The DNA Damage Response Signaling Cascade Regulates Proliferation of the Phytopathogenic Fungus *Ustilago maydis* in Planta

Carmen de Sena-Tomás, Alfonso Fernández-Álvarez, William K. Holloman, and José Pérez-Martín

**INTRODUCTION**

The sexual life cycle starts with fusion of two different haploid cells, proceeds to the formation of a diploid, and ends with meiosis to generate four haploid cells (Chen et al., 2007). However, the way in which haploid cells are brought together and the predominance of the haploid or diploid phase varies between species. In fungal cells, mating, the process equivalent to fertilization, brings together two haploid nuclei in the same cytoplasm. In some species of fungi, this process is followed by meiosis, resulting in a diploid nucleus that enters meiosis. In fungal cells, maintenance of the dikaryotic state requires an intricate cell division process that often involves the formation of a structure known as the clamp connection as well as the sorting of one of the nuclei to this structure to ensure that each daughter dikaryon inherits a balance of each parental genome. Here, we describe an atypical role of the DNA damage checkpoint kinases Chk1 and Atr1 during pathogenic growth of *U. maydis*. We found that Chk1 and Atr1 collaborate to control cell cycle arrest during the induction of the virulence program in *U. maydis* and that Chk1 and Atr1 work together to control the dikaryon formation. These findings uncover a link between a widely conserved signaling cascade and the virulence program in a phytopathogen. We propose a model in which adjustment of the cell cycle by the Atr1-Chk1 axis controls fidelity in dikaryon formation. Therefore, Chk1 and Atr1 emerge as critical cell type regulators in addition to their roles in the DNA damage response.

Establishment and maintenance of dikaryotic growth in Basidiomycete fungi is controlled by information specified at the Mating Type (MAT) loci, specialized regions of fungal genomes akin to the sex chromosomes of larger eukaryotes (Lee et al., 2010). Although the specific contribution of the MAT locus components to dikaryon formation varies among species characterized thus far, a central element common to all of them is the activation of a specific transcriptional cascade controlled by a heterodimeric homeodomain transcription factor, with components derived from the MAT locus of each parent. Dikaryotic maintenance involves an intricate cell division process to ensure that each dikaryon inherits a balance of each parental genome. In many dikaryotic basidiomycetes (with some exceptions, such as various Uredinomycetes; for instance, see Ikeda et al., 2003) cell division involves the formation of a specialized structure known as the clamp connection as well as the sorting of one of the nuclei to this structure. In this way, nuclear division occurs in a synchronous and independent fashion in two distinct subcellular compartments. Once nuclear division is finished, the clamp connection is resolved to reconstitute the dikaryotic status of daughter cells (Brown and Casselton, 2001; Gladfelter and Berman, 2009). Studies with *Coprinopsis cinerea* and *Schizophyllum commune* indicated that the MAT genes encoding homeodomain transcription factors govern nuclear pairing as well as clamp formation (Casselton and Olesnick, 1998; Kües, 2000); therefore, a connection between MAT genes and cell cycle control is predicted, although the details behind these connections are largely unknown.
In *U. maydis*, the dikaryotic state dominates the period of growth occurring during the infectious phase. This state is dependent upon the *MAT*-encoded homeprotein called the b-complex, whose subunits, bW and bE, are provided by each mating partner (Feldbrügge et al., 2004; Brefort et al., 2009). During induction of the virulence program in *U. maydis*, an infectious dikaryotic hypha is produced on the plant surface as a result of mating of a pair of compatible haploid budding cells. The infectious hypha or filament is composed of a single dikaryotic cell that is arrested in the cell cycle at G2 phase (Mielnichuk et al., 2009), and its formation is dependent on synthesis of the b-complex. The arrest is transient, and eventually the filament manages to enter the plant tissue, where it starts to proliferate while maintaining its dikaryotic status. Since mutations that abolish this transient cell cycle arrest also impair dikaryon formation (Mielnichuk et al., 2009), it is thought that b-induced cell cycle arrest is required for dikaryon establishment in *U. maydis*. In our efforts to describe the mechanisms behind this transient cell cycle arrest, we recently reported that the widely conserved Chk1 protein kinase is required for this cell cycle arrest (Mielnichuk et al., 2009). Chk1 is better known as a key signal transducer within the DNA damage response cascade (Chen and Sanzhez, 2004) in a broad range of eukaryotes, including *U. maydis* (Pérez-Martin, 2009). Here, we investigated the response of Chk1 to b-complex formation. We present findings showing that Chk1 is activated through phosphorylation by the conserved upstream activating kinase Atr1 and that the Atr1-Chk1 regulatory axis serves in maintenance of dikaryotic status in planta. Because Chk1 controls cell cycle progression, we propose that b-complex–mediated activation of the Atr1-Chk1 axis is part of the mechanism responsible of coordination during a dikaryotic cell cycle.

**RESULTS**

b-Dependent Cell Cycle Arrest Requires Chk1 Activating Phosphorylation

Assembly of the heterodimeric b-complex during dikaryon formation in *U. maydis* is concomitant with transient cell cycle arrest as well as with accumulation of phosphorylated forms of Chk1 and translocation of Chk1 into the nucleus, two hallmarks of Chk1 activation (Mielnichuk et al., 2009). Previous research (Pérez-Martin, 2009) established that in response to DNA damage, Chk1 activation results in G2 cell cycle arrest and involves phosphorylation at two residues (Thr-394 and Ser-448) located in the regulatory domain (Figure 1A). Mutant isoforms containing Ala in place of these residues could not be activated in response to DNA damage signals (Pe´ rez-Martı´n, 2009). To determine whether these phosphorylation sites were also important during the control of the inducible *nar1* promoter that is induced by the addition of nitrate to medium (Brachmann et al., 2001). As a control, a T7-tagged wild-type chk1 allele was used. Upon induction, Chk1 T394A S448A protein did not show the reduced electrophoretic mobility observed with wild-type Chk1 protein after induction of heterodimeric b protein (Figure 1B). Moreover, the cells carrying the nonphosphorylatable Chk1 allele were
impaired in cell cycle arrest just as were cells deleted for \textit{chk1} (Figure 1C). We also analyzed the ability of compatible haploid cells carrying the \textit{chk1T394A S448A} allele to infect plants and found that the nonphosphorylatable mutant showed similar virulence defects as those observed in cells lacking Chk1 protein (Figure 1D). In summary, these results show that the \textit{chk1T394A S448A} mutant phenocopies all defects observed in the \textit{chk1} null mutant, implying that phosphorylation of Chk1 at these residues is integral to the mechanism of b-dependent activation of Chk1.

\textbf{Atr1 Is Required in the Response to DNA Damage in \textit{U. maydis}}

In other model systems, Chk1 phosphorylation in response to DNA damage is performed by two phosphatidylinositol 3-kinase-related kinases (PIKKs), ATM and ATR (Zhou and Elledge, 2000; Nyberg et al., 2002). Putative orthologs of these two PIKKs are present in \textit{U. maydis} genome (Figure 2A; see Supplemental Data Set 1 online). The genes encoding Atm1 (Um15011) and Atr1 (Um01110) were identified as entries (noted in parentheses) in the manually annotated Munich Information Center for Protein Sequences \textit{U. maydis} database (see http://mips.helmholtz-muenchen.de/genre/proj/ustilago/). To further analyze the relationships between these putative kinases and Chk1, gene deletion of the corresponding genes was attempted. Constructions containing a hygromycin resistance cassette flanked by regions located upstream and downstream of the \textit{atr1} and \textit{atm1} open reading frames were transformed in haploid cells. Only mutants lacking the \textit{atr1} gene were obtained. As this result suggested that \textit{atm1} was essential, a diploid/meiotic analysis protocol was employed. We successfully inactivated one \textit{atm1} allele in the diploid FBD11 strain, replacing it with the hygromycin resistance cassette, generating the \textit{atm1} \textit{D} null allele. After sporulation, we analyzed the meiotic progeny of this strain, and we...
Figure 3. Atr1 Is Required for Activation of Chk1 in Response to DNA Damage in *U. maydis*.

(A) Epistasis analysis between *chk1*, *atr1*, and *brh2*. Survival curves against UV irradiation of the indicated single and double mutants were obtained. Cells were grown to late log phase, adjusted to a density of $2 \times 10^7$ cells per mL, and irradiated with UV. Survival was determined by counting colonies visible after incubation for 2 to 3 d. wt, wild type.

