified using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de). The percentages inside each box represent the sequence identity when compared to the *U. maydis* sequence. (B) Dendrogram of Wee1-like proteins. The tree was reconstructed using the ClustalW method (http://www.ebi.ac.uk/clustalw/). Bar: 0.05 substitutions per aa. (C) Comparison of the catalytic domain of *U. maydis* Wee1 kinase with that of related Wee1-like proteins. The roman numerals indicate the catalytic subdomains as designated by Hanks (Hanks, 1991). The shaded residues are amino acids characteristic of Wee1 family kinases. (D) Protein levels at different stages of the cell cycle. Extracts from UMC38 cells carrying a myc-tagged copy of Wee1 and arrested at S or M phase, G1 phase enriched or cells growing asynchronously (As) were immunoblotted. The same filters were probed with anti-myc and anti-PSTAIRE antibodies and Cdk1 levels were used as loading controls. (E) Levels of *wee1* expression at different cell cycle stages. RNA extracted from wild-type FB1 cells arrested at S phase or M phase, or enriched in G1 phase and growing asynchronously (As), was analyzed by northern blotting. The filter was hybridized with probes for *wee1* and 18s rRNA as a control of loading.

Wee1 is essential for the survival of *U. maydis*

To further analyze the function of Wee1, we inactivated one *wee1* allele in the diploid FBD11 strain, replacing it with a hygromycin-resistance cassette, generating the Δ*wee1* null allele. When the meiotic progeny of this strain were analyzed after sporulation, no hygromycin-resistant cells were found, indicating that *wee1* is an essential gene. Therefore, we constructed another strain in which the Wee1 protein could be conditionally ablated. This was achieved using the *U. maydis nar1* promoter that is induced by growing the cells in nitrate as the nitrogen source, and strongly repressed when the cells are grown in rich medium (YPD) (Brachmann et al., 2001). A chimeric allele (*wee1*^{nar}) was constructed by fusing the *nar1* promoter to the coding region of *wee1* and the native allele was

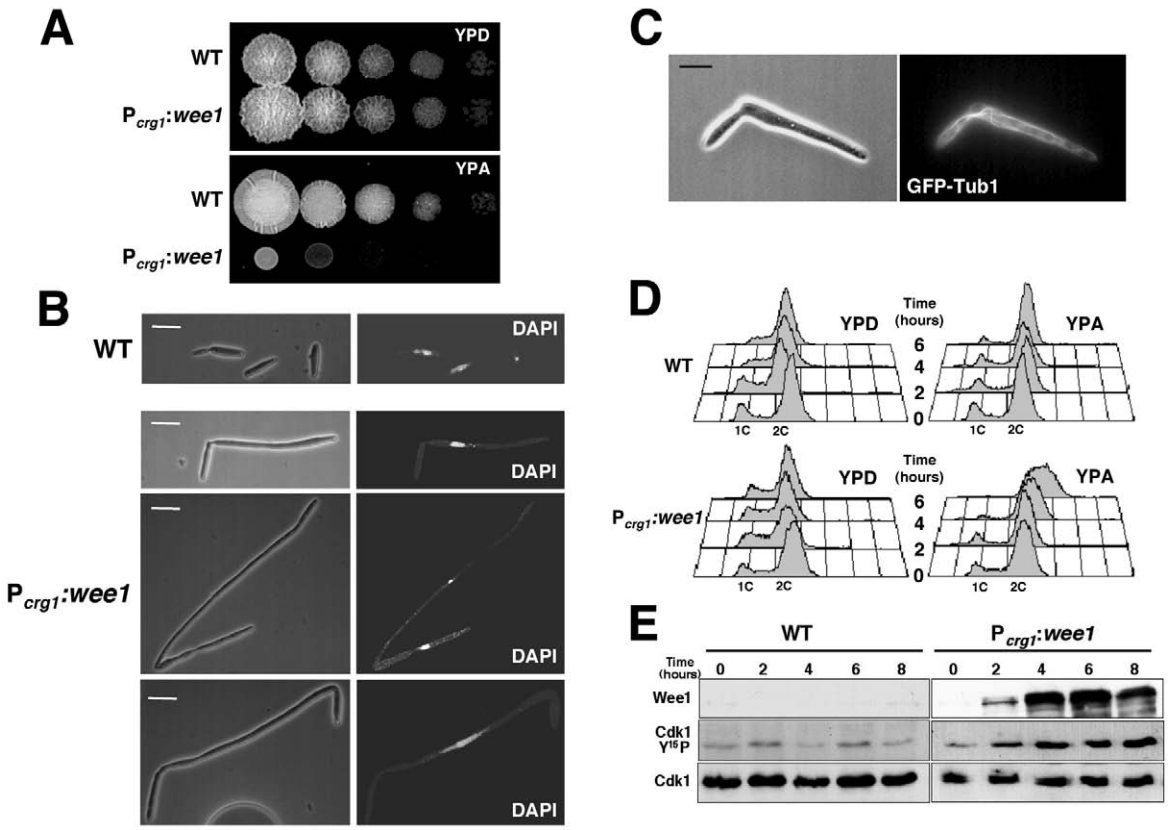


Fig. 4. Ectopic expression of *wee1*. (A) The growth of cells expressing an ectopic copy of the *wee1* gene was examined by spotting serial dilutions of exponential cultures of FB1 (wild type) and UMC33 (P_{crg1}:*wee1*) strains in solid rich medium with either 2% glucose (YPD, non-inducing conditions) or 2% arabinose (YPA, inducing conditions). Plates were incubated for 3 days at 28°C. (B) Micrographs showing the cell morphology of FB1 and UMC33 cells after 6 hours of growth in YPA liquid cultures (inducing conditions). Note the elongated shape and the presence of a single nucleus (DAPI staining). Scale bars: 20 μm. (C) Microtubule network of UMC41 cells, carrying an α-tubulin-GFP fusion and expressing high levels of *wee1* (GFP-Tub1 epifluorescence; Scale bar: 25 μm. (D) FACS analysis of cell DNA content of FB1 and UMC33 in non-inducing (YPD) and inducing conditions (YPA). Samples were removed 0, 2, 4 and 6 hours after transfer to conditional medium. The shift to a DNA content higher than 2C observed in UMC33 cells incubated in CMA for 6 hours was due to mitochondrial DNA staining. (E) Western analysis of inhibitory phosphorylation after *wee1* overexpression. Protein extracts from the FB1 and UMC33 cultures incubated in inducing conditions for the times indicated were obtained (YPA; in hours). The overexpressed *wee1* allele was myc-tagged and detected with an anti-MYC antibody. Cdk1 was visualized with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies.

replaced by this conditional allele. In the conditional mutant, a three-fold increase was observed in *wee1* mRNA with respect to the levels in wild-type cells in permissive (minimal medium containing nitrate, $MMNO_3$) but not in restrictive conditions (YPD medium; Fig. 5A). Conditional mutant cells grew on solid medium containing nitrate at a rate similar to control wild-type cells. However, in accordance with the essential role of the Wee1, they were unable to form colonies when shifted to solid YPD medium (Fig. 5B).

We analyzed the growth of this conditional strain in liquid medium. In restrictive conditions the conditional cells generated chains of rounded cells that divided by septation (Fig. 6). The cell compartments were shorter than normal (6 μm on average versus 17 μm in wild-type *U. maydis* cells), and contained at least one nucleus. Nevertheless, when the DNA content was analyzed by FACS we found that in restrictive conditions, $wee1^{nar}$ cells accumulated DNA in genome sized multiples (Fig. 4D). Cell analysis using microdensitometry (Snetselaar and McCann, 1997) indicated that the relative intensity of nuclei fluctuate between two peaks, presumably corresponding to 1C and 2C DNA content (not shown), which is consistent with normal DNA replication. Accordingly, these cell aggregates were reminiscent of those seen in cells expressing the constitutively unphosphorylated $cdk1^{AF}$ allele. Our interpretation is that impairing the inhibitory phosphorylation of Cdk1 promoted premature entry into mitosis, resulting in the inability to produce a bud and followed by division through septation.

Strikingly, when grown in permissive conditions the conditional strain did not display a wild-type phenotype. Mother and bud cells were more elongated than the wild-type cells (Fig. 5E), and no clear G1 phase was observed (Fig. 5D). We believe that the unscheduled expression of the $wee1^{nar}$ allele augmented Wee1 activity to above normal levels (compare the amount of *wee1* mRNA in wild-type and $wee1^{nar}$ cells growing in permissive conditions, Fig. 5A). This increase in Wee1 activity could have delayed entry into mitosis resulting in an increase in cell size at division and as a consequence, a shorter G1. An additional phenotype found in these conditional cells was that about 30% of the budding cells had lateral buds.

Wild-type cells bud in a polar manner whereby the daughter cell emerges from one end of the mother cell. During exponential growth, less than 1% of wild-type cells had lateral buds. Thus, although preliminary, this result suggests that correct Wee1 activity could be required for bud site selection in *U. maydis*.

To correlate Wee1 function with inhibitory phosphorylation in Cdk1, wild-type and conditional cells were grown in permissive conditions until the mid-exponential phase and then the cultures were shifted to restrictive conditions. Samples were collected at different times and the degree of Tyr^{15} phosphorylation was analyzed by Western blotting with the anti-Cdc2-$Y^{15}P$ antibody. A dramatic reduction in the levels of Tyr^{15} phosphorylation was clearly evident in extracts obtained from conditional cells growing in restrictive conditions (Fig. 5C).

Taken together these results support the notion that Wee1-mediated inhibitory phosphorylation of Cdk1 controls entry into mitosis and hence the length of the G2 phase in *U. maydis*.

Wee1 controls the Cdk1-Clb2 complex

We previously demonstrated the specificity of the B-type cyclin Clb2 to the G2/M transition where it appears to be rate-limiting for entry into mitosis in *U. maydis* (García-Muse et al., 2004). Cell growth was arrested in G2 phase when Clb2 function was

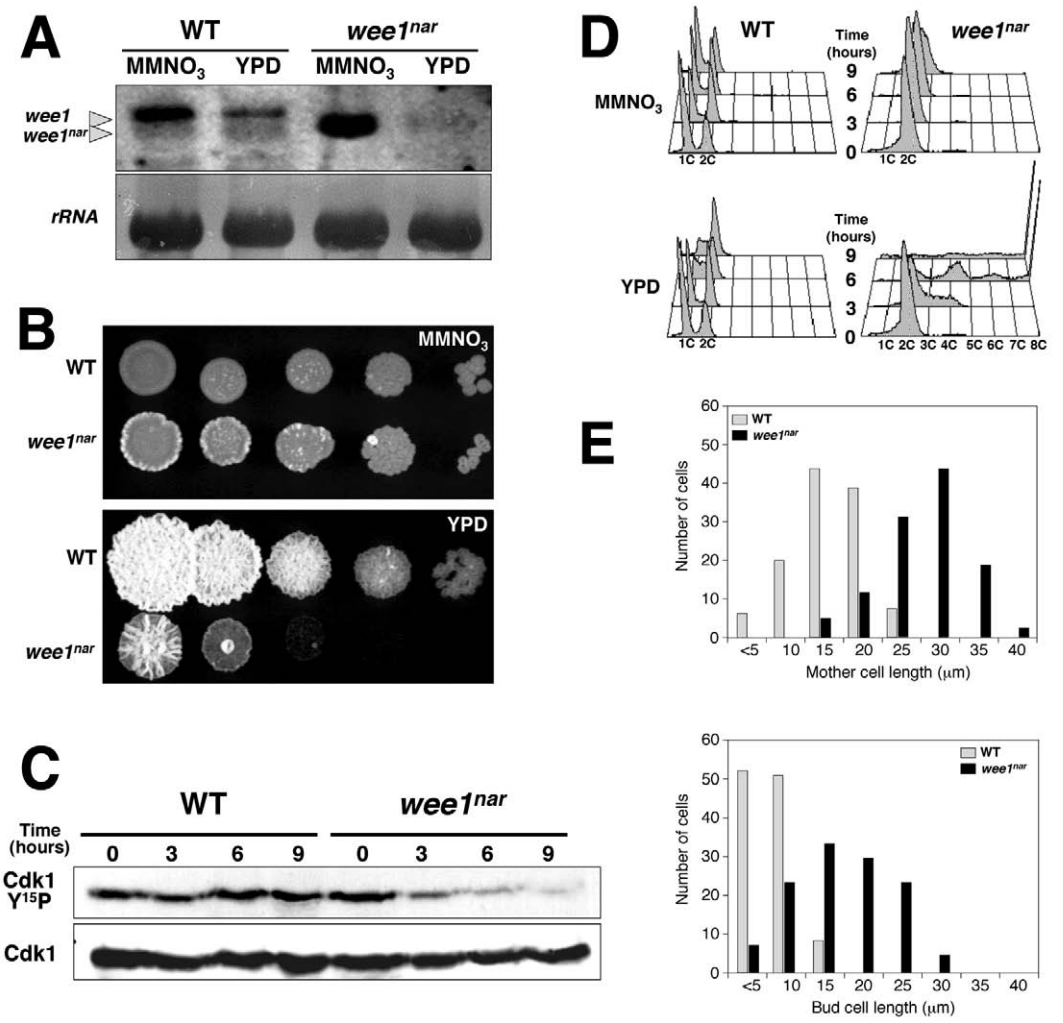


Fig. 5. Conditional removal of Wee1. (A) Levels of *wee1* mRNA in the conditional strain. The wild-type FB1 and conditional UMC23 (*wee1*^{nar}) strain were grown for 8 hours in permissive (minimal medium with nitrate, $MMNO_3$) or restrictive conditions (rich medium, YPD). The RNA was extracted and analyzed by northern blotting, loading 10 μg total RNA per lane. 18s rRNA was used to control for loading. (B) Growth of conditional strain in solid medium. Serial tenfold dilutions of FB1 (WT) and UMC23 (*wee1*^{nar}) cultures were spotted in solid rich medium (YPD) and minimal medium with nitrate ($MMNO_3$). YPD plates were incubated for 2 days and the nitrate plates for 4 days at 28°C. (C) Western blotting of inhibitory phosphorylation following *wee1* depletion. Protein extracts from the strains indicated were obtained after incubation in repressive conditions (YPD) at the times indicated (in hours). The Cdk1 levels were determined with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies. (D) Flow cytometry of wild-type and UMC23 cells grown in permissive and restrictive conditions. Cells grown in $MMNO_3$ were centrifuged, washed twice in minimal medium without nitrogen, and resuspended in the appropriate medium. Samples were taken for FACS analysis at the times indicated. (E) Length of wild-type and UMC23 cells growing in permissive conditions ($MMNO_3$). In the upper plot, the length of the major axis of FB1 and UMC23 mother cells was measured and plotted as function of the number of cells. Below, the length of the buds was measured from the same population. A sample of 110 cells was used for each measurement.

impaired, these cells developing an elongated bud that displayed active polarized growth, a phenotype resembling that generated by *wee1* overexpression. In contrast, high levels of *clb2* expression resulted in short cells that divide by septation, a phenotype reminiscent of cells that do not express *wee1* or in which a constitutively unphosphorylated *cdk1*^{AF} allele was overexpressed. Together, these observations suggest that the Cdk1-Clb2 complex could be a target of Wee1.

To evaluate whether Wee1 antagonizes the Cdk1-Clb2 complex, we generated a double conditional mutant *wee1*^{nar} *clb2*^{nar}. When we compared this mutant with the respective single *wee1*^{nar} and *clb2*^{nar} conditional mutants in restrictive conditions, the concurrent down-regulation of *wee1* and *clb2* transcription produced a phenotype identical to that caused by the down-regulation of *clb2* expression alone (Fig. 7A). This epistatic relationship located *clb2* genetically downstream of *wee1*. We then analyzed whether high levels of Wee1 could overcome the effects of high levels of Clb2 on cell morphology. Cells with high constitutive levels of *clb2* expression (replacing the native *clb2* promoter with the strong constitutive *hsp70* promoter) and carrying an ectopic arabinose-inducible *wee1* allele expressed the phenotype of cells overexpressing *clb2* when *wee1* expression was not induced (YPD, Fig. 7B). However, when these cells were transferred to arabinose-containing medium, the cells elongated, resembling wild-type cells that overexpress *wee1* (Fig. 7B, YPA). This result provides further evidence of antagonism between *clb2* and *wee1*.

Finally, we directly tested whether the Cdk1/Clb2 complex is a target of inhibitory phosphorylation in *U. maydis*. Using strains that carry epitope-tagged *U. maydis* cyclins [the B-type cyclins Clb1 and Clb2 (García-Muse et al., 2004) and the G1 cyclin, Cln1 (S. Castillo-Lluva and J.P.-M., unpublished)], these cyclins were immunoprecipitated and the levels of Tyr^{15}P-Cdk1 with which they were associated were analyzed. We found that Cdk1 was not phosphorylated on the inhibitory residues when complexed with the G1 cyclin, Cln1. However, when Cdk1 was associated with either Clb1 or Clb2 it was recognized by the phospho-specific antibody (Fig. 7C). Thus, an important determinant for Wee1 substrate recognition appears to be the presence of the Clb1 and Clb2 subunits, but not the Cln1 subunit.

Transcriptional regulation of *wee1* is important to adapt cell size to different nutritional conditions

Fungal cells adjust their cell cycle depending on environmental conditions. For instance, in response to poor nutritional conditions, this adjustment results in an enlargement of the generation time avoiding the generation of abnormally small daughter cells (Rupes, 2002). In *U. maydis*, this enlargement takes place by increasing the length of the G1 phase until the cell reaches a minimum size to enter a new S phase (Snetselaar and McCann, 1997). We have previously shown that the APC adaptor Cru1 is important in *U. maydis* to determine the length of G1 and cell size in response to nutritional conditions. Indeed, the mRNA levels of *cru1* are regulated by the quality of the growth medium (Castillo-Lluva et al., 2004). In this sense, cell size control in *U. maydis* seems to be more similar to *S. cerevisiae*, in which it takes place mainly in G1, than to

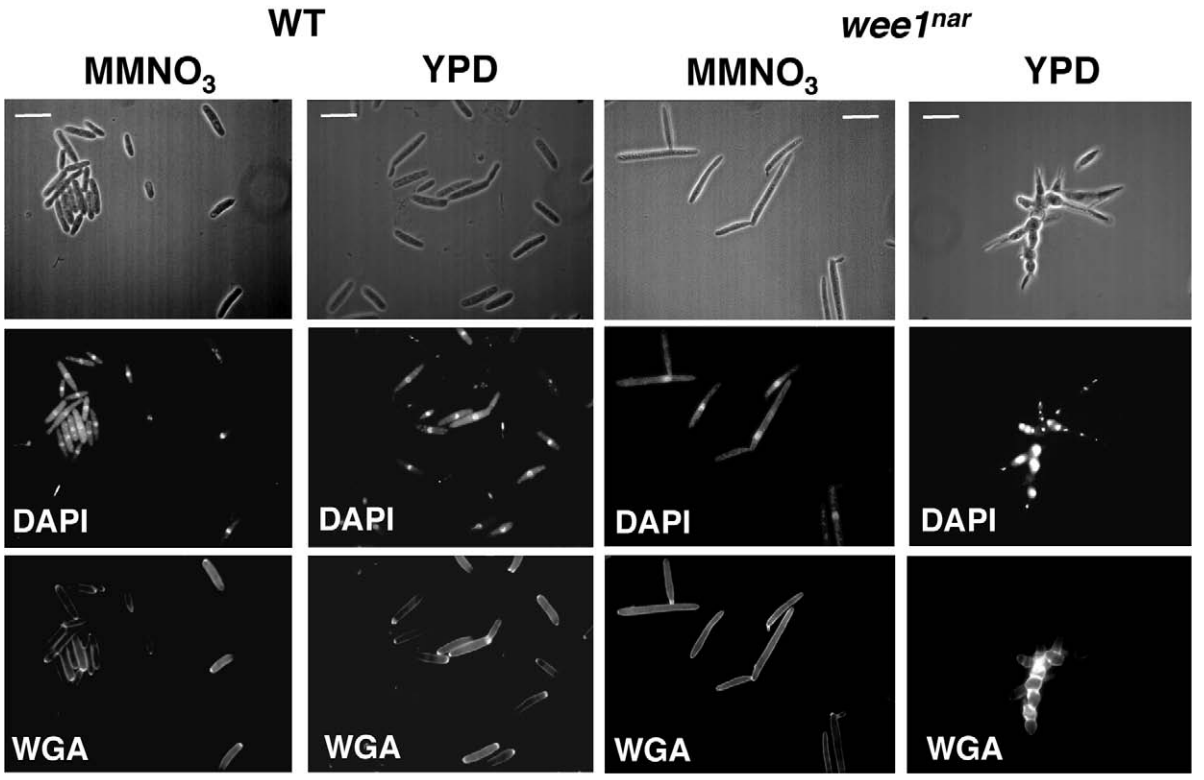


Fig. 6. Morphology of *wee1* conditional cells. (A) Wild-type and mutant cells in permissive and restrictive conditions. FB1 (wild type) and UMC23 (*wee1*^{nar}) cells were incubated for 8 hours in $MMNO_3$ or YPD and then stained with DAPI and FITC-WGA to visualize their nuclei and cell walls, respectively. Scale bars: 20 μm.

