Technical advance

Conditional gene expression reveals stage-specific functions of the unfolded protein response in the *Ustilago maydis*–maize pathosystem

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SUMMARY

*Ustilago maydis* is a model organism for the study of biotrophic plant–pathogen interactions. The sexual and pathogenic development of the fungus are tightly connected since fusion of compatible haploid sporidia is prerequisite for infection of the host plant, maize (*Zea mays*). After plant penetration, the unfolded protein response (UPR) is activated and required for biotrophic growth. The UPR is continuously active throughout all stages of pathogenic development in planta. However, since development of UPR deletion mutants stops directly after plant penetration, the role of an active UPR at later stages of development remained to be determined. Here, we established a gene expression system for *U. maydis* that uses endogenous, conditionally active promoters to either induce or repress expression of a gene of interest during different stages of plant infection. Integration of the expression constructs into the native genomic locus and removal of resistance cassettes were required to obtain a wild-type-like expression pattern. This indicates that genomic localization and chromatin structure are important for correct promoter activity and gene expression. By conditional expression of the central UPR regulator, Cib1, in *U. maydis*, we show that a functional UPR is required for continuous plant defence suppression after host infection and that *U. maydis* relies on a robust control system to prevent deleterious UPR hyperactivation.

Keywords: conditional gene expression, pathogenicity, unfolded protein response, *Ustilago*.

INTRODUCTION

The phytopathogenic basidiomycete *Ustilago maydis* causes the smut disease on maize (*Zea mays*) and is a well-established model organism to study sexual fungal development and biotrophic fungal–plant interactions, but also basic cellular processes such as DNA recombination and vesicular transport (Bakkeren et al., 2008; Banuett, 1995; Dean et al., 2012; Kehmann and Kämper, 2004; Lanver et al., 2018).

The available genome sequence, a broad range of molecular techniques and tools, as well as a highly efficient homologous recombination system enable the precise genetic manipulation of *U. maydis* (Brachmann et al., 2008; Kämper, 2004; Kämper et al., 2006; Schuster et al., 2016; Terfrüchte et al., 2014). Common and frequently used ways to characterize gene functions are available, including deletion or overexpression of genes, as well as the generation of gene fusions for fluorescence microscopy or epitope tagging. Polymerase chain reaction (PCR)-based methods for gene replacement via homologous recombination as well as promoters for constitutive, inducible or titratable (over)expression of genes like the *tef*, *otef*, *nar1*, *crg1* or *tet-Off* promoter are also available (Banks et al., 1993; Bottin et al., 2002; Brachmann et al., 2004; Kämper, 2004; Spellig et al., 1996; Zarnack et al., 2008). These promoters can be fused to a gene of interest and are integrated either in the native gene locus or into the locus of the succinate dehydrogenase-encoding gene (*UMAG_00844, sdh2*; *ip* locus) by homologous recombination, conferring carboxin resistance (Keon *et al.*, 1991). However, gene expression analysis using metabolism-dependent promoters may result in pleiotropic effects due to metabolic changes and unwanted overexpression of the gene of interest. Other conditional gene expression systems in fungi include, for example, oestrogen-, orzearalenone- or light-inducible expression systems for *Aspergillus* sp.* (Pachlinger *et al.*, 2005), *Gibberella zeae* (Lee *et al.*, 2010) or *Neurospora crassa* (Salinas *et al.*, 2018), respectively (see Kluge *et al.*, 2018 for a comprehensive overview). These systems are all suitable to control gene expression under axenic culture conditions. However, tools to address
the function of genes specifically during the process of organismal interactions, such as fungal–plant interactions, are not yet well established.

*Ustilago maydis* is a dimorphic fungus, specifically infecting its host plant maize. Its sexual and pathogenic development are interconnected because plant infection requires cell–cell fusion of compatible haploid sporidia to generate the infectious, dikaryotic filament. Development of the fungus, including mating, filamentous growth, plant penetration and biotrophic growth in planta, is controlled by a tetrapolar mating-type system ( Bölker , 2001; Feldbrügge et al. , 2004; Hartmann et al. , 1996; Wahl et al. , 2010). The a-mating type locus encodes a pheromone-receptor system that regulates cell–cell recognition and fusion ( Bölker et al. , 1992), whereas all subsequent steps of pathogenic development are controlled by the bE/bW-heterodimer encoded by the b-mating type locus ( Heimel et al. , 2010a; Kämper et al. , 1995; Schulz et al. , 1990; Wahl et al. , 2010). After penetration of the plant surface, *U. maydis* establishes a compatible biotrophic interaction with the host plant by secreting effectors that suppress plant defence reactions ( Lanver et al. , 2017; Lo Presti et al. , 2015a). Expression of effector-encoding genes is specifically induced during the fungal–plant interaction ( Kämper et al. , 2006; Lanver et al. , 2018), resulting in increased stress imposed on the endoplasmic reticulum (ER). Activation of the unfolded protein response (UPR) is critical to counteract elevated ER stress levels and for efficient secretion of effector proteins ( Hample et al. , 2016; Lo Presti et al. , 2015b; Pinter et al. , 2019). The UPR is controlled by a key regulatory basic leucine zipper (bZIP) transcription factor termed Hac1 in Saccharomyces cerevisiae, XBP1 in higher eukaryotes and Cib1 in *U. maydis* ( Cox and Walter , 1996; Heimel et al. , 2013; Kawahara et al. , 1998; Rüegsegger et al. , 2001). The UPR is activated by unconventional cytoplasmic splicing of the HAC1/cib1/XBP1 messenger RNA (mRNA), generating the processed form of the mRNA (e.g. cib1s) that is translated into the active transcription factor. Hence, the effects of genetic UPR activation can be analysed by expression of the cib1s mRNA without drug-induced side-effects.

In fungal human and plant pathogens, a functional UPR is necessary for disease development ( Cheon et al. , 2011; Heimel et al. , 2013; Joubert et al. , 2011; Kong et al. , 2015; Richie et al. , 2009; Yi et al. , 2009). In *U. maydis*, the UPR is specifically activated after plant penetration and remains constantly active during all subsequent stages of biotrophic growth inside the host plant ( Heimel et al. , 2013). This suggests that the UPR is constantly required for efficient protein secretion and regulation of pathogenic growth. However, since cib1 mutant strains are arrested early after plant infection, the relevance of a functional UPR at later stages of biotrophic development in planta remains to be addressed.

Here, we established a system for conditional and stage-specific gene expression during pathogenic growth of *U. maydis* in planta. Based on previously published time-resolved transcriptome data of fungal gene expression during biotrophic growth ( Lanver et al. , 2018), genes with desired in planta expression patterns were identified and their promoters were used for conditional gene expression. Importantly, we observed that maintenance of the genomic context and removal of resistance marker cassettes are required for correct promoter activity and conditional gene expression. To address the function of the UPR regulator Cib1 at later stages of biotrophic development, we used conditional promoters to repress, induce or overexpress cib1 at specific stages of biotrophic growth in planta. We thereby demonstrate that *U. maydis* is resistant to UPR hyperactivation after plant penetration, suggesting effective strategies to prevent or cope with deleterious ER stress. By contrast, repression of cib1 expression at 2 or 4 days post-inoculation (dpi) revealed that a functional UPR is not only essential for establishment of biotrophy, but also required for colonization and continuous suppression of the plant defence at later stages of development in planta.

## RESULTS

### Genomic localization and the presence of resistance marker cassettes affect the activity of promoters specifically expressed in planta

In previous studies, promoters of *U. maydis* mig (maize induced genes) genes that are specifically expressed in planta were used for conditional gene expression during infection ( Lo Presti et al. , 2015b; Scherer et al. , 2006; Wahl et al. , 2010). In addition to the mig1 gene ( Basse et al. , 2000), mig genes include the mig2 gene cluster harbouring five highly homologous genes, all of which are plant-specifically expressed but not involved in the virulence of *U. maydis* ( Basse et al. , 2002). The mig2 genes (mig2_1, mig2_2, mig2_3, mig2_4 and mig2_5) differ in their strength and temporal dynamics of expression. Thus, their promoters represent suitable targets for controlled and plant-specific expression/over-expression of a gene of interest.

To address the effect of overexpressing the spliced version of the cib1 mRNA (cib1s in the following text), encoding the UPR regulator Cib1, during pathogenic development in planta, we integrated a P_mig2_1:cib1s promoter fusion into the ip locus of the solopathogenic SG200 strain ( Kämper et al. , 2006). The ip or cbx locus is commonly used for integration of linear DNA into the *U. maydis* genome by homologous recombination, conferring resistance against carboxin ( Brachmann et al. , 2001). Since the virulence of strain SG200P_mig2_1::cib1s was severely attenuated in plant infection experiments (Fig. 1A), we investigated at which stage pathogenic development was blocked. Our analysis revealed the inability of SG200P_mig2_1::cib1s to induce filamentous growth under axenic conditions and on the plant surface (Fig. 1B). Since filamentous growth is crucial for all subsequent steps of pathogenic development, such as appressoria formation and...
penetration of the leaf surface, our data suggest that the pathogenesis process is abrogated before plant infection.

We have previously shown that constitutive expression of cib1 is inhibits the formation of infectious filaments (Heimel et al., 2013). Hence, we tested if integration of the P mig2_1:cib1s construct into the ip locus might result in increased expression levels of cib1 during growth in axenic culture. Indeed, levels of cib1 were significantly increased in strain SG200P mig2_1:cib1s compared to the SG200 control strain (Fig. 1C). Since elevated cib1 levels might result either from increased activity of the cib1 wild type (WT) open reading frame (ORF) that is also present in SG200P mig2_1:cib1s or from 'leaky' P mig2_1-driven expression, we used the Δcib1 background for further analyses. To study if this effect is specific for the ip locus, we generated U. maydis strain FB1Δcib1∆mig2_1::cib1s (mig2_1 locus, −NatR) by replacing the mig2_1 ORF with the cib1s gene. To exclude potential effects.

![Fig. 1](image)

**Fig. 1** The locus of integration and presence of a resistance cassette influence promoter activity. (A) Plant infection assay with the *Ustilago maydis* solopathogenic strain SG200 and a derivative strain. Strains SG200 and SG200 P mig2_1:cib1s (ip locus) were inoculated into 8-day-old maize seedlings. Disease symptoms were rated 8 days post-inoculation and grouped into categories as shown in the figure. n = number of inoculated plants. Significance was calculated using the Mann–Whitney test. ***P < 0.001 (B) Analysis of b-dependent filament formation on PD-CC solid medium and on the leaf surface. Strains SG200 and SG200P mig2_1:cib1s (ip locus) were spotted on PD-CC solid medium. Photographs were taken after 24 h at 28 °C. White fuzzy colonies indicate the formation of filaments. Fungal hyphae were stained 24 h after inoculation with Calcofluor White to visualize the cells. Scale bar = 10 µm. (C) RT-qPCR analysis of cib1 gene expression when integrated in different loci and after removal of the resistance cassette. Primers specifically detecting the cib1 transcript were used. RNA was isolated from exponentially growing U. maydis strains SG200, SG200 P mig2_1:cib1s (ip locus integration), FB1Δcib1∆mig2_1::Pcib1s (mig2_1 locus, +NatR) and FB1Δcib1∆mig2_1::cib1s (mig2_1 locus, −NatR). eIF2b was used for normalization. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent the SEM. Statistical significance was calculated using the Student’s t-test. *P < 0.05, **P < 0.01. (D) RT-qPCR analysis of pit1 and pit2 gene expression when integrated in different loci and after removal of the resistance cassette. RNA was isolated from exponentially growing U. maydis strains SG200, SG200 P pit1/2::pit1/2 (ip locus integration), SG200 P pit1/2::pit1/2 (pit1 locus, +NatR), SG200 P pit1/2::pit1/2 (pit2 locus, +HygR) and P pit1/2::pit1/2 (pit1 locus, −HygR). eIF2b was used for normalization. Expression values represent the mean of three biological replicates with two technical duplicates each. Error bars represent the SEM. Statistical significance was calculated using the Student’s t-test. n.s., not significant, **P < 0.01 and ***P < 0.001.
of the resistance cassette used for integration, the nourseothricin (NatR) resistance cassette was removed by flippase/flippase recombination target (FLP/FRT) recombination (Khrunyk et al., 2010). This revealed that elevated cib1+ levels indeed resulted from aberrant Pmig2_1 activity and only strains in which the nourseothricin resistance marker was removed [mig2_1 locus
Fig. 2 Overexpression of cib1 in planta does not affect pathogenicity of Ustilago maydis. (A) Endoplasmic reticulum stress assay of strains FB1, FB1Δcib1, FB1Δcib1 Δmig1::cib1 and FB1Δcib1Δmig2_1::cib1. Serial 10-fold dilutions were spotted on YNBG solid medium supplemented with tunicamycin (TM) (1.0 μg/mL) or dithiothreitol (DTT) (1 mM). Pictures were taken after 48 h of incubation at 28 °C. (B) Mating assay with compatible mixtures of FB1, FB2, FB1Δcib1, FB2Δcib1, FB1Δcib1Δmig1::cib1 and FB1Δcib1Δmig2_1::cib1. Mixtures were spotted on charcoal-containing potato dextrose (PD-CC) solid medium as shown in the figure. Photographs were taken after 24 h at 28 °C. White fuzzy colonies indicate the formation of filaments. (C) Plant infection assay with compatible mixtures of FB1 and FB2, FB1Δcib1, FB2Δcib1, FB1Δcib1Δmig1::cib1 and FB1Δcib1Δmig2_1::cib1. Eight-day-old maize seedlings were co-inoculated with the indicated strain mixtures. Disