Crosstalk between the Unfolded Protein Response and Pathways That Regulate Pathogenic Development in *Ustilago maydis*

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The unfolded protein response (UPR) is a conserved eukaryotic signaling pathway regulating endoplasmic reticulum (ER) homeostasis during ER stress, which results, for example, from an increased demand for protein secretion. Here, we characterize the homologs of the central UPR regulatory proteins Hac1 (for Homologous to ATF/CREB1) and Inositol Requiring Enzyme1 in the plant pathogenic fungus *Ustilago maydis* and demonstrate that the UPR is tightly interlinked with the b mating-type-dependent signaling pathway that regulates pathogenic development. Exact timing of UPR is required for virulence, since premature activation interferes with the b-dependent switch from budding to filamentous growth. In addition, we found crosstalk between UPR and the b target Clampless1 (Clp1), which is essential for cell cycle release and proliferation in planta. The unusual C-terminal extension of the *U. maydis* Hac1 homolog, Cib1 (for Clp1 interacting bZIP1), mediates direct interaction with Clp1. The interaction between Clp1 and Cib1 promotes stabilization of Clp1, resulting in enhanced ER stress tolerance that prevents deleterious UPR hyperactivation. Thus, the interaction between Cib1 and Clp1 constitutes a checkpoint to time developmental progression and increased secretion of effector proteins at the onset of biotrophic development. Crosstalk between UPR and the b mating-type regulated developmental program adapts ER homeostasis to the changing demands during biotrophy.

INTRODUCTION

Development of eukaryotic pathogens is accompanied by drastic changes in morphology, lifestyle, nutrient acquisition, and growth behavior (Heitman, 2006; Sexton and Howlett, 2006). Therefore, pathogens contain robust control systems to maintain cellular physiology and homeostasis and to adapt to changing host environments.

During its life cycle, the phytopathogenic basidiomycete *Ustilago maydis* passes through different developmental stages (Christensen, 1963). In the saprophytic phase, haploid cells propagate by budding. The biotrophic stage is initiated by fusion of two haploid cells via a pheromone-based recognition system encoded by the a mating-type locus (Böker et al., 1992). The resulting filamentous dikaryon is characterized by a G2 cell cycle block and is thus unable to proliferate (Scherer et al., 2006; Mielnichuk et al., 2009; Heimel et al., 2010a). Before plant penetration, the cell cycle–arrested filaments elongate by polar tip growth. During extended polar growth, the most apical compartment of the hyphae contains cytoplasm, while distal compartments are devoid of any cytoplasm and sealed off via septation. Filament formation, maintenance of cell cycle arrest and the ability to infect maize (*Zea mays*) plants are controlled by the heterodimeric bE/bW transcription factor encoded by the b mating-type locus (Schulz et al., 1990; Kämper et al., 1995). The b-heterodimer regulates a hierarchical, transcriptional network affecting more than 340 genes (Heimel et al., 2010b). After penetration of the host plant, the Clampless1 (Clp1) protein triggers release of the b-mediated cell cycle block, resulting in proliferation of the filamentous dikaryon. Clp1 suppresses the b-pathway by physical interaction with the b-heterodimer and with Rbf1 (for Regulator of b-Filament1), the major transcriptional regulator downstream of the b-heterodimer (Heimel et al., 2010b). Although the clp1 transcript is present in earlier stages, Clp1 protein can be detected only after penetration of the host plant, suggesting posttranscriptional regulation of clp1 (Scherer et al., 2006).

During the biotrophic interaction, *U. maydis* secretes effector proteins to suppress plant defense responses and to redirect nutrient fluxes (Kämper et al., 2006; Skibbe et al., 2010; Djamei and Kahlmann, 2012). Many of these secreted effectors are predicted to be glycosylated (Fernandez-Alvarez et al., 2012). Therefore, at this stage, an enormous stress is imposed on the endoplasmic reticulum (ER) and the secretory machinery.
Homeostasis of the ER during ER stress is maintained by the unfolded protein response (UPR) elicited by misfolded proteins in the ER (Walter and Ron, 2011). Misfolded proteins in the lumen of the ER are recognized by the membrane-localized kinase/RNase Inositol Requiring Enzyme1 (Ire1) (Gardner and Walter, 2011). This triggers oligomerization of Ire1 and results in the activation of both its kinase and ribonuclease activity, which resides on the cytoplasmic portion of Ire1 (Sidrauski and Walter, 1997; Korennykh et al., 2009; Li et al., 2010; Rubio et al., 2011). The RNase activity causes unconventional cytoplasmic splicing of an mRNA encoding a bZIP transcription factor, termed Hac1p (for Homologous to ATF/CREB 1) in yeast and X-Box Binding Protein1 (XBP1) in mammals (Cox and Walter, 1996; Kawahara et al., 1997; Korennykh et al., 2009; Li et al., 2010; Rubio et al., 2011). The spliced transcript gives rise to an active transcription factor, which regulates the expression of genes encoding proteins that facilitate relief of ER stress, including ER chaperones and proteins that mediate lipid synthesis and ER expansion (Cox et al., 1997; Travers et al., 2000). Although the UPR is important for survival under stress conditions, constitutive activation of the UPR is deleterious for cell growth (Cox and Walter, 1996; Kawahara et al., 1997; Chawla et al., 2011; Rubio et al., 2011) and can even lead to UPR-triggered cell death in mammals (Shore et al., 2011; Tabas and Ron, 2011; Hetz, 2012). The UPR pathway has been reported to be required for virulence in various biological systems, including yeast (Cox and Walter, 1996; Kawahara et al., 1997; Korennykh et al., 2009; Li et al., 2010; Rubio et al., 2011). The mechanisms that coordinate the UPR and infection-related development are not well understood.

Here, we report that the homolog of the central UPR regulator Hac1 in U. maydis Cib1 (for Clp1 interacting bZip1) not only triggers UPR but also coordinates biotrophic development. Cib1 was previously identified as Clp1-interacting protein and is required for proliferation of U. maydis in the host plant (Heimel et al., 2010a). The interactions between the UPR and the b-mediated developmental pathway affect different developmental transitions during the U. maydis life cycle. We show that premature activation of UPR interferes with the morphogenetic transition from yeast-like growth to the infectious filament. However, during penetration of the plant, UPR is required for Cib1 accumulation and to establish the biotrophic interaction. Our data provide a molecular basis for the development-specific activation of UPR and demonstrate how UPR signaling is integrated into a regulatory network coordinating fungal development, pathogenicity, and adaptation to the host environment.

RESULTS

Cib1 Is the Homolog of the Central UPR Regulator Hac1 and Is Required for ER Stress Resistance

The bZIP transcription factor Cib1 has been identified as an interacting protein of the developmental regulator Clp1. After successful entry of U. maydis into the plant, the cib1 transcript is subject to posttranscriptional regulation via alternative splicing, resulting in expression of an active bZIP transcription factor (Heimel et al., 2010a). This suggests that Cib1 might be homologous to the central UPR regulator Hac1. Sequence comparison revealed that the intron/exon borders of cib1 perfectly match the hac1-consensus splice sites CNG/CNG (Hooks and Griffiths-Jones, 2011) (Figure 1A; see Supplemental Figure 1A and Supplemental References 1 online). In addition, the predicted secondary structure of cib1 mRNA contains stem-loop structures that are typical of mRNAs encoding homologs of Hac1 (see Supplemental Figure 1B). We verified the function of Cib1 as a Hac1 homolog by complementation analysis in the yeast Saccharomyces cerevisiae. Expression of either spliced or unspliced U. maydis cib1 in an S. cerevisiae strain deleted for hac1 restored ER stress resistance of the Δhac1 mutant (see Supplemental Figure 2A). In addition, microscopy analysis revealed the expected nuclear localization of Cib1:3xGFP (for 3xGreen Fluorescent Protein) and foci formation of Ire1-GFP upon DTT treatment (see Supplemental Figures 2B and 2C online).

To further analyze the function of Cib1 as regulator of UPR signaling in U. maydis, we analyzed splicing of cib1 mRNA after induction of ER stress in the haploid pathogenic strain SG200 (Kämper et al., 2006). Abundance of spliced cib1 mRNA increased considerably when cells were treated with the ER stress inducing compounds tunicamycin (Tm) or DTT (Figure 1B). In addition, we found increased expression of five conserved UPR target genes upon Tm or DTT treatment: um15034, which encodes a predicted homolog of the ER-resident chaperone Karyogamy Gene2/Binding Protein1 (Bip1; e-value: 0) (herein referred to as bip1) (Normington et al., 1989; Rose et al., 1989); um00904, which encodes a predicted homolog of yeast ER chaperone Luminal Hsp70 1 (Lhs1p) (e-value: 1.0e-49); um05352, which encodes a predicted homolog of yeast protein disulfide isomerase Multicopy Suppressor of PDH1 deletion1 (Mpd1p) (e-value: 6.0e-25); um10287, which encodes a predicted homolog of the yeast glycoprotein chaperone Calnexin1 (Cne1p) (e-value: 3.0e-65) (Travers et al., 2000); and um02729, which encodes a predicted homolog of the Plasmodium falciparum signal peptide peptidase Ssp1 (e-value: 3.0e-46), known to be involved in the ER-associated degradation pathway in P. falciparum, Drosophila melanogaster, and Arabidopsis thaliana (Martinez and Chrispeels, 2003; Casso et al., 2012; Harbut et al., 2012) (Figure 1B; see Supplemental Figures 3A and 3B online).

As hac1 mRNA splicing depends on Ire1 (Cox and Walter, 1996), we deleted the U. maydis gene um03481, encoding a protein with significant similarity to the Ire1 protein from S. cerevisiae (e-value: 1.6e-95). Deletion of um03481 abolished splicing of cib1 mRNA completely, demonstrating that Um03481 is the functional homolog of Ire1 in U. maydis (Figure 1B). We also observed that expression of a functional Cib1-3xGFP fusion protein was strongly induced upon DTT treatment, while expression of Cib1-3xGFP was not detectable in Δire1 strains (Figure 1C).

When Δcib1 and Δire1 strains were tested for growth on solid media supplemented with DTT or Tm, both mutants showed severe growth defects (Figure 1D). Since ire1 is required for splicing of cib1 mRNA during UPR, we expressed the spliced version of cib1 (cib1Δ) under control of its endogenous promoter in wild-type and Δire1 cells. In wild-type cells, expression of
cib1Δ did not affect ER stress resistance or growth in axenic culture, indicating that Ire1-independent activation of the UPR pathway is not deleterious under these conditions (Figure 1D). In Δire1 strains, the introduction of cib1Δ suppressed hypersensitivity to ER stress at least partially (Figure 1D). The incomplete suppression of ER stress hypersensitivity by cib1Δ in Δire1 strains might be caused by altered cib1Δ gene expression in the Δire1 background. Thus, we measured mRNA levels of unspliced transcript of cib1Δ (cib1u), cib1Δ, and the UPR-induced marker genes bip1, lhs1, mpd1, cne1, and spp1 via quantitative RT-PCR (qRT-PCR). Expression levels of cib1Δ, cib1Δ, and bip1 were comparable to those of all tested genes were highly similar under nonstressed conditions (see Supplemental Figures 3A and 3B online). Immunoblot analysis revealed no obvious differences in protein levels of Cib1-3xGFP expressed in wild-type or Δire1 strains (Figure 1E). Hence, we assume that additional Ire1-dependent functions are required for full expression of UPR marker genes and full ER stress resistance.

Interestingly, we were not able to express cib1Δ in Δcib1 mutants, suggesting a potential deleterious effect of cib1Δ in cells that lack cib1Δ. Indeed, induced expression of cib1Δ under control of the inducible crg promoter (Bottin et al., 1996) resulted in strong growth inhibition in Δcib1Δ cells, but not in wild-type cells. Growth suppression was also observed in wild-type strains but only if multiple copies of the expression construct were integrated (see Supplemental Figure 4 online). This suggests that unspliced cib1Δ counteracts the deleterious effect of
cib1+ expression in a dose-dependent manner and explains the highly deleterious effects of cib1- in the absence of cib1+.

Correct Timing of the UPR upon Entry into the Plant Is Required for Biotrophic Development of U. maydis

We previously demonstrated development-specific Cib1 expression at the onset of biotrophic development (Heimel et al., 2010a). In addition, we observed significant increased expression levels of all five UPR marker genes (bip1, lhs1, mdp1, cne1, and spp1) during biotrophic growth when compared with axenic growth in liquid culture (see Supplemental Figure 5 online). This indicates that in U. maydis UPR is activated during biotrophic growth.

Therefore, we determined the importance of UPR function and the development-specific activation of UPR in U. maydis in maize infection assays. We used the haploid pathogenic strain SG200, which is able to infect plants without the need of a compatible mating partner due to the presence of a chimeric b locus (bE1/bW2) (Kämper et al., 2006). Deletion of either cib1 or ire1 resulted in strongly attenuated virulence and only weak symptoms, such as chlorosis (Figure 2A), indicating that a functional UPR is required for pathogenic development in U. maydis. Quantification of fungal proliferation following plant penetration revealed that proliferation of Δire1 and Δcib1 mutants was significantly reduced. Hyphae of both mutant strains were found predominantly in the epidermal cell layer even 48 h after inoculation (HAI) (Figures 2B and 2C). By contrast, wild-type hyphae had already colonized eight to 10 cells by 24 HAI, progressing into the mesophyll layer.

U. maydis secretes a large battery of effectors to suppress plant defense responses (Djamei and Kahmann, 2012). Since

Figure 2. Mutants with Defective UPR Are Nonpathogenic.

(A) The haploid pathogenic strain SG200 (wild type [WT]) and derivatives were inoculated into 7-d-old maize seedlings. Disease symptoms were rated 10 d after inoculation and grouped into color-coded categories depicted on the right. n represents the number of inoculated plants. Statistical significance of alteration in disease rating (compared with wild-type infection) was calculated using the Wilcoxon-signed-rank test. ***P value < 0.001.

(B) Fungal proliferation was quantified at 24 and 48 HAI by microscopy analysis of WGA-AF488 and propidium iodide–stained infected plant tissue. At least 30 hyphae were counted in three independent samples. Note that significance was only calculated for the 24-HAI time point. Proliferation of hyphae with a single seed could not be fully resolved in strains SG200, cib1+, and Δire1 cib1+ at 48 HAI. SG200Δclp1 was used as an additional control. Error bars represent the sd. Statistical significance was calculated using Students t test. *P value < 0.05.

(C) Microscopy analysis of fungal development after infection of maize plants. Fungal and plant material were stained with WGA-AF488 (green) and propidium iodide (magenta), respectively. Confocal projections were derived from leaves 48 HAI. SG200Δclp1 served as an additional control. Bars = 250 µm for overview images and 25 µm for higher magnified images depicting hyphal morphology.
U. maydis UPR mutant strains might be compromised in effector secretion, we investigated whether expression of defense-associated genes in maize was induced upon infection with UPR mutants. We quantified expression of pathogenesis-related (PR) genes pr1, pr3, and pr5 upon inoculation with Δcib1 and Δire1 strains relative to the wild-type control. pr1 and pr5 are typical markers for salicylic acid–induced defense responses (van Loon et al., 2006; van der Linde et al., 2012). pr3 expression is induced after inoculation of maize plants with U. maydis at prepenetration stages (12 HAI) but is suppressed after plant invasion (Doehrleman et al., 2008b). Compared with plants infected with wild-type strains, we observed at 48 HAI significantly increased expression levels of all three PR genes in plants inoculated with Δcib1, but not in plants infected with Δire1 strains (Figure 3A). We analyzed penetration efficiency in Δire1, Δcib1, and wild-type strains (Freitag et al., 2011) to assess if differences in PR gene expression result from inefficient penetration of the leaf surface in Δire1 strains. Indeed, these strains showed a significantly reduced penetration efficiency compared with wild-type and Δcib1 strains (Figure 3B). Most probably, the absence of PR gene induction upon inoculation with the Δire1 strain is caused by the reduced number of infected maize cells. Consequently, we assume that deletion of ire1 affects additional processes that are required for plant infection, emphasizing again a function of Ire1 independent from cib1 splicing. In line with the results obtained in ER stress assays, expression of cib1Δ led only to partial suppression of the virulence defect of SG200Δire1 (Figure 2A). This finding is consistent with observations in the human pathogenic fungus Aspergillus fumigatus, where ireA, which encodes a homolog of Ire1, has additional functions required during pathogenic development (Feng et al., 2011).

Although cib1Δ-mediated constitutive activation of the UPR as shown by increased expression levels of cib1Δ and all five UPR marker genes (bip1, lhs1, mpd1, cne1, and spo1) (see Supplemental Figures 3A and 3B online) did not interfere with growth or ER stress resistance in axenic culture, integration of the cib1Δ allele into SG200 affected pathogenic development, as tumor formation was drastically reduced in SG200 cib1Δ (Figure 2A). Constitutive activation of UPR appears to affect primarily stages prior to plant penetration, since proliferation of cib1Δ and Δire cib1Δ cells in planta was indistinguishable from that of wild-type hyphae (Figures 2B and 2C). In addition, hyphal morphology of SG200cib1Δ and Δire cib1Δ appeared to be unaffected (Figure 2C). This indicates that the exact timing of UPR activation is important for pathogenic development of U. maydis.

**UPR Activation Attenuates b Matting-Type-Dependent Filament Formation**

Penetration of the plant epidermis by U. maydis requires the expression of filamentous cells that develop appressoria at their tips. Filament formation is dependent on the heterodimeric bE/bW transcription factor, which is formed when the two proteins originate from different b alleles (Schulz et al., 1990; Kämper et al., 1995). In SG200, the b-heterodimer triggers formation of filamentous cells on charcoal containing media. Deletion of ire1 in SG200 resulted in reduced filamentous growth, while deletion of cib1 had no effect (Figure 4A), suggesting that a functional UPR is not required for filament formation. Remarkably, constitutive activation of UPR by expression of cib1Δ suppressed filament formation in SG200, SG200Δire1, and SG200ΔireΔ (Figure 4A; see Supplemental Figure 6 online). This phenotype was even more pronounced when multiple copies of cib1Δ were integrated in SG200 [cib1ΔΔ] (Figure 4B), resulting in increased cib1Δ mRNA levels (see Supplemental Figure 7 online). Expression of bE, bW, and, as a consequence, of the bE/bW target gene rbf1 was significantly reduced in cib1Δ and even more so in cib1ΔΔΔ cells (Figure 4C). As rbf1 is required and sufficient for filament formation (Heimel et al., 2010b), reduced rbf1 levels are likely to account for cib1Δ-mediated attenuation of filament formation.

To further characterize the effect of cib1Δ on development preceding plant penetration, we tested if cib1Δ-mediated constitutive activation of the UPR disturbs formation of appressoria. The hydrophobic surface of Parafilm combined with the application of hydroxy-fatty acid serves as a potent trigger for the formation of b-dependent filaments with differentiated appressoria (Mendoza-Mendoza et al., 2009). As expected, the number of filaments formed in SG200 cib1Δ was drastically reduced compared with SG200 (Figure 4D). However, formation of appressoria was only slightly reduced when normalized to the number of filaments (Figure 4E). We conclude that constitutive activation of the UPR appears to interfere primarily with the transition from budding to hyphal growth. Thus, inoculating more cells should compensate for the virulence defect of SG200 cib1Δ. The infection symptoms of plants inoculated with SG200 at either 5 × 10^5 (OD_{600} = 1) cells or 5 × 10^6 (OD_{600} = 3) cells were comparable, indicating saturation for SG200 infections.
with 5 × 10^5 cells (see Supplemental Figure 8 online). By contrast, infection symptoms of plants inoculated with SG200 cib1^s were significantly increased using the higher cell numbers (see Supplemental Figure 8 online). These data further support that the transition to filamentous growth rather than fungal proliferation in planta is affected by the cib1^s-dependent premature activation of the UPR.

**cib1^s-Mediated Inhibition of Filamentous Growth Is Partially Compensated for by Overexpression of bE/bW**

Since bE/bW expression was significantly reduced in dependence of cib1^s in SG200, we next asked whether increased expression of the b-heterodimer could suppress cib1^s-mediated inhibition of filament formation. In strain AB31, bE1 and bW2 are under the control of the Ara-inducible crg promoter (Brachmann et al., 2001) that triggers bE/bW expression at ∼5-fold higher levels than in SG200 (Figure 5A). Constitutive expression of cib1^s in AB31 did not interfere with filament formation upon induced bE1/bW2 expression on Ara-containing charcoal medium. Filament formation was slightly reduced only if cib1^s was integrated in multiple copies [AB31 cib1^s(x)] (Figure 5B). Similar results were obtained when filament formation was followed in liquid culture: While AB31 cib1^s was indistinguishable from AB31, multiple integration of constitutively active cib1^s led to reduced filamentous growth (Figure 5C). Spotting assays on charcoal containing Ara/Glc gradient plates revealed that filament formation in AB31 is suppressed by cib1^s in a dose-dependent manner (Figure 5E).
Importantly, expression of \( bE \), \( bW \), and \( rbf1 \) was not altered in AB31 upon single or multiple integration of \( cib1 \) (Figure 5D). Thus, inhibition of \( b \)-dependent filament formation occurs by at least two independent mechanisms, including UPR-mediated decrease of \( b \)-gene expression and a second, yet unidentified mechanism. As the expression levels of \( cib1^+ \), \( cib1^{++} \), and the UPR target genes \( bip1 \), \( lhs1 \), \( mpd1 \), \( cne1 \), and \( spp1 \) were found to be similar in the SG200 and AB31 backgrounds (see Supplemental Figures 7A and 7B online), we can exclude the possibility that strain-specific differences in UPR activation can account for the partial suppression of \( cib1^+ \)-dependent inhibition of filament formation by enhanced \( bE/bW \) levels in AB31.

Interaction between Clp1 and Cib1 Modulates UPR

Clp1 is one of the major regulators of pathogenic development and is required to release the \( b \)-mediated cell cycle block after penetration of the host plant (Heimel et al., 2010a). As Cib1 and Clp1 physically interact, and as protein levels of both proteins increase simultaneously during pathogenic development (Heimel et al., 2010a), we addressed the role of Clp1 with regard to UPR signaling in \( U. \text{maydis} \). Deletion of \( clp1 \) neither affected ER stress resistance nor interfered with the inhibitory effect of \( cib1^+ \) on filament formation (Figure 1D; see Supplemental Figure 6 online). However, induced expression of Clp1 significantly increased resistance to Tm-induced ER stress, suggesting a role for Clp1 in the modulation of UPR (Figure 6A). This prompted us to characterize the interaction between Clp1 and Cib1 in more detail (Figure 6B). Interaction studies employing the yeast two-hybrid system demonstrated that the interaction domain resides in the C-terminal part of Cib1 (Heimel et al., 2010a). Deletion of the last 140 amino acids of Cib1 (Cib1\(^{1433}\)) completely abolished interaction with Clp1, while up to 100 C-terminal amino acids (Cib1\(^{1474}\), Cib1\(^{1508}\), and Cib1\(^{1541}\)) could be deleted without affecting the Cib1/Clp1 interaction (Figure 6B). Comparison of Cib1 with other fungal homologs of Hac1p revealed that the interaction domain is located within a C-terminal extension that occurs only in \( U. \text{maydis} \) and the closely related smut fungi \( Sporisorium \text{reilianum} \) and \( Ustilago \text{hordei} \) (see Supplemental Figure 9 online).

Figure 5. Increased Expression of \( b \) Restores \( cib1^+ \) Suppressed Filamentous Growth.

(A) qRT-PCR analysis of \( bE \) and \( bW \) in strains SG200 and AB31. Actin was used for normalization. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent SD.

(B) \( U. \text{maydis} \) strain AB31 and derivatives were spotted on CM charcoal plates supplemented with Ara to induce the expression of the \( bE1/bW2 \) genes under control of the Ara-responsive \( crg1 \) promoter.

(C) The ratio between filaments and budding cells of AB31 and derivatives was determined 6 h after \( bE1/bW2 \) induction in Ara-containing liquid medium. At least 300 cells were counted in three independent experiments. Error bars indicate SD. Suppression of filament formation by \( cib1^+ \) occurs in a dose-dependent manner. Significance was calculated using Student’s \( t \) test. **\( P \) value < 0.01.

(D) qRT-PCR analysis of \( bE \), \( bW \), and \( rbf1 \) expression in strain AB31 and derivatives harboring \( cib1^+ \) as a single copy (\( cib1^+ \)) or multiple integration (\( cib1^{l+x} \)). Actin (\( um11232 \)) was used for normalization. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent SD.

(E) \( U. \text{maydis} \) strain AB31 and derivatives were spotted on CM charcoal plates supplemented with Ara or Glc (left and right, respectively) to induce expression of the \( bE1/bW2 \) genes under control of the Ara-responsive \( crg1 \) promoter. The plate in the middle contains a reciprocal gradient of Ara and Glc to modulate \( bE/bW \) expression.
To test whether direct interaction between Clp1-Cib1 is necessary for the Clp1-mediated increase of ER stress resistance, we expressed Clp1 in a strain that harbors the cib1433 allele (lacking the interaction domain) instead of full-length cib1. The Δcib1 cib1433 strain showed no altered ER stress resistance under conditions when clp1 was not induced. However, in contrast with the strain harboring full-length cib1, induced expression of clp1 did not alter the sensitivity of the Δcib1 cib1433 strain on Tm-containing medium (Figure 6A). Taken together, these data demonstrate that direct interaction between Clp1 and Cib1 is required to increase ER stress resistance.

Next, we analyzed whether Clp1 affects the transcriptional response after UPR activation. To our surprise, overexpression of Clp1 reduced the overall transcript level of cib1 both under UPR (+Tm) and non-UPR-inducing conditions (control) (Figure 6C). Importantly, these changes in gene expression were dependent on the Clp1/Cib1 interaction, since none of these alterations were observed in strains expressing truncated Cib1433.

Figure 6. Clp1 Modulates UPR via Interaction with Cib1.

(A) ER stress assay in response to increased levels of clp1. Serial 10-fold dilutions of U. maydis strain JB1 (wild type [WT]) and derivatives were spotted on YNB solid medium supplemented with the carbon source indicated. clp1 is expressed under the control of the Ara-inducible crg1-promoter (Pcrg). Tm was added at the indicated concentrations. Plates were incubated for 48 h at 28°C.

(B) Quantification of Clp1 interaction with Cib1 and truncated derivatives of Cib1 in the yeast two-hybrid system. Plasmid pGBKT7-Clp1 and pGAD-DS, expressing full-length cib1 cDNA lacking the unconventional intron (Cib1), or the respective C-terminal truncated Cib1 alleles, were cotransformed in yeast strain AH109. pGBKTT7-Clp1 and pGAD-BW (Heimel et al., 2010a) served as the positive control (+), and pGBKTT7 and pGAD-Cib1 served as the negative control (−). Results shown (ONPG-based determination of β-Gal activity in Miller units) represent the mean of five biological replicates with three technical replicates each. Error bars represent SD.

(C) qRT-PCR analysis of UPR genes. U. maydis strain JB1 (WT) and derivatives were cultured in YNB medium with Glc to an OD600 = 0.2 and shifted to YNB medium containing Ara to induce clp1 expression in strains containing Pcrg:clp1. Tm was added to a final concentration of 5 µg/mL, and strains were incubated for 4 h. Expression of the spliced cib1 mRNA (cib1s), the unspliced cib1 mRNA (cib1u), and the UPR target bip1 was measured in response to Tm treatment and clp1 expression in the wild type or Δcib1 cib1433 genetic background. The Δcib1 strain was used as negative control to test for unspecific activation of bip1 expression. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent SD. Statistical significance was calculated relative to the wild type using Student’s t test. *P value < 0.05; **P < 0.01, and ***P < 0.001.
These data indicate that expression of clp1 leads to reduced activity of the UPR pathway, resulting in increased tolerance to high levels of ER stress in U. maydis.

### Activation of UPR Enhances Clp1 Protein Stability

To further characterize the biological function of Clp1 during UPR, we analyzed the effect of UPR activation on Clp1 abundance. We previously demonstrated that Clp1 is subject to posttranscriptional regulation (Scherer et al., 2006). Although clp1 mRNA is highly induced in hyphae growing on the plant surface, Clp1 is only detectable after penetration of the plant and appears simultaneously with Cib1 (Heimel et al., 2010a). To visualize Clp1 expression upon UPR, we used an AB31 derivative harboring a functional Clp1-GFP fusion protein (Scherer et al., 2006). In this strain, transcription of clp1:gfp can be triggered by induced expression of the b-heterodimer (Scherer et al., 2006; Heimel et al., 2010b). Interestingly, cells exposed to DTT displayed a strong nuclear signal of Clp1-GFP, while non-induced cells showed only diffuse cytoplasmic staining (Figure 7A). We next tested whether elevated Clp1-GFP levels correlate with increased expression of cib1s or were an indirect effect of DTT treatment. To this end, we integrated cib1s in single or multiple copies in AB31 clp1-gfp. While clp1 transcript levels were not significantly different (Figure 7B), the abundance of Clp1-GFP protein was elevated in cib1s and was even more

![Figure 7. Clp1 Is Stabilized by Cib1.](image-url)

(A) Clp1-GFP was monitored by fluorescence microscopy 3 h after induction of the b-heterodimer in strain AB31clp1-gfp. DTT was added at a final concentration of 3 mM at the time point of b-induction. A nuclear-localized GFP signal can be observed in cells treated with DTT, indicating expression of the Clp1-GFP fusion protein. Exposure time was 250 ms. DIC, differential interference contrast. Bars = 10 µm.

(B) Analysis of clp1 expression upon cib1s-mediated UPR activation. Strain AB31clp1-GFP (wild type [WT]) and cib1s derivatives were transferred to CM liquid medium containing Ara as carbon source to induce Pcrg-driven expression of bE1/bW2. Incubation was continued for 6 h, and clp1 levels were determined by qRT-PCR. clp1 expression is not significantly altered by cib1s-mediated UPR activation (P value > 0.1). elf2b (um04869) was used for normalization. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent SD.

(C) Analysis of Clp1-GFP expression upon cib1s-mediated UPR activation. The same cells that were used in (B) were subjected to immunoblot analysis. Clp1-GFP levels were determined using a GFP-specific antibody. Clp1-GFP levels are higher in cib1s and cib1s(x) than in the wild-type control. Coomassie blue-stained bands served as loading control (LC).

(D) Schematic overview of time course experiment. Strain AB31clp1-GFP (WT) and derivatives were transferred to CM liquid medium containing Ara as the carbon source to induce Pcrg-driven expression of bE1/bW2 (b-on). After 3 h, DTT was added (3 mM) to activate the UPR. Incubation was continued for 3 h, and 100 µg/mL of cycloheximide was added to inhibit protein biosynthesis. Protein samples were taken before the addition of cycloheximide (T0) and 15 min (T15) or 45 min (T45) after cycloheximide treatment, respectively.

(E) Immunoblot analysis of (D) to follow Clp1-GFP expression in response to DTT-mediated UPR activation. Coomassie blue-stained bands served as loading control. Clp1-GFP was visualized using a GFP-specific antibody.

(F) To analyze the stability of Clp1-GFP in response to UPR activation, normalized Clp1-GFP levels at T15 and T45 were determined relative to T0 using ImageJ. Values represent the mean of four biological replicates and error bars indicate SD. Statistical significance was calculated using Student’s t test. **P value < 0.001.

[See online article for color version of this figure.]
increased in cib1ΔΔ strains (Figure 7C). Thus, Clp1 accumulation correlates with increased cib1Δ expression, indicating that cib1Δ-dependent UPR activation regulates Clp1 expression post-transcriptionally.

To test if increased protein levels of Clp1-GFP after DTT-induced UPR activation result from stimulated translation or enhanced protein stability, we analyzed the stability of Clp1-GFP by inhibiting protein biosynthesis through the addition of cycloheximide (Figure 7D). Three hours after DTT-induced UPR activation, Clp1-GFP protein level was increased in AB31clp1-gfp (wild type) (Figure 7E). Comparison of Clp1-GFP decay showed that the induction of UPR significantly stabilized the Clp1 protein (Figure 7F). This stabilization depended on cib1 and required the C-terminal interaction domain of Cib1 (Figure 7F). ER stress–inducing conditions were confirmed by qRT-PCR analysis of cib1Δ and bip1 expression. cib1Δ levels in DTT-treated AB31clp1-gfp (wild-type) cells were similar to those in strain AB31clp1-gfp cib1Δ without DTT treatment. Elevated cib1Δ levels indicated successful UPR activation under these conditions. Moreover, cib1Δ and bip1Δ levels were similar in the wild type and the Δcib1 cib1Δ background, demonstrating that the transcription factor function of Cib1ΔΔ is not altered by the C-terminal truncation (see Supplemental Figure 10 online). Taken together, our data show that stabilization of Clp1 is UPR dependent and requires the physical interaction of Clp1 and Cib1. This suggests that plant-specific UPR activation contributes to Clp1 accumulation, which then triggers fungal proliferation within the host plant.

Interaction between Clp1 and Cib1 Affects Virulence of U. maydis

Clp1 and Cib1 are specifically induced during plant penetration and both genes are critical for pathogenic development (Scherer et al., 2006; Heimel et al., 2010a). To determine whether also the interaction between Cib1 and Clp1 is required for virulence, we introduced the truncated cib1ΔΔ allele into SG200Δcib1. Although the ER stress resistance of the resulting strain SG200Δcib1 cib1ΔΔ was not affected (Figure 8A), we observed a decrease in virulence compared both to the corresponding wild-type strain SG200 and to the complemented mutant SG200Δcib1 cib1ΔΔ (Figure 8B). This suggests that direct interaction between Clp1 and Cib1 is required for full virulence.

We then expressed the C-terminal extension of Cib1, which contains the Clp1 interaction domain of Cib1 (Cib1ΔΔΔΔΔ), under control of its endogenous promoter in the haploid pathogenic strain CL13 (Bölker et al., 1995). We used strain CL13 as background, as it is less virulent than SG200 and is suitable for monitoring more subtle changes in symptom development (Bölker et al., 1995; Di Stasio et al., 2009; Djamei et al., 2011). Expression of Cib1ΔΔΔΔΔ did not alter ER stress resistance (see Supplemental Figure 11A online) but resulted in increased symptom development when compared with CL13 (see Supplemental Figure 11B online), indicating a dominant effect of the isolated domain. Importantly, expression of Cib1ΔΔΔΔΔ in CL13Δcib1 did not rescue ER stress resistance and loss of pathogenicity in C13Δcib1 strains (see Supplemental Figures 12A and 12B online). Together, our results suggest that the decreased virulence of strains expressing Cib1ΔΔΔΔΔ is associated with the absence of the Clp1–Cib1 interaction.

DISCUSSION

Here, we show that the key features of UPR regulation are conserved in U. maydis and that the UPR is crucial for pathogenic development. Deletion of either cib1Δ or ire1Δ resulted in a block of fungal development immediately following plant penetration and no fungal proliferation in planta could be observed. Similarly, deletion of the Hac1 homolog affected virulence in the necrotrophic plant pathogenic fungus Alternaria brassicicola and in the human pathogenic fungi A. fumigatus and Cryptococcus neoformans (Richie et al., 2009; Cheon et al., 2011; Joubert et al., 2011). In addition, the Kar2/Bip1-associated ER chaperone Lhs1 has been shown to be required for pathogenicity of the hemibiotrophic fungus Magnaporthe oryzae (Yi et al., 2009). Thus, a functional UPR appears to be commonly required for fungal virulence. Whereas deletion of Hac1 homologs in A. brassicicola and A. fumigatus or of Lhs1 in M. oryzae affected growth and/or conidiation in axenic culture, no such phenotype was observed upon deletion of cib1Δ in U. maydis.
It has been assumed that the UPR is required to ensure efficient secretion of proteins required during pathogenic development or to cope with changing environmental conditions, such as the enhanced temperature upon infection of humans (Richie et al., 2011). It is conceivable that particularly biotrophic pathogens need a well-regulated and efficient secretory system to promote proper signal exchange with the host plant. Consistent with a potential function of the UPR in the efficient secretion of effector proteins, we observed increased expression of defense-related PR genes in maize plants inoculated with Δcib1 mutants. For U. maydis, a set of 554 secreted proteins has been predicted (Mueller et al., 2008), and several of these proteins have been shown to function as effectors crucial for biotrophic development (Kämper et al., 2006; Djamei et al., 2011; Hemetsberger et al., 2012). In addition, the ER-mannosidase Gas1 and the O-mannosyltransferase Pmt4 are required for virulence of U. maydis (Schirawski et al., 2005; Fernandez-Alvarez et al., 2009, 2012). Thus, not only secretion, but also glycosylation of effector proteins, is important for their function during biotrophic development; both pathways are closely interconnected with the UPR.

In addition to these recognized functions, we now demonstrate that the UPR is tightly connected to the b-dependent developmental cascade that controls sexual and pathogenic development in U. maydis. We observed that activation of UPR leads to reduced b-gene expression in SG200 and thereby attenuates b-dependent filament formation in a dose-dependent manner. It remains to be determined whether this is achieved via direct binding of Cib1 to the promoter region of the b-genes or via regulation of other genes upstream of b, such as Prf1 (Hartmann et al., 1996; Kafkamik et al., 2003; Zammack et al., 2008). We suppose that this effect is indirect, since we were unable to identify UPR elements (Mori et al., 1992; Kohn et al., 1993; Fordyce et al., 2012) in the promoter region of bE and bW. However, as inhibition of b-dependent filament formation also occurred in strain AB31, which is not affected in b-gene expression, we assume that at least one additional mechanism by which cib1 affects b-dependent filament formation exists.

In S. cerevisiae, activation of the UPR interferes with the nitrogen starvation–induced morphogenetic switch to pseudohyphal growth (Schröder et al., 2000). However, in U. maydis, activation of the UPR does not only interfere with, but is also required for, developmental transitions of U. maydis: The temporally defined activation of UPR during plant penetration appears to be critical for development of infectious hyphae. During pathogenic development, Cib1-dependent inhibition of the b-pathway could support the release of the b-dependent G2 cell cycle block after plant penetration (Figure 9). The link between the UPR and developmental control is further accomplished via direct interaction between Cib1 and Clp1, leading to stabilization of Clp1. As Clp1 is crucial for cell cycle release and hyphal proliferation in planta (Scherer et al., 2006; Heimel et al., 2010a), it is conceivable that UPR-dependent stabilization of Clp1 acts as a trigger for proliferation. Thus, the Clp1/Cib1 interaction might serve as a checkpoint to adjust cellular physiology at the onset of biotrophic development.

However, as the interaction between Cib1 and Clp1 is not essential for pathogenic development, additional posttranscriptional mechanisms that promote Clp1 expression must exist. One potential mechanism can be deduced from the observation that activation of the UPR in strains expressing cib1ΔΔΔ still resulted in higher levels of Clp1-GFP, although Clp1 is not stabilized (Figures 7E and 7F). Thus, it is possible that, in addition to the stabilization of Clp1 by the Cib1/Clp1 interaction, UPR itself positively affects Clp1 levels. However, the underlying molecular details require further investigation.

In mammals, it has been shown that the UPR is regulated in a stress-, tissue-, or development–specific manner; in addition, fine-tuning mechanisms exist that modulate UPR signaling. One of the known fine-tuning mechanisms involves Xbp1 sp protein resulting from unspliced XBP1 mRNA, which functions as a negative regulator of Xbp1α, which results from spliced XBP1 mRNA. This negative effect of Xbp1α on Xbp1α is thought to delimit the threshold of UPR activation and thereby confers a switch–like behavior on UPR (Byrd and Brewer, 2012). We show that a similar regulation also exists in lower eukaryotes
and provide evidence for an additional role of cib1+/ transcript or Cib1+ protein in preventing UPR hyperactivation. U. maydis cells overexpressing cib1+ require cib1+ to tolerate high levels of Cib1 protein and to suppress the deleterious effects imposed by a hyperactive UPR. This indicates that in U. maydis, unspliced cib1 mRNA or the resulting protein Cib1+ is required to attenuate Cib1 function.

We also observed in U. maydis the development-specific regulation of UPR, currently known only for higher eukaryotes. In mammals, differentiation of B cells into actively secreting plasma cells requires activation of Xbp1 (Calfon et al., 2002). Protein secretion in these professional secretory cells depends on the UPR but also requires modulation of the UPR pathway during constitutive UPR activation (Tabas and Ron, 2011; Hetz, 2012; Moore and Hollien, 2012). We propose that a similar scenario occurs in U. maydis hyphae during biotrophic growth. Expression of cib+ is highly induced throughout the biotrophic stage (Heimel et al., 2010a), indicating a constitutively active UPR. We consider it likely that the large amounts of secreted proteins expressed during biotrophic development bear the risk of extensive UPR activation, which negatively affects cell growth. We showed that this could be prevented by induced clp1 expression, which resulted in elevated ER stress tolerance. Clp1 alleviates the expression of cib and also of the UPR target bpl1, suggesting that Clp1 relieves the harmful impact of hyperactive UPR.

During the life cycle, this kind of regulation is restricted to planta growth by combined transcriptional and post-transcriptional regulation of clp1. clp1 is only transcribed in cells with an active bE/bW heterodimer, and our current data strongly suggest that subsequent UPR activation during plant penetration triggers accumulation of the Clp1 protein. The integration of UPR signaling and the b-dependent developmental control pathway via the C-terminal extension of Cib1 constitutes a direct mechanism to adjust UPR to the specific demands of a biotrophic fungus. After plant penetration, activation of UPR results in accumulation of Clp1, leading to cell cycle release and fungal proliferation. In parallel, Clp1 might increase ER stress tolerance to allow for increased effector secretion and constitutive UPR activation (Figure 9). Coupling of UPR signaling to complex developmental programs appears to be a common feature in both higher and lower eukaryotes (Schröder and Kaufman, 2005; Wu and Kaufman, 2006). Since this coupling involves the interaction of organism-specific proteins with conserved key regulators of UPR signaling, it could explain the low level of sequence conservation in Hac1 homologs of related fungal species, even though their main function is highly conserved.

In summary, our study provides insight into the extensive crosstalk between UPR signaling and the developmental pathways that establish the biotrophic stage of U. maydis (Figure 9). Currently it is unclear which signal(s) activates Ire1 upon entry into the plant. Activation of Ire1 during infection could either be the consequence of increased protein secretion or be triggered by plant-derived molecules. Intriguingly, the latter mode of activation would allow the coordination of host perception with the exactly timed delivery of high amounts of effectors.

METHODS

Strains and Growth Conditions

Escherichia coli strain TOP10 (Invitrogen) was used for cloning purposes and amplification of plasmid DNA. Ustilago maydis cells were grown at 28°C in yeast-extract-peptone-Suc (YPEPS) light medium (Tsukuda et al., 1988), complete medium (CM) (Holliday, 1974), or yeast nitrogen base (YNB) medium (Mahier et al., 2006; Freitag et al., 2011). Growth conditions and media used to induce crg1 promoter-driven gene expression followed the protocol of Brachmann et al. (2001). Mating assays were performed as described (Brachmann et al., 2001); ER stress resistance was assayed on YNB media containing the indicated concentrations of DTT or Tm (Sigma-Aldrich). Cultivation and yeast two-hybrid analyses were performed according to the Matchmaker 3 manual (Clontech).

U. maydis and yeast strains used in this study are listed in Supplemental Table 2 online.

DNA and RNA Procedures

Molecular methods followed described protocols (Sambrook et al., 1989). DNA isolation from U. maydis and transformation procedures were performed according to Schulz et al. (1990). Homologous integration of constructs was verified by PCR and DNA gel blot analyses. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and further column purified (RNeasy; Qiagen). RNA integrity was checked using a Bioanalyzer with an RNA 6000 Nano LabChip kit (Agilent). For all gene deletions, we used a PCR-based approach (Kämper, 2004). Generation of strains expressing GFP fusion proteins at their endogenous genomic locus was performed according to Brachmann et al. (2004). qRT-PCR analysis was performed as described before (Heimel et al., 2010a).

Plasmid Constructions

For construction of plasmid pCib3, the cib1 open reading frame, including 1.5 kb 5′-region (promoter) and 0.5 kb 3′-region (terminator), was PCR amplified from genomic DNA, adding attB1-site and attB2-site to the 5′- and 3′-ends, respectively. The PCR product was used for one tube BP/ LR Gateway cloning into the destination vector p1233bb-GW (M. Vranes, unpublished data). p1233bb-GW is a p123 derivative (Aichinger et al., 2003) in which the Puel·GFP cassette has been replaced with a chloramphenicol resistance cassette that is flanked by attR1 and attR2 recombination sites for Gateway cloning.

The resulting vector pCib3 was linearized with SspI and integrated into the p locus of the respective U. maydis strains. Truncated alleles of cib1 (cib1433 and cib1434-574 [Clp-ID]) were generated using standard PCR techniques, as described (Higuchi, 1990) and were also integrated into vector p1233bb-GW to produce plasmids pCib1433 and pCib1434-574, respectively.

pRU11-(not6476):cib1cDNA was generated by PCR amplification of cib1 cDNA and integration of the PCR product via Ndelt-Notl restriction sites into the vector pRU11-(Notl)-6474 (Pneo-driven expression) (Heimel et al., 2010b). pRU11-(not6476):clp1 was constructed by inserting full-length clp1 from pRU-ATG1 (Scherer et al., 2006) into pRU11-(not6476) via Ndelt-Notl restriction sites. To construct pCib1435-crg:clp1, a PCR-amplified fragment from pCib1435 encompassing the cib1 promoter, the cib1 allelle, and the cib1 terminator, was subcloned into pCRII-Topo (Invitrogen), excised via EcoRI restriction sites, and inserted into pRU11-(not6476):clp1, opened with EcoRI. Vectors were linearized with SspI and integrated into the p locus of the respective U. maydis strains via homologous recombination.

Truncated Cib1 alleles used for yeast two-hybrid analyses were PCR amplified from cib1 cDNA (lacking the
unconventional intron) and inserted into pGAD-DS (Dualsystems Biotech) via Sfi restriction sites.

The plasmid harboring cib1Δ under control of its natural promoter was generated with recombinatorial cloning in Saccharomyces cerevisiae (Colot et al., 2006). The vector backbone pRS426 (Sikorski and Hieter, 1989) allows amplification in E. coli and S. cerevisiae. To facilitate the expression of cib1Δ in U. maydis, a PvuII-PvuII fragment containing cib1Δ under control of its natural promoter was cloned into the PvuII-MscI sites of pETEF-GFP-MMXN (Böhmer et al., 2008), resulting in pPcib:cib1Δ. pPcib:cib1Δ was used as a template to construct pPcib:cib1Δ-3xGFP, which was generated by amplifying the truncated version of cib1Δ. The resulting fragment was digested with KpnI and NotI and inserted into the KpnI-NotI sites of pETEF-GFP-MMXN. Primers used are listed in Supplemental Table 3 online. All plasmids were verified by sequencing.

pPcib:cib1Δ was used as template to construct pPcib:cib1Δ-3xGFP, which was generated by amplifying the cib1Δ promoter and open reading frame, lacking the intron and stop codon. The resulting fragment was digested with KpnI and SfiI and integrated in a three-fragment ligation with a 3xGFP SfiI-NotI fragment isolated from plasmid pMFS-4 h (Baumann et al., 2012) into the KpnI-NotI sites of pETEF-GFP-MMXN.

For the complementation analysis in yeast, either cib1Δ or cib1Δ was PCR amplified and integrated into p414MET25, opened with Spel and ClaI, by recombinatorial cloning (Colot et al., 2006). Plasmids were re-isolated, sequence verified, and transformed in yeast strain RH3351 (Herzog et al., 2013).

For expression of Ire-GFP, the ire1 open reading frame was PCR amplified and integrated as an NcoI fragment into pETEF-GFP-MMXN. The resulting plasmid, pire1-GFP, was linearized with SspI and integrated into the jpr locus of strain SG200.

Microscopy

Microscopy analysis was performed using an Axioimager equipped with an AxioCam MRm camera or a Lumar V12 equipped with an AxioCam HRC (Zeiss). Images were processed with Axiovision (Zeiss). Confocal microscopy was performed using a TCS-SP5 confocal microscope (Leica Microsystems). Images were processed using LAS-AF software (Leica Microsystems). Analysis of appressoria and filament formation was performed according to Mendoza-Mendoza et al. (2009). Briefly, U. maydis strains were grown to an OD600 = 0.8, washed, and resuspended in 2% YEPS light to an OD600 = 0.2 and supplemented with 100 µM 16-hydroxyhexadecanoic acid (Sigma-Aldrich). After spraying on Paraflor, fungal hyphae were stained with WGA-AF488 and propidium iodide as described above. Fungal proliferation was determined by microscopy and propidium iodide as described above, and subjected to microscopy analysis. For the complementation analysis in yeast, either cib1Δ or cib1Δ was PCR amplified and integrated as an NcoI fragment into pETEF-GFP-MMXN. Primers used are listed in Supplemental Table 3 online. All plasmids were verified by sequencing.

For expression of Ire-GFP, the ire1 open reading frame was PCR amplified and integrated as an NcoI fragment into pETEF-GFP-MMXN. The resulting plasmid, pire1-GFP, was linearized with SspI and integrated into the jpr locus of strain SG200.

Quantification of PR Gene Expression

Infected leaf tissue was harvested and 10 samples were pooled and frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent and used for qRT-PCR analysis as described above. Expression levels of maize (Zea mays) pr1, pr3, and pr5 were determined and normalized to GAPDH expression levels.

Plant Infections

The haploid strains SG200 and CL13 and their respective derivatives were grown to an OD600 of 0.8 in YEPS light medium and concentrated to an OD600 of 1 in water; 0.5 mL of the resulting suspension was used to inoculate 7-d-old maize seedlings of the variety Early Golden Bantam (Olds Seeds). Symptoms were scored according to disease rating criteria reported by Kämper et al. (2006). Three independent experiments for each plant infection were performed, and the average scores for each symptom are shown in the respective diagrams. The significance of the phenotypes was calculated using the Wilcoxon-Rank-Sum as described previously (Freitag et al., 2011).

Protein Procedures

Protein isolation and immunoblot analysis were performed according to Lanver et al. (2010).

To detect GFP-tagged proteins, commercially available rabbit anti-GFP (Sigma-Aldrich) was used at a 1:4000 dilution. Tubulin was detected with mouse antitubulin (Calbiochem) antibody at a 1:3000 dilution. For stability measurement of Clp1-GFP in relation to DTT-mediated UPR activation, cells were grown in complete medium supplemented with 1% Glc (CMG) to an OD600 = 0.25, washed with complete medium containing 1% Ara (CMA), and resuspended in complete medium containing 1% Ara to an OD600 = 0.25 to induce b-heterodimer activated Clp1-GFP expression. Three hours after b-induction, cells were treated with 3 mM (final concentration) DTT to induce the UPR. Three hours after UPR induction, protein synthesis was blocked by the addition of 100 µg/mL (final concentration) of cycloheximide, and cells were sampled after 0, 15, and 45 min and quick frozen in liquid nitrogen, and protein extracts were prepared as described (Lanver et al., 2010). Clp1-GFP levels were quantified using ImageJ (http://rsb.info.nih.gov/ij/) and normalized to Coomassie Brilliant Blue-stained bands. Detection of tubulin levels with tubulin-specific antibody (Calbiochem) resulted in oversaturated signals and did not allow for accurate quantification of relative protein levels. Stability of proteins was calculated as the decrease of Clp1-GFP protein at t = 15 and t = 45 min, relative to t = 0. Experiments were performed in four biological replicates. Statistical significances (P value) were calculated using Student’s t test.

Ortho-nitrophenyl-β-galactosidase (ONPG) assay was performed according to Miller (1972) as described in the Yeast Protocols Handbook (Clontech). Briefly, pellets of a 5-M cell overnight culture were washed with 3 mL of breaking buffer (100 mM Tris-Cl, pH 8, and 20% [v/v] glycerol) and resuspended in 250 µL of breaking buffer supplemented with 1 mM DTT (final concentration). After the addition of glass beads, cells were disrupted by bead beating using a Retsch MM200 cell mill for 5 min at 30 Hz. Then, Z-buffer (100 mM Na2PO4, 1 mM MgSO4, and 10 mM KCl, pH 7.0) supplemented with 1 mM DTT was added to 10 to 100 µL of prepared lysate, to a total volume of 800 µL. After equilibration to 28°C, the enzymatic reaction was started by the addition of 160 µL ONPG stock solution (4 mg/mL) and stopped by the addition of 400 µL of 1 M Na2CO3 stock solution. Miller units were calculated using the formula (OD540 × 1000): (volume × Δt)/time × OD600. Results were shown as mean of three technical replicates of five biological replicates each and the SD thereof.
Statistical Analysis

Statistical significance was calculated using Student’s t test. In plant infection experiments, statistical significance was calculated using the Wilcoxon-signed-rank test as described previously (Freitag et al., 2011). Results were considered significant if the P value was <0.05.

Accession Numbers

Sequence data from this article can be found at the Munich Information Center for Protein Sequences Ustilago maydis Database and GenBank/EMBL databases under the following accession numbers: actin (um11232), XP_762364; bET1 (um12051), XP_756724.1; bip1 (um15034); bW2 (M84182); clp1 (um02438), XP_758885; cib1 (um11782), XP_759656; elf2b (um04869), XP_761016; rbf1 (um03172), XP_79319; pr1 (Zm.15280.1), BM351351; pr3 (Zm.1085.1), BM393991; pr5 (Zm.6659.1), BM075306; GAPDH (NM00111943); S. cerevisiae Hac1p NP_116622.1; Candida albicans Hac1 ABS83487; Trichoderma reesei Hac1 Q8TF3.1; Aspergillus nidulans AN9397 XP_682666.1; Aspergillus fumigatus HacA ACJ61678.1; Cryptococcus neoformans Hx1 (CNAG006143.2), AFR98359.1; and Spongopodium reilianum (Sr14500) CBQ73912.1. Please note that U. maydis gene models deposited at GenBank/EMBL databases were not subject to manual annotation. For manually curated gene models, refer to http://mips.helmholtz-muenchen.de/genre/proj/ustilago/.

Supplemental Data

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

Supplemental Figure 1. Overview of Unconventional Splice Sites in Fungal Hac1 Homologs.

Supplemental Figure 2. Complementation of the Yeast Hac1 Mutant with cib1 and Microscopy Analysis of Cib1-GFP and Ire1-GFP Localization.

Supplemental Figure 3. UPR Gene Expression of Strain SG200 and Derivatives in Response to ER Stress.

Supplemental Figure 4. Overexpression of cib1+ Inhibits Cell Growth.

Supplemental Figure 5. UPR Gene Expression during Biotic Growth.

Supplemental Figure 6. Suppression of Filament Formation by Cib1+ Does Not Require clp1.

Supplemental Figure 7. Comparison of cib1+, cib1+1, and bip1 Expression Levels in SG200 and AB31 on Charcoal-Containing Solid Media.

Supplemental Figure 8. Plant Infection Assay in Dependence of Inoculated Cell Numbers.

Supplemental Figure 9. Schematic Overview of Protein Structures of Hac1p and Homologs from Various Fungal Species.

Supplemental Figure 10. UPR Gene Expression upon DTT-Mediated ER Stress Induction.

Supplemental Figure 11. The Clp1 Interaction Domain of Cib1 Affects Virulence.

Supplemental Figure 12. The Clp1 Interaction Domain Is Not Sufficient for cib1+ Complementation.

Supplemental Table 1. Numeric Values of Expression Data Depicted in Supplemental Figure 3.

Supplemental Table 2. Strains Used in This Study.

Supplemental Table 3. Primers Used in This Study.

Supplemental References 1. References Cited in Supplemental Data.

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AUTHOR CONTRIBUTIONS

The experiments were carried out in equal parts in the Heimel, Böker, and Kämper labs. K.H., J.F., M.B., and J.K. conceived and designed the experiments. K.H., J.F., M.H., and J.A. performed the experiments. K.H. and J.F. analyzed the data. K.H., J.F., M.B., and J.K. wrote the article.

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REFERENCES