(B) In vivo phosphorylation of Chk1 in response to agents that induce DNA damage depends on Atr1. Wild-type (UMP162) and *atr1Δ* (UMP207) cells carrying an endogenous Chk1-T7 allele were grown with no treatment (control) or in the presence of 0.5 mM HU or 50 ng/mL phleomycin (Phleo) for 6 h. Protein extracts were immunoprecipitated with a commercial anti-T7 antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-T7 antibody.

(C) Atr1 is required to localize Chk1 at the nucleus. Cell images of wild-type (UMP111) and *atr1Δ* (UCS15) strains carrying a Chk1-3GFP fusion protein after 3 h of incubation in the presence of HU or phleomycin (Phleo). DIC, differential interference contrast. Bar = 10 μm.

(D) Quantification of the cell response to DNA damage as the percentage of cells carrying a clear nuclear GFP fluorescence signal. The graph shows the result from two independent experiments, counting more than 100 cells each. Means and SDs are shown.
found no hygromycin resistant cells, indicating that \textit{atm1} was an essential gene. We decided to focus on the characterization of Atr1. Sequence comparisons of \textit{U. maydis} Atr1 with orthologs from different organisms revealed the presence of conserved domains, including the PIKK-specific domains FAT and FATC (Bosotti et al., 2000), suggesting that \textit{U. maydis} Atr1 is a bona fide Atr1 ortholog (Figure 2B). Consistently, we found that cells lacking Atr1 were extremely sensitive to several genotoxic insults: UV irradiation, which induces pyrimidine dimers in DNA; hydroxyurea (HU), which inhibits ribonucleotide reductase and therefore affects replication by depletion of deoxynucleotide triphosphates, causing replication fork stalling and collapse; methyl methanesulfonate, which induces DNA alkylation; phleomycin, a radiomimetic drug that causes double-strand breaks in

\textbf{Figure 4.} Atr1 is Required for b-Dependent Cell Cycle Arrest.

\textbf{(A)} Cell images of control (UCS20) and a derived strain lacking the \textit{chk1} gene (\textit{chk1\Delta}, UMP112) or \textit{atr1} (\textit{atr1\Delta}, UCS21) incubated for 8 h in inducing conditions (MM-NO3). Strains carried a NLS-GFP fusion under control of the \textit{b}-dependent \textit{dik6} promoter to detect the nucleus. Bar = 15 \textmu m.

\textbf{(B)} Percentage of cells producing mononucleated filaments (i.e., cell cycle arrested) after 24 h of incubation. The graph shows the result from two independent experiments, counting more than 100 cells each. Means and SDs are shown.

\textbf{(C)} Dependence on \textit{atr1} for in vivo phosphorylation of Chk1 during \textit{b}-dependent filamentation. Control (UCS31) and \textit{atr1\Delta} (UCS32) AB33-derived cells carrying an endogenous \textit{chk1-T7} allele were incubated in MM-NO3 for the indicated time (in hours). Protein extracts were immunoprecipitated with a commercial anti-T7 antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-T7 antibody.

\textbf{(D)} Atr1 is required to localize Chk1 at the nucleus in response to \textit{b}-induction. Cell images of control (UMP133) and \textit{atr1\Delta} strain (UMP208) carrying a Chk1-3GFP fusion protein after 6 h in inducing conditions (MM-NO3). Bar = 30 \textmu m.

\textbf{(E)} Distribution of cells showing nuclear GFP accumulation in function of cell length. Quantification is the result of measurement of two independent experiments, counting more than 100 cells each. Mean and SD are shown.
Atr1 Phosphorylates and Activates Chk1 in Response to DNA Damage in U. maydis

To define relationships between atr1 and chk1, we constructed double mutants of chk1 and atr1 and analyzed sensitivity to UV. As control for this epistasis analysis, we also constructed an atr1 brh2 double mutant strain. Our genetic results suggest that atr1 acts in the same pathway as chk1, since double chk1 atr1 mutants showed the same sensitivity as atr1 mutant, while brh2 seems to work in a distinct pathway than atr1 since their effects were additive (Figure 3A).