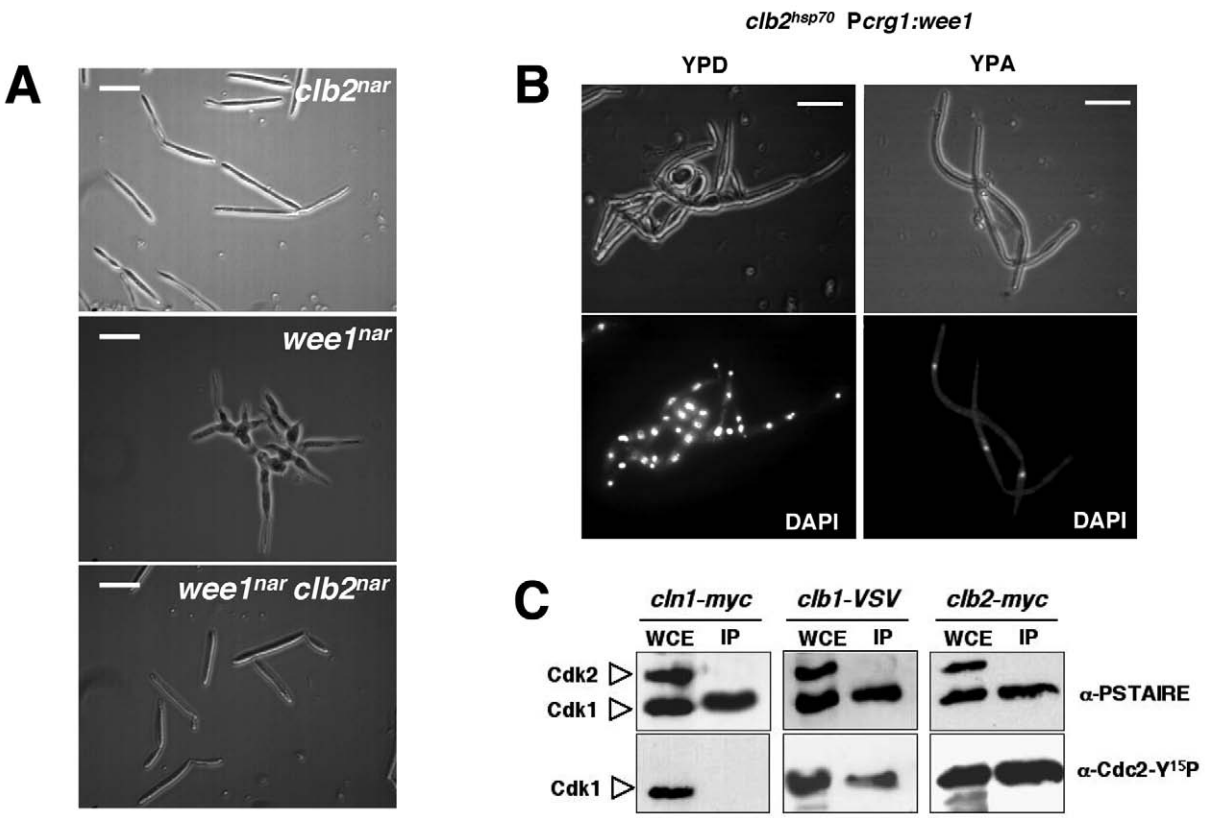


Fig. 7. Relationship between Clb2 and Wee1. (A) Epistatic analysis of *clb2* and *wee1*. The single mutant TAU42 (*clb2*^{nar}) and UMC23 (*wee1*^{nar}) strains, and the double mutant strain UMP40 (*clb2*^{nar}*wee1*^{nar}) were grown in restrictive conditions (YPD) for 8 hours. Note that the phenotype of the double mutant in these conditions is similar to the phenotype of the *clb2*^{nar} cells. Scale bars: 20 μm. (B) Overexpression of *wee1* overcomes the morphological effects imposed by high levels of Clb2. UMC45 cells that express constitutively high levels of *clb2* and carry the ectopic arabinose-inducible P$_{crg}$:*wee1* allele, grow as filaments with short cell compartments in non-inducing conditions (YPD). Transferring the same cells to inducing conditions (YPA) produces elongated cells that resemble wild-type cells overexpressing *wee1*. Scale bars: 20 μm). (C) Inhibitory phosphorylation of B cyclin-associated Cdk1. Protein extracts from cells expressing epitope-tagged versions of Cln1 (SONU58), Clb1 (UMP19) and Clb2 (UMP27) were immunoprecipitated with anti-myc (Cln1 and Clb2 extracts) or anti-VSV (Clb1 extract). Whole cell extracts (WCE) and immunoprecipitates (IP) were immunoblotted with anti-phospho-Cdc2 (Tyr15) and anti-PSTAIRE antibodies. Cdk2 is the *U. maydis* homolog of *S. cerevisiae* Pho85, which is also recognized by the anti-PSTAIRE antibody.

fission yeast, in which G2/M transition is the primary cell size control point (Rupes, 2002). However, the above results showed that the levels of Wee1 seem to be important in determining the length of the G2 phase and hence cell size. Therefore, to examine whether Wee1 has a role in coordinating cell growth, cell division and nutritional conditions in *U. maydis*, we first addressed whether the levels of *wee1* mRNA were affected by the growth medium. Total RNA was extracted from wild-type cells cultured in YPD, CMD and MMD, and the levels of *wee1* mRNA analyzed. It was readily apparent that *wee1* mRNA levels increased as the quality of the medium decreased (Fig. 8A).

How important is *wee1* transcriptional regulation for cells to adapt to different nutritional conditions? To address this question we exchanged the native *wee1* promoter with the *scp* promoter, a weak *U. maydis* constitutive promoter that produces low levels of expression (Bölker et al., 1995a). The abundance of the mRNA produced by this promoter did not vary as a function of the nutritional conditions, and was similar to the *wee1* levels produced in wild-type cells growing in rich medium (YPD, Fig. 8A). Moreover, the cells carrying the constitutive *wee1*^{scp} allele were smaller than wild-type cells (Fig. 8B), and this difference was more obvious in less nutritional medium. This result suggests that up-regulation of Wee1 levels play some role in the adjustment of cell cycle in response to nutritional conditions, particularly in less favorable medium. Analysis of the FACS profile of wild-type and mutant *wee1*^{scp} cells grown in the different media (Fig. 8C) showed an increase in the number of cells with 1C DNA content (i.e. cells in G1 phase). This bias towards cells in G1, suggests that the G1 period must be lengthened in these small cells until the minimum size required for overriding the Start-restraint is attained One possible interpretation of these results is that *U. maydis* uses two different cell size controls at the same time in response to nutritional conditions: one in G1 phase (related with the APC-Cru1 complex) and other in G2/M (related with the Wee1 levels), and that the absence of one of these controls must be compensated by the other. To reinforce this interpretation, we tried unsuccessfully to delete the *cru1* gene in cells carrying the *wee1*^{scp} allele. To determine if the deletion of the *cru1* gene in *wee1*^{scp} cells was lethal, we crossed haploid Δ*cru1* (UMP9, marked with a hygromycin-resistance gene)

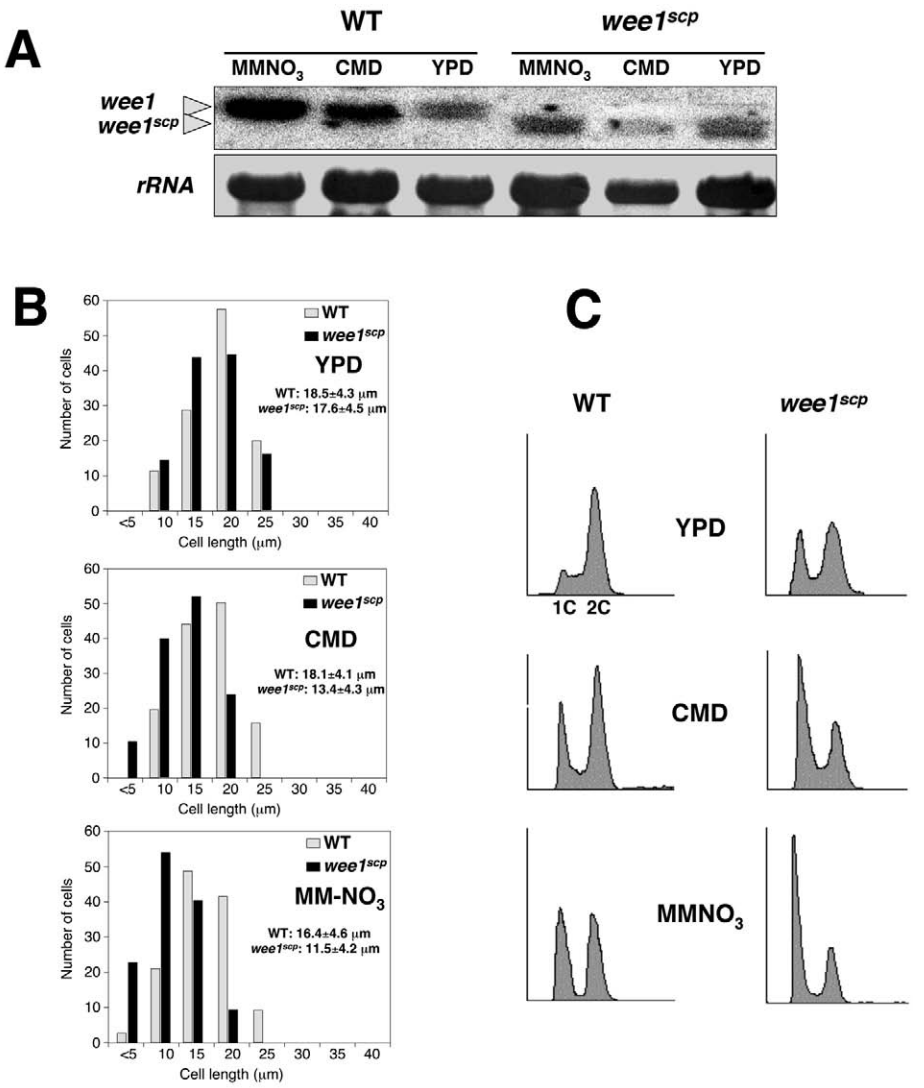


Fig. 8. Cells in which *wee1* is transcriptionally deregulated, do not adapt to different nutritional conditions. (A) The levels of *wee1* mRNA are transcriptionally regulated by nutritional conditions. Wild-type FB1 cells (WT) and the mutant UMC44 strain (*wee1*^{scp}) in which the endogenous *wee1* promoter has been exchanged for the *scp* promoter, were grown in nutrient-rich (YPD), complete (CMD) and minimal medium ($MMNO_3$) to OD_{600} 0.5. Their RNA was isolated and 10 μg of RNA was loaded per lane, using 18s rRNA as a loading control. (B) Length of wild-type and *wee1*^{scp} cells growing in different media. The length of the major axis of 120 cells was measured and plotted as a function of the number of cells. (C) DNA content of wild-type and *wee1*^{scp} cells growing in different media.

cells with compatible haploid *wee1*^{scp} cells (UMC44, marked with a carboxine resistance gene) and afterwards we analyzed the meiotic progeny. From 100 analyzed resulting haploid cells, none was found to carry the two mutant alleles, indicating a synthetic lethality.

Inhibitory phosphorylation could be required for polar growth during the infection process

Several results suggested an involvement of the inhibitory phosphorylation of Cdk1 in the maintenance of polarized growth in *U. maydis*. The conditional depletion of Wee1 or the expression of the *cdk1*^{AF} allele generated cell aggregates composed of cells that often lost polarity and became almost spherical. In contrast, the overexpression of *wee1* generated a highly polarized growth. The initiation of pathogenic development in *U. maydis* involves the activation of a strong polarized growth, as yeast-like budding cells switch, after mating, to tip-growing hyphae, so-called infective tubes (Kahmann et al., 2000). Therefore, we were interested in determining whether cells deficient in inhibitory Cdk1 phosphorylation were able to produce the infective tube. Since *wee1* is essential in *U. maydis*, we took advantage of the cells expressing the *cdk1*^{AF} allele to mimic defective inhibitory phosphorylation. The induction of infective tube formation can be easily scored by co-spotting compatible strains (i.e. with different *a* and *b* loci) on solid medium containing charcoal. On these plates, cell fusion and the development of an infective dikaryotic filament results in the formation of a white layer of aerial hyphae on the surface of the growing colony [Fuz$^+$ phenotype (Holliday, 1974)]. We generated compatible strains that expressed an ectopic copy of the *cdk1*^{AF} allele under the control of the *nar1* promoter. Mixtures of compatible wild-type and mutant strains were spotted on charcoal-containing minimal medium with ammonium (non-inducing conditions) or nitrate (inducing conditions) as a nitrogen source. Mixtures of wild-type cells produced a clear Fuz$^+$ phenotype in both conditions. In contrast, when compatible strains carrying the *cdk1*^{AF} allele were spotted or mixed with wild-type compatible strains, the Fuz$^+$ phenotype did not develop (Fig. 9A). Furthermore, microscopic observation of the mutant mixtures growing on nitrate-containing charcoal plates confirmed the absence of dikaryotic hyphae (not shown). These results indicated that inhibitory phosphorylation is required for the formation of the dikaryotic infective filament. However, dikaryotic hyphal formation requires the fusion of compatible cells, and we therefore considered it possible that this phenotype is a secondary consequence of a defect in mating. To circumvent the need for cell fusion we generated the respective mutations in the solo-pathogenic strain SG200 (Bölker et al., 1995b). This strain, which carries the information of the two mating types, is able to form infective tubes without a mating partner. Consistently with the charcoal assay, SG200 cells expressing the *cdk1*^{AF} allele were unable to produce the infective filament (Fig. 9B).

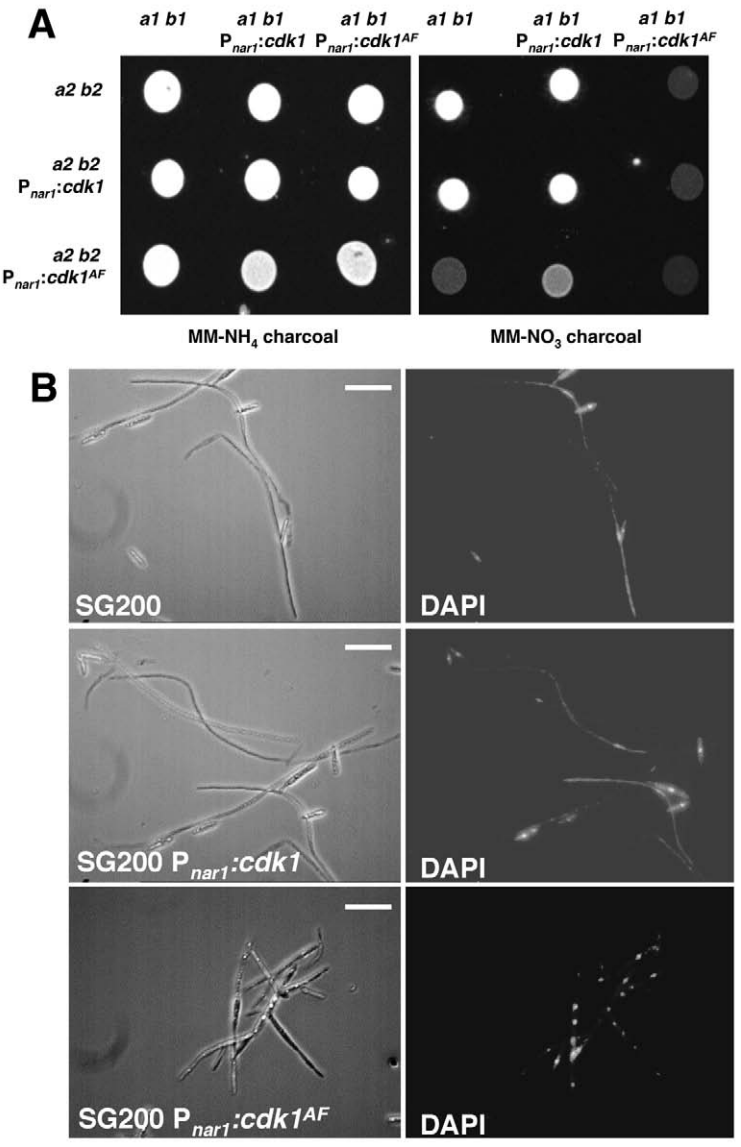
Once it has entered the plant, *U. maydis* proliferates in a hyphal form. Therefore, we wished to address whether the impaired inhibitory phosphorylation of Cdk1 also affected the

Table 3. Pathogenicity assays

Inoculum (relevant phenotype)	Chlorosis		Anthocyanin formation		Tumor formation	
	Total	Percentage	Total	Percentage	Total	Percentage
a1b1 × *a2b2*	54/58	93	54/58	93	54/58	93
a1b1 P_{tef1}*:cdk1* × *a2b2* P_{tef1}*:cdk1*	62/67	92	62/67	92	61/67	91
a1b1 P_{tef1}*:cdk1*^{AF} × *a2b2* P_{tef1}*:cdk1*^{AF}	0/84	0	0/84	0	0/84	0
a1b1 P_{mig1}*:cdk1* × *a2b2*	49/50	98	49/50	98	49/50	98
a1b1 P_{mig1}*:cdk1*^{AF} × *a2b2*	72/75	96	2/75	0.02	0/75	0

ability to grow inside the plant. Since plants inoculated with a mixture of *cdk1*^{AF} compatible strains were asymptomatic and indistinguishable from uninoculated plants (Table 3), most likely because of the inability of mutant fungal cells to invade the plant, we constructed an *a1 b1* strain carrying an ectopic copy of the *cdk1*^{AF} allele under the control of the *mig1* promoter (as well as a control strain with the wild-type *cdk1* allele). This promoter directs expression only when the fungus is growing inside the plant (Basse et al., 2000). These strains were crossed with wild-type *a2 b2* compatible cells. Strikingly, the plants inoculated with the mixture carrying the ectopic constitutively unphosphorylated *cdk1*^{AF} allele showed evidence of chlorosis emanating from the point of inoculation, but fungal infection was unable to induce either anthocyanin stains or tumor formation (Fig. 10A). In contrast, the plants inoculated with mixtures carrying an ectopic wild-type *cdk1* allele under *mig1* promoter control displayed all the usual symptoms of infection (Banuett and Herskowitz, 1996). To analyze the fungus growing inside the plant, symptomatic leaves were stained and examined microscopically. In contrast to control infections that showed the typical hyphal growth inside the plant, the fungal cells expressing the *cdk1*^{AF} allele were present in clumps composed of long chains of rounded cells (Fig. 10B).

Taken together, these results strongly suggest that inhibitory phosphorylation of Cdk1 plays an important role at different points of the infection process and that this role could be related with the ability to support a sustained polarized growth.



Discussion

The main objective of this study was to examine the inhibitory effect that phosphorylation of Tyr^{15} of Cdk1 has on *U. maydis* cells. To pursue this goal, we produced a *cdk1* mutant allele refractory to inhibitory phosphorylation, which promoted aberrant morphogenesis when expressed in *U. maydis* cells. Since this kind of mutation makes the CDK catalytic subunit resistant to negative regulation by Wee1-related kinases in other organisms, we isolated the Wee1-related kinase gene for *U. maydis*. This gene encodes a polypeptide containing features common to all protein kinases, as well as certain motifs specific to the Wee1 kinase family. As expected, the *U. maydis wee1* gene functions as a dose-dependent inhibitor of mitosis. When *wee1* was overexpressed in *U. maydis*, uninuclear cells with a 2C DNA content underwent arrest apparently during G2 phase. The activity of Wee1 as a

Fig. 9. Inhibitory phosphorylation of Cdk1 is required for infective tube formation. (A) Mixtures of the strains indicated were spotted on $MMNH_4$-charcoal (non-inducing) and $MMNO_3$-charcoal (inducing) plates, and incubated for 48 hours at room temperature. Fuzziness (gray colonies) was an indication of successful infective tube formation.
(B) Morphology of the solopathogenic strain SG200 and its derivatives, UMC14 (SG200 P_{nar1}*:cdk1*) and UMC13 (SG200P_{nar1}*:cdk1*^{AF}), which were incubated in inducing conditions (liquid $MMNO_3$ medium) for 8 hours. Scale bars: 20 μm.

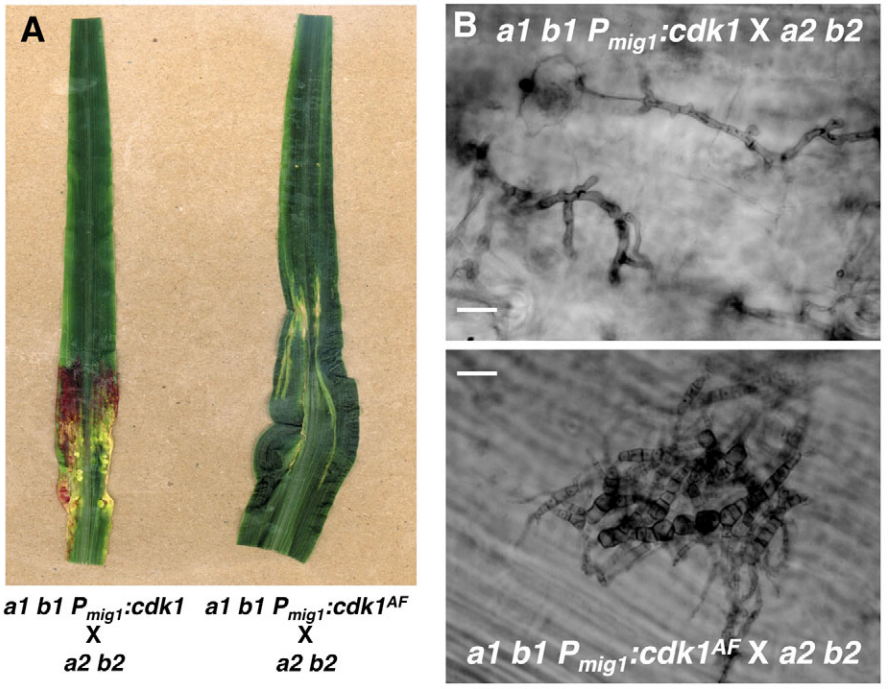


Fig. 10. Impaired inhibitory phosphorylation of Cdk1 affects the ability of *U. maydis* to produce a successful plant infection. (A) Disease symptoms on the leaf blades of young maize plants 14 days post-inoculation with the strains indicated. Control infection (*a1 b1* P_{mig1}*:cdk1* × *a2 b2*) produced the characteristic symptoms of disease (observe the red stain produced by the anthocyanin streaking and small tumors at the leaf margin). In contrast, infection with cells expressing the *cdk1* allele refractory to Tyr^{15} phosphorylation (*a1 b1* P_{mig1}*:cdk1*^{AF} × *a2 b2*) did not induce symptoms other than chlorosis (observe the yellowing of the leaf tissue). (B) Hyphal development of *a1 b1* P_{mig1}*:cdk1* × *a2 b2* (upper panel) and *a1 b1* P_{mig1}*:cdk1*^{AF} × *a2 b2* (bottom panel) cells inside the plant tissue. Symptomatic leaves were removed one week after inoculation and the presence of fungal cells was detected by staining with Chlorazole Black E. Observe the dramatic difference in growth between the *a1 b1* P_{mig1}*:cdk1*^{AF} × *a2 b2* hyphae (bottom panel) and the controls, where two branching hyphae can clearly be seen (upper panel). Scale bars: 20 μm.

mitotic inhibitor seems to be related to its ability to phosphorylate Cdk1. There is a clear correlation between the degree of Cdk1 Tyr^{15} phosphorylation and Wee1 activity. In addition, the G2 cell cycle arrest induced by Wee1 overexpression is largely suppressed in strains expressing a copy of the *cdk1*^{AF} allele (J.P.-M., unpublished).

The fact that *wee1* overexpression causes arrest of *U. maydis* cells in G2 suggests that the Cdk1-cyclin complexes targeted by Wee1 are required for the G2/M transition. In *U. maydis*, the G2/M transition requires the participation of both the Cdk1-Clb1 and the Cdk1-Clb2 complexes (García-Muse et al., 2004). Accordingly, we have shown that the phosphorylated Cdk1 is associated to these B-type cyclins. The Tyr^{15} phosphorylated Cdk1 cannot be found in complexes with Cln1, indicating that as in other organisms, different cyclins target CDKs for distinct negative regulatory controls in *U. maydis* (Devault et al., 1992; Booher et al., 1993; Watanabe et al., 1995). We believe that the primary target for Wee1 is the Cdk1-Clb2 complex. While Cdk1-Clb1 is required for the G1/S and G2/M transitions, Cdk1-Clb2 appears to be specific for G2/M transition and it seems to be a rate-limiting for entry into mitosis (García-Muse et al., 2004). Indeed, *clb2* overexpression produces a phenotype that resembles those produced by overexpression of the *cdk1*^{AF} allele or by downregulation of *wee1*. In contrast, overexpression of *clb1* produces lethal chromosome missegregation (García-Muse et al., 2004). Furthermore, we found that high levels of Wee1 overcome the effects on cellular morphology imposed by high levels of Clb2. In contrast, the toxic effect of high levels of Clb1 (García-Muse et al., 2004) cannot be suppressed by an abundance of Wee1 (J.P.-M., unpublished). However, since the Cdk1-Clb1 complex is also subjected to Tyr^{15} phosphorylation (albeit apparently less than the Cdk1-Clb2 complexes), we believe a fraction of the Cdk1-Clb1 complexes involved in the G2/M transition, are also targeted by the Wee1 kinase. As such, we assume that elements specific to the G1/S transition modify the capacity of the Wee1 kinase to phosphorylate the Cdk1-Clb1 complex.

Inhibitory phosphorylation seems to be essential for cell cycle regulation in *U. maydis*. This is similar to *S. pombe* in which two related kinases (Wee1 and Mik1) phosphorylate Cdc2, the deletion of either gene being lethal (Lundgren et al., 1991). In contrast, the gene encoding the Wee1-like kinase in *S. cerevisiae* (*SWE1*) is dispensable for growth (Booher et al., 1993). The requirement in *U. maydis* for *wee1* reflects the importance of controlling G2 phase length in this organism. Once DNA replication has occurred, *U. maydis* cells must decide whether to bud or to enter into a mating program, a decision that is taken in response to external stimuli (García-Muse et al., 2003). Controlling the length of G2 seems to be primordial for *U. maydis* to make the correct decision. In addition, our data are in accordance with an interesting model proposed by Kellog (Kellog, 2003), which suggests that Wee1-related kinases monitor the total amount of polar growth that occurs. Fission yeast and *U. maydis* are rod-shaped cells and all growth occurs at the ends of the cell (polar growth). In contrast, *S. cerevisiae* cells undergo a brief period of polar growth during bud emergence, but then growth occurs over the entire surface of the bud (isotropic growth). In agreement with Kellog (Kellog, 2003), the loss of *wee1* function causes a more severe phenotype in *U. maydis* than in *S. cerevisiae*, despite the fact that both organisms divide by budding. We believe that this may be because *U. maydis* relies almost entirely on polar growth that occurs during G2, whereas budding yeast only undergo a brief period of polar growth and then switch to isotropic growth.

Also, again in contrast with *S. cerevisiae*, two observations reported here also suggest that inhibitory phosphorylation of Cdk1 could be involved in the modulation of a S phase checkpoint response. We found that the amount of Tyr^{15} phosphorylation increases after treatment with HU, and that

cells expressing the *cdk1*^{AF} allele were hypersensitive to sub-lethal concentrations of HU (i.e. the S phase checkpoint defect is presumably the cause of the HU hypersensitivity).

Progression through the cell cycle is regulated principally before the onset of S phase and of mitosis. In both cases, a critical cell mass must be attained before progression occurs (Nurse, 1975; Fantes, 1977). Recently, we described that in *U. maydis*, the absence of the APC adaptor Cru1 resulted in the inability to adjust G1 phase and hence in smaller than normal cells (Castillo-Lluva et al., 2004). Here, our results show that there is a close relationship between *wee1* expression and cell size. We generated two different *wee1* alleles, *wee1*^{nar} and *wee1*^{scp}, that are transcribed more or less than the wild-type allele. Altering the expression of *wee1* produced cells that were larger and smaller than normal. Wild-type cells can adjust their size to the exterior environment, such as the availability of nutrients (Fantes and Nurse, 1978). Thus, by regulating Wee1 function, growth signals could conceivably produce such an effect. We found that *wee1* mRNA levels increased as the quality of the medium decreased. In poor medium cells spend longer in G1 phase than in rich medium (Castillo-Lluva et al., 2004) and *wee1* seems to be expressed preferentially in G1 phase. Hence, the differences in mRNA levels imposed by the growth medium could be easily explained without having to invoke mechanisms by which *wee1* transcription is directly controlled by the nutritional conditions. Nevertheless, this latter possibility cannot be discarded.

Although minimal medium has been shown to extend the G1 phase of the *U. maydis* cell cycle (Castillo-Lluva et al., 2004), we propose that poorer medium may also delay progression to G2, and that this delay is dependent on Tyr^{15} phosphorylation. Such coordination in the length of the G1 and G2 phases is not only useful in conditions of limited nutrition but also to compensate for defects in each phase. For instance, G1 phase is not appreciable in cells carrying the *wee1*^{nar} allele, which leads to a delay in mitosis and oversized buds (compare the FACS profile of *wee1*^{nar} cells with wild-type cells growing in MM-NO_3, Fig. 4B). In contrast, in *wee1*^{scp} cells, which have low levels of *wee1* mRNA and are small, there is a clear increase in the time spent by the cell in G1 phase. Furthermore, Cru1 is indispensable for the growth of *wee1*^{scp} cells, probably because these double mutants cannot lengthen the G1 period to restrain S phase until the critical size to override the Start control is attained.

In addition to the influence on cell size, the *cdk1*^{AF} allele or the down-regulation of *wee1* had a marked effect on septation and cell pattern formation during vegetative growth. This indicates that septation in axenic cultures of *U. maydis* is normally prevented through a mechanism involving tyrosine phosphorylation of Cdk1. Indeed, septation in *A. nidulans* is regulated *via* tyrosine phosphorylation of *nimX*^{cdc2} (Harris and Kraus, 1998; Kraus and Harris, 2001). Another possibility is that the failure to inhibit Cdk1 through phosphorylation impairs the coordination between cell cycle regulation and the transition to the budding mode of growth. In *S. cerevisiae* a checkpoint system monitors the emergence of the bud. This morphogenetic checkpoint coordinates bud emergence with the cell cycle through the activity of the Swe1 protein kinase and tyrosine phosphorylation of Cdc28 (Lew and Reed, 1995; Sia et al., 1996). This system is linked to the organization of the cytoskeleton and septin function through a protein kinase cascade that eventually inhibits Cdc28 by tyrosine phosphorylation (Carroll et al., 1998; Longtine et al., 1998; Barral et al., 1999). If such a system were operative in *U. maydis*, it may become critical in axenic cultures where *U. maydis* cells grow by budding. The failure to inhibit Cdk1 by phosphorylation may prevent the normal synchronization and eventually contribute to the developmental defects we have observed.

One of the aims of this work was to define the interaction between the inhibitory phosphorylation of the catalytic subunit of mitotic CDKs and the program of virulence in *U. maydis*. We found that the expression of a constitutively unphosphorylated Cdk1 abolishes the ability of *U. maydis* to produce infective filaments. This inability could be explained assuming a role of Wee1 protein in the regulation of polarized growth. Alternatively, since the infective filament is cell cycle arrested in G2 phase (C.S. and J.P.-M., unpublished), it is tempting to speculate that G2 cell cycle arrest is mediated by the inhibition of Cdk1 by phosphorylation, and that the failure to induce arrest of the cell cycle impairs the formation of the infective tube. This interesting possibility will be clarified in future studies. By restricting the expression of the constitutively unphosphorylated *cdk1* allele to fungi growing inside the plant, we bypassed the requirement of inhibitory phosphorylation for plant penetration. Hence, we were able to show that Tyr^{15} phosphorylation is also required for infection to progress inside the plant, since the symptoms of mutant cell infection do not progress beyond chlorosis. Interestingly, fungal cells expressing high levels of *clb2* (García-Muse et al., 2004) showed a similar infection defect. Taken together, these data indicate that the accurate control of G2/M transition seems to be important for successful infection by *U. maydis*. It is worth bearing in mind that *U. maydis* cells grow in yeast-like unicellular form in saprophytic conditions. The induction of the pathogenic phase requires two compatible haploid cells to fuse and the generation of an infective dikaryotic filament that invades and proliferates inside the plant (Kahmann et al., 2000). Studies of pseudohyphal development in *S. cerevisiae* have revealed the importance of controlling the G2/M transition for the production of the filamentous growth (for a review, see Rua et al., 2001).

In summary, the results presented here reinforce the connections between the cell cycle and the induction of the pathogenic program in *U. maydis*. Moreover, these data highlight the potential significance of cell cycle regulation in microbial pathogenesis.

We thank Prof. Correa-Bordes for advice regarding the phosphorylation western blots. This work was supported by Grant BIO2002-03503 from MCyT and Grant 07B/0040/2002 from CAM.

References

Aligue, R., Wu, L. and Russell, P. (1997). Regulation of *Schizosaccharomyces pombe* Wee1 tyrosine kinase. *J. Biol. Chem.* **272**, 13320-13325.

Banuett, F. and Herskowitz, I. (1989). Different *a* alleles are necessary for maintenance of filamentous growth but not for meiosis. *Proc. Natl. Acad. Sci. USA* **86**, 5878-5882.

Banuett, F. and Herskowitz, I. (1996). Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*. *Development* **122**, 2965-2976.

Banuett, F. and Herskowitz, I. (2002). Bud morphogenesis and the actin and

microtubule cytoskeletons during budding in the corn smut fungus, *Ustilago maydis*. *Fungal Genet. Biol.* **37**, 149-170.

Basse, C. W. and Steinberg, G. (2004). *Ustilago maydis*, model system for analysis of the molecular basis of fungal pathogenicity. *Mol. Plant Pathol.* **5**, 83-92.

Basse, C. W., Stumpferl, S. and Kahmann, R. (2000). Characterization of a *Ustilago maydis* gene specifically induced during the biotrophic phase: evidence for negative as well as positive regulation. *Mol. Cell. Biol.* **20**, 329-339.

Bölker, M., Böhnert, H. U., Braun, K. H., Görl, J. and Kahmann, R. (1995a). Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* **248**, 547-552.

Bölker, M., Genin, S., Lehmler, C. and Kahmann, R. (1995b). Genetic regulation of mating and dimorphism in *Ustilago maydis*. *Can. J. Bot.* **73**, 320-325.

Booher, R. N., Deshaies, R. J. and Kirschner, M. W. (1993). Properties of *Saccharomyces cerevisiae* Wee1 and its differential regulation of $p34^{CDC28}$ in response to G1 and G2 cyclins. *EMBO J.* **12**, 3417-3426.

Bottin, A., Kämper, J. and Kahmann, R. (1996). Isolation of a carbon source-regulated gene from *Ustilago maydis*. *Mol. Gen. Genet.* **253**, 342-352.

Brachmann, A. (2001). Die frühe infektionsphase von *Ustilago maydis*: Genregulation durch das bW/bE-Heterodimer. PhD Dissertation, Ludwig-Maximilians-Universität, München.

Brachmann, A., Weinzierl, G, Kämper, J. and Kahmann, R. (2001). Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. *Mol. Microbiol.* **42**, 1047-1063.

Brachmann, A., Schirawski, J., Mülller, P. and Kahmann, R. (2003). An unusual MAP kinase is required for efficient penetration of the plant surface by *Ustilago maydis*. *EMBO J.* **9**, 2199-2210.

Barral, Y., Parra, M., Bidlingmaier, S. and Snyder, M. (1999). Nim1-related kinases coordinate cell cycle progression with the organization of the peripheral cytoskeleton in yeast. *Genes Dev.* **13**, 176-187.

Carroll, C. W., Altman, R., Schieltz, D., Yates, J. R. and Kellog, D. (1998). The septins are required for the mitosis-specific activation of the Gin4 kinase. *J. Cell Biol.* **143**, 709-717.

Castillo-Lluva, S., García-Muse, T. and Pérez-Martín, J. (2004). A member of the Fizzy-related family of APC activators is required at different stages of plant infection by *Ustilago maydis*. *J. Cell Sci.* **117**, 4143-4156.

Cerutti, L. and Simanis, V. (2000). Controlling the end of the cell cycle. *Curr. Opin. Genet. Dev.* **10**, 65-69.

De Souza, C. P., Ye, X. S. and Osmani, S. A. (1999). Checkpoint defects leading to premature mitosis also cause endoreplication of DNA in *Aspergillus nidulans*. *Mol. Biol. Cell* **10**, 3661-3674.

Devault, A., Fesquet, D., Cavadore, J. C., Garrrigues, A. M., Labbé, J. C., Lorca, T., Picard, A., Philippe, M. and Dorée, M. (1992). Cyclin A potentiates maturation-promoting factor activation in the early *Xenopus* embryo *via* inhibition of the tyrosine kinase that phosphorylates Cdc2. *J. Cell Biol.* **118**, 1109-1120.

Fantes, P. A. (1977). Control of cell size and cycle time in *Schizosaccharomyces pombe*. *J. Cell Sci.* **24**, 51-67.

Featherstone, C. and Russell, P. (1991). Fission yeast $p107^{wee1}$ mitotic inhibitor is a tyrosine/serine kinase. *Nature* **349**, 808-811.

Fleig, U. N. and Gould, K. L. (1991). Regulation of Cdc2 activity in *Schizosaccharomyces pombe*: the role of phosphorylation. *Semin. Cell Biol.* **2**, 195-204.

García-Muse, T., Steinberg, G. and Pérez-Martín, J. (2003). Pheromone-induced G2 arrest in the phytopathogenic fungus *Ustilago maydis*. *Eukaryot. Cell* **2**, 494-500.

García-Muse, T., Steinberg, G. and Pérez-Martín, J. (2004). Characterization of B-type cyclins in the smut fungus *Ustilago maydis*: roles in morphogenesis and pathogenicity. *J. Cell Sci.* **117**, 487-506.

Garrido, E. and Pérez-Martín, J. (2003). The *crk1* gene encodes an Ime2-related protein that is required for morphogenesis in the plant pathogen *Ustilago maydis*. *Mol. Microbiol.* **47**, 729-743.

Garrido, E., Voß, U., Müller, P., Castillo-Lluva, S., Kahmann, R. and Pérez-Martín, J. (2004). The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. *Genes Dev.* **18**, 3117-3130.

Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F. and Kirschner, M. W. (1991). Cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. *Cell* **67**, 197-211.

Gould, K. L. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast $cdc2^{+}$ protein kinase regulates entry into mitosis. *Nature* **342**, 39-45.

Gillissen, B., Bergemann, J., Sandmann, C., Schrör, B., Bölker, M. and Kahmann, R. (1992). A two-component regulatory system for self/non-self recognition in *Ustilago maydis*. *Cell* **68**, 647-657.

Hanks, S. K. (1991). Eukaryotic protein kinases. *Curr. Opin. Struct. Biol.* **1**, 369-383.

Harris, S. D. (1997). The duplication cycle in *Aspergillus nidulans*. *Fungal Genet. Biol.* **22**, 1-12.

Harris, S. D. and Kraus, P. R. (1998). Regulation of septum formation in *Aspergillus nidulans* by a DNA damage checkpoint pathway. *Genetics* **148**, 1055-1067.

Harvey, S. L. and Kellogg, D. R. (2003). Conservation of mechanisms controlling entry into mitosis: budding yeast Wee1 delays entry into mitosis and is required for cell size control. *Curr. Biol.* **13**, 264-275.

Holliday, R. (1974). *Ustilago maydis*. In *Handbook of Genetics* (ed. R. C. King), pp. 575-595. New York, USA: Plenum Press.

Jorgensen, P., Nishikawa, J. L., Breitkreutz, B. J. and Tyers, M. (2002). Systematic identification of pathways that couple cell growth and cell division in yeast. *Science* **297**, 395-400.

Kahmann, R., Steinberg, G., Basse, C., Feldbrügge, M. and Kämper, J. (2000). *Ustilago maydis*, the causative agent of corn smut disease. In *Fungal Pathology* (ed. J. W. Kronstad), pp. 347-371. Dodrecht, The Netherlands: Kluwer Academic Publishers.

Kellogg, D. R. (2003). Wee1-dependent mechanisms required for coordination of cell growth and cell division. *J. Cell Sci.* **116**, 4883-4890.

Kraus, P. R. and Harris, S. D. (2001). The *Aspergillus nidulans snt* genes are required for the regulation of septum formation and cell cycle checkpoints. *Genetics* **159**, 557-569.

La Valle, R. and Wittenberg, C. (2001). A role for the Swe1 checkpoint kinase during filamentous growth of *Saccharomyces cerevisiae*. *Genetics* **158**, 549-562.

Lew, D. J. (2000). Cell cycle checkpoints that ensure coordination between nuclear and cytoplasmic events in *Saccharomyces cerevisiae*. *Curr. Opin. Genet. Dev.* **10**, 47-53.

Lew, D. J. and Reed, S. I. (1995). Cell cycle checkpoints monitors cell morphogenesis in budding yeast. *J. Cell Biol.* **129**, 739-749.

Lew, D. J. and Kornbluth, S. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* **8**, 795-804.

Longtine, M. S., Fares, H. and Pringle, J. R. (1998). Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *J. Cell Biol.* **143**, 719-736.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991). Mik1 and Wee1 cooperate in the inhibitory tyrosine phosphorylation of Cdc2. *Cell* **64**, 1111-1122.

Martínez-Anaya, C., Dickinson, R. J. and Sudbery, P. E. (2003). In yeast, the pseudohyphal phenotype induced by isoamyl alcohol results from the operation of the morphogenesis checkpoint. *J. Cell Sci.* **116**, 3423-3431.

McGowan, C. and Russell, P. (1995). Cell cycle regulation of human Wee1. *EMBO J.* **14**, 2166-2175.

Millar, J. B., McGowan, C. H., Lenaers, G., Jones, R. and Russell, P. (1991). $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. *EMBO J.* **10**, 4301-4309.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. *Nature* **256**, 547-551.

Parker, L. L., Atherton-Fessler, S. and Piwnica-Worms, H. (1992). $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. *Proc. Natl. Acad. Sci. USA* **89**, 2917-2921.

Rey, A. L., Herald, H. A. and Lew, D. J. (1996). Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Mol. Biol. Cell* **7**, 1657-1666.

Rua, D., Tobe, B. T. and Kron, S. J. (2001). Cell cycle control of yeast filamentous growth. *Curr. Opin. Microbiol.,* **4**, 720-727.

Rupes, I. (2002). Checking cell size in yeast. *Trends Genet.* **18**, 479-485.

Russell, P. and Nurse, P. (1986). $cdc25^{+}$ functions as an inducer in the mitotic control of fission yeast. *Cell* **45**, 145-153.

Russell, P. and Nurse, P. (1987). Negative regulation of mitosis by $wee1^{+}$, a gene encoding a protein kinase homolog. *Cell* **49**, 559-567.

Sherman, F., Fink, G. R. and Hicks, J. B. (1986). *Laboratory Course Manual For Methods In Yeast Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sia, R. A., Herald, H. A. and Lew, D. J. (1996). Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Mol. Biol. Cell* **11**, 1657-1666.

Snetselaar, K. M. and McCann, M. C. (1997). Using microdensitometry to correlate cell morphology with the nuclear cycle in *Ustilago maydis*. *Mycologia* **89**, 689-697.

Spellig, T., Bottin, A. and Kahmann, R. (1996). Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Mol. Gen. Genet.* **252**, 503-509.

Steinberg, G., Wedlich-Söldner, R., Brill, M. and Schulz, I. (2001). Microtubules in the fungal pathogen *Ustilago maydis* are highly dynamic and determine cell polarity. *J. Cell Sci.* **114**, 609-622.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple alignment aided by quality analysis tools. *Nucleic Acid Res.* **25**, 4876-4882.

Tsukuda, T., Carleton, S., Fotheringham, S. and Holloman, W. K. (1988). Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. *Mol. Cell. Biol.* **8**, 3703-3709.

Watanabe, N., Broome, M. and Hunter, T. (1995). Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. *EMBO J.* **14**, 1878-1891.

Wolkow, T. D., Harris, S. D. and Hamer, J. E. (1996). Cytokinesis in *Aspergillus nidulans*is controlled by cell size, nuclear positioning and mitosis. *J. Cell Sci.* **109**, 2179-2188.