We also analyzed whether Atr1 was required for the accumulation of phosphorylated forms of Chk1 in response to DNA damage agents. Our current view of DNA damage–dependent Chk1 activation in U. maydis is that there are two main signals to be detected by DNA surveillance systems: DNA double-strand breaks (induced here by phleomycin treatment) and single-strand DNA tracts as a hallmark of replication stress (caused by HU treatment). We challenged cultures of wild-type and atr1 mutant strains carrying the Chk1-T7 allele with HU or phleomycin and found that absence of Atr1 abolished the mobility shift in the presence of HU, while in the presence of phleomycin, the shift was only partially abrogated. Distinct behavior in Chk1 phosphorylation with respect to these two DNA damaging agents was previously noted (Pérez-Martin, 2009), suggesting that these two stimuli are transmitted by different signaling pathways, and our results support the idea that at least one of them seems to be totally dependent on Atr1.

Given that phosphorylation seemed to be required for Chk1 activation and that in response to DNA damage Chk1 accumulated in the nucleus, we also analyzed the ability of Chk1 to translocate to the nucleus in response to DNA damage in the absence of Atr1. We examined the subcellular localization of green fluorescent protein (GFP)-tagged Chk1 in the presence of HU and phleomycin (Figure 3C). While control cells showed a clear nuclear accumulation of the fluorescent signal in the presence of these DNA damaging agents, in the absence of Atr1, GFP-tagged Chk1 failed to accumulate in the nucleus. This response was dramatic in the presence of HU but somewhat less obvious in the presence of phleomycin, mirroring the above phosphorylation results (Figure 3D).

Atr1-Dependent Phosphorylation of Chk1 Is Required for b-Dependent Cell Cycle Arrest

We analyzed whether Atr1 was required for b-induced cell cycle arrest. For this, we deleted the atr1 gene in strains expressing the homeoprotein b-complex heterodimer (AB33 background). We observed that deletion of atr1 in this genetic background resulted in elongated cells. To distinguish the b-induced filaments from such a cell population background, the haploid strains used expressed a GFP fusion to a nuclear localization signal under control of the dik6 promoter, which is specifically activated by the b-complex heterodimer (Flor-Parra et al., 2006). In this way, only cells expressing the b-dependent program produced a fluorescent signal. We found that in the control strain, almost the totality of the b-expressing cell population carried a single nucleus, while chk1Δ and atr1Δ filaments frequently carried more than two nuclei (Figures 4A and 4B), which was consistent with a defect in the ability to arrest entry into mitosis. These results suggest that most likely Atr1 is the PIKK kinase involved in the activation of Chk1 in response to b-induction.

To gain additional support for this idea, we investigated whether Atr1 was required for the accumulation of phosphorylated forms of Chk1 in response to b-induction.

DNA; and ionizing radiation (IR), which generates double-strand breaks (Figure 2C; see Supplemental Figure 2 online). As comparisons we used mutant cells lacking the chk1 gene as well as cells lacking brh2, which encodes a BRCA2-like protein required for homology-directed recombinational repair (Kojic et al., 2002).
of Chk1 in response to the induction of the β-dependent program. Consistently, we found that there was no Chk1 phosphorylation in cells lacking Atr1 after β-induction (Figure 4C). In addition, given that phosphorylation seemed to be required for Chk1 activation and nuclear accumulation, the relationships between phosphorylation and nuclear localization in response to the induction of the β-dependent program were also investigated. When AB33-derived cells carrying a Chk1-GFP fusion were induced to produce filaments, we observed a clear GFP signal accumulation in the nucleus (Figure 4D). As we already noted, Chk1 activations seems to be a transient response and the nuclear GFP signal disappears in long filaments (Mielnichuk et al., 2009). To quantify this apparently transient response, we measured cells (n = 100 cells, two independent experiments) producing filaments of different length and plotted against the presence or not of a nuclear GFP signal (Figure 4E), confirming that only shorter filaments (i.e., early stages) showed nuclear accumulation of Chk1. When similar measures were performed in AB33-derived cells carrying a Chk1-GFP fusion but lacking the atr1 gene, we found that no cells showed accumulation of nuclear GFP signal at any stage (Figures 4D and 4E).

Atr1 and Chk1 Have Roles during Pathogenic Growth in Planta

U. maydis infection of maize results in anthocyanin pigment production by the plant and the formation of tumors that are filled with proliferating fungal cells that eventually differentiate into black teliospores (Banuett and Herskowitz, 1996). We tested strains defective either in Chk1 or Atr1 for pathogenicity during the infection process. We found that >25% of the plants showed no or minor symptoms such as chlorosis (Figure 5A). This result can be easily attributed to impaired formation of a functional infective filament, as a consequence of impaired cell cycle arrest during dikaryon establishment (Mielnichuk et al., 2009). However, once plants were infected with these mutant strains, they rarely showed big tumors, and even in these rare occasions, no teliospores were found in these tumors. A striking feature observed in tumors induced by chk1D and atr1D strains is the development of small, shootlike structures (Figure 5B). Such structures were described previously in plants infected by U. maydis mutants exhibiting overactivation of cAMP cascade and were attributed to a defective fungal development inside the plant (Krüger et al., 2000).

These results strongly suggest roles of Chk1 and Atr1 beyond the initial steps of infection. A possible explanation for these results might be in the compromised ability of the mutant cells to deal with DNA damage occurring during proliferation inside the plant in response to the plant defense system (i.e., reactive oxygen and/or nitrogen species). However, this explanation seems unlikely since the DNA damage-sensitive brh2 mutant cells complete the life cycle at levels similar to wild-type cells (Figure 5A). This suggests that the Atr1-Chk1 axis has some additional role in the pathogenic process beyond signaling repair of DNA damage.

Chk1 and Atr1 Are Required to Maintain the Dikaryotic Growth

We tested whether proliferation inside the plant was affected by the disruption of Atr1-Chk1. Infected plant tissue was stained with Alexa-Fluor-labeled wheat germ agglutinin (WGA-AF488), a
lectin that binds to chitin, enabling detection of fungal cell walls, and with propidium iodide, to visualize plant membranes (Doehlemann et al., 2009). *U. maydis* grows as a dikaryon during its pathogenic state. Previous work (Scherer et al., 2006) showed that clamp-like structures were involved in the distribution of nuclei in hyphae and thereby in the ability to proliferate inside the plant. Consistently, we found that in wild-type and *brh2*Δ filaments, single clamp-like structures were placed at the position of septum formation. However, *atr1*Δ and *chk1*Δ mutants were impaired in proliferation, and hyphae showed aberrant formation and variable distribution of clamp-like structures (Figures 6A and 6B). To quantify these defects, individual hyphae were counted and sorted in relation to the number of clamp-like structures observed at the three more apical septa (S1, S2, and S3). Control (wild type) and brh2Δ filaments showed the expected distribution of one clamp-like structure per septum. Filaments defective in *chk1* or *atr1* showed a clear deviation of this pattern. The graph shows the result from three independent experiments, counting more than 29 filaments each. Means and SDs are shown. Top images exemplify typical wild-type (left) or mutant (right) hyphae.

(B) Examples of wild-type and the indicated mutant hyphae expressing a nuclear localized 3xGFP to detect nuclei. Wild-type hyphae contain two nuclei per cell as observed for *brh2*Δ. The *atr1*Δ and *chk1*Δ hyphae contain multiple nuclei per cell compartment. The distribution of the nuclei with respect to the clamp-like structures is given in the lateral illustrations.

(C) Quantification of the number of nuclei per cellular compartment in hyphae from infected plant tissue with the indicated strains. The graph shows the result from three independent experiments, counting more than 29 filaments each. Means and SDs are shown.

Figure 7. *Atr1* and *Chk1* Are Required for Dikaryon Maintenance in Planta.

(A) Distribution of clamp-like structures in mutant cells. Infected plant tissue (obtained 2 to 3 d after infection [dpi]) with the indicated strains was stained with WGA-AF488 to detect hyphae and PI to mark maize cells. Then individual hyphae were counted and sorted in relation to the number of clamp-like structures observed at the three more apical septa (S1, S2, and S3). Control (wild type) and brh2Δ filaments showed the expected distribution of one clamp-like structure per septum. Filaments defective in *chk1* or *atr1* showed a clear deviation of this pattern. The graph shows the result from three independent experiments, counting more than 29 filaments each. Means and SDs are shown. Top images exemplify typical wild-type (left) or mutant (right) hyphae.
U. maydis nucleus into the daughter bud during vegetative cell division in the clamp cell is reminiscent of the movement of the diploid before mitosis. Moreover, the movement of one of the nuclei into most likely, as it happens after nuclear DNA duplication and cell cycle stage these processes take place, but G2 phase occurs in two distinct cell compartments. It is not known in which other divides in the main hypha, with the result that mitosis enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments. Most likely these processes take place during G2 phase. Since such a nuclear ballet will need an extended G2 period, we propose that the role of Atr1 and Chk1 during dikaryotic cell cycle is to synchronize nuclei as well as provide an extended window during G2 phase to allow the above-mentioned process.

These observations suggest that Chk1 and Atr1 have roles in appropriate nuclear segregation and therefore that in their absence, dikaryotic cells replicate aberrantly, most likely causing the observed proliferation defects in planta.

**DISCUSSION**

Dikaryon cell division involves nuclear migration and sorting of the divided nuclei to ensure that each daughter cell inherits a balance of each parental genome (Casselton, 2002). This cell cycle relies on a synchronized nuclear division and the development of clamp connections, a specialized projection formed close to the position of the future septum formation. One nucleus enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments. It is not known in which cell cycle stage these processes take place, but G2 phase is most likely, as it happens after nuclear DNA duplication and before mitosis. Moreover, the movement of one of the nuclei into the clamp cell is reminiscent of the movement of the diploid nucleus into the daughter bud during vegetative cell division in *U. maydis*, a process occurring at the G2/M transition (Straube et al., 2005). Such a nuclear ballet most likely will need an extended G2 period to proceed. One of the roles of the Atr1-Chk1 axis during DNA damage response is to provide an elongated G2 phase to cells to allow time to repair DNA and resolve other problems before mitosis starts (Toettcher et al., 2009). On this basis, we propose that the role of the Atr1-Chk1 axis during the dikaryotic cell cycle is to synchronize nuclei as well as to provide an extended window during G2 phase to enable the above-mentioned process (Figure 8). It could be well that in the absence of the Atr1-Chk1 axis, the nuclear cycle, clamp cell formation, and cytokinesis occur asynchronously resulting in the defects such as observed in mutant hyphae inside the plant.

Since b proteins are required for synchronization between cell cycle and cytokinesis (Wahl et al., 2010), we propose that in normal conditions it is the timely activation of the Atr1-Chk1 axis by the b homeodomain protein that produces accurate control of cell cycle. We have no clue about how the b protein activates this cascade. Our preliminary attempts to correlate expression of compatible b alleles with an increase in *chk1* or *atr1* transcription were negative (data not shown). Chk1 activation is linked to DNA damage in vegetative cells, and our results indicated that Atr1 is also required for the response to DNA damage. Therefore, it is tempting to speculate that b-induction could be associated with some class of DNA damage that triggers this kinase cascade during b-dependent filament formation. It is worth noting that b proteins activate the transcription of a gene encoding a putative DNA polymerase β (Brachmann et al., 2001), which belongs to the family X of DNA polymerases that are described to be involved in a number of DNA repair processes (Raman et al., 2004). On the other hand, several lines of evidence suggest that it is unlikely that activation of the Atr1-Chk1 cascade during b-induction is related to DNA damage. First, in previous studies using the formation of Rad51 foci as reporter for active DNA repair, there was no evidence for DNA damage associated with the induction of the infective hyphae (Mielnichuk et al., 2009). Second, we observed no defect in the ability to arrest the cell cycle (see Supplemental Figure 4 online) or to infect plants (Figure 5A) in cells lacking *Brh2*, which is required for DNA repair by homologous recombination (Kojic et al., 2002). Finally, preliminary research indicated that cells lacking the PolX polymerase were able to arrest cell cycle at levels comparable to wild-type cells (see Supplemental Figure 4 online). Two recent reports showed that activation of the DNA damage response cascade can be triggered in the absence of DNA damage by stable association of elements of the cascade with chromatin (Bonilla et al., 2008; Soutoglou and Misteli, 2008). Whether a similar mechanism could explain our observations in *U. maydis* will require additional research.

There are increasing numbers of reports indicating that the ability of the DNA damage response cascade to modulate cell cycle progression can be used during developmental processes. Some of these processes were linked with limited DNA damage as happens in B cell differentiation (Sherman et al., 2010) or even in the absence of apparent DNA damage, such as in the midblastula transition in *Drosophila melanogaster* embryos (Sibon et al., 1997) or in the asynchronous division at two-cell-stage *Caenorhabditis elegans* embryos (Brauchle et al., 2003). The surprising finding that a regulatory cascade involved in DNA damage responses plays a role in a fungal developmental process mirrors these previous results and reinforces the emerging idea that checkpoint cascades may have roles beyond cell surveillance by virtue of their ability to interact with cell cycle machinery.

This article is based on the following figures:

**Figure 8. Working Model of the Role That Atr1-Chk1 Play during Pathogenic Growth of *U. maydis* inside the Plant.**

During dikaryon cell division, one nucleus enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments. Most likely these processes take place during G2 phase. Since such a nuclear ballet will need an extended G2 period, we propose that the role of Atr1 and Chk1 during dikaryotic cell cycle is to synchronize nuclei as well as provide an extended window during G2 phase to allow the above-mentioned process.

These observations suggest that Chk1 and Atr1 have roles in appropriate nuclear segregation and therefore that in their absence, dikaryotic cells replicate aberrantly, most likely causing the observed proliferation defects in planta.
**METHODS**

*Ustilago maydis* Genetic Methods

*U. maydis* strains are listed in Table 1. Manipulations with *U. maydis*, culture methods, gene disruption and gene transfer procedures, survival after DNA damage, and plant infections have been described previously (Castillo-Lluva and Pérez-Martín, 2005; Flor-Parr et al., 2007; Mielnichuk and Pérez-Martín, 2008; Mielnichuk et al., 2009). Protein extracts, immunoprecipitations, and immunoblot analysis were performed as described previously (García-Muse et al., 2004; Sgarlata and Pérez-Martín, 2005).

Null mutants were constructed by replacing the entire open reading frame were amplified and ligated to a gene cassette expressing resistance to antibiotics by standard methodology (Brachmann et al., 2004). Briefly, a pair of DNA fragments flanking the *atr1* open reading frame were amplified and ligated to a gene cassette encoding hygromycin resistance and flanked by *Sfi*I sites. The 5' fragment spans from nucleotide +967 to nucleotide +29962. The flanking 3' fragment spans from nucleotide +7912 to nucleotide +9962.

**Table 1. Strains Used in This Work**

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**Microscopy**

To visualize fungal hyphae in plant by wheat germ agglutinin-Alexa fluor 488 (WGA-AF488; Invitrogen) and propidium iodide (PI) (Sigma-Aldrich), samples 2 to 3 d after infection of maize (*Zea mays*) leaves were incubated in staining solution (1 μg/mL PI, 10 μg/mL WGA-AF488, and 0.02% Tween 20) for 30 min and washed in 1× PBS, pH 7.4 (Doehlemann et al., 2009). Analysis of the infection stages was done using a DeltaVision wide-field microscope (Applied Precision). Image deconvolution was performed using z-series of between 10 and 15 focal planes, acquired at 0.5-μm intervals. Image processing was performed using Adobe Photoshop CS2 and Canvas 8.0 (Deneba).

To quantify the clamp-like structures in fungal hyphae, plant leaves 2 to 3 d after infections were analyzed. Individual hyphae where the hyphal tip as well as the penetration points could be observed were analyzed, quantifying the number of clamp-like structures. To quantify the number of nuclei per cellular compartment, maize leaves 2 to 3 d after infections were analyzed. Only nuclei in individual hyphae were quantified.

**Sequence Analysis**

Alignments were made with ClustalW (Thompson et al., 1997). Phylogenetic dendrograms were constructed using MEGA 2.1 (Kumar et al., 2001).
with the minimum evolution or maximum parsimony algorithm and gap deletion option.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers JF690671 (Atm1) and JF690672 (Atr1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phosphorylation of Chk1 Is Required for Normal in Planta Proliferation of Hyphae.

Supplemental Figure 2. Complementation of chk1 and atr1 Deletion Mutants.

Supplemental Figure 3. Single Phosphorylation Mutants in Chk1 Showed Wild-Type Phenotypes with Respect to Cell Cycle Arrest and Infection.

Supplemental Figure 4. Absence of Bh2 or PoIX Does Not Affect the Ability of the b-Complex to Arrest Cell Cycle.

Supplemental Data Set 1. Sequence Alignment of Atr- and Atm-Like PI3K Kinases.

ACKNOWLEDGMENTS

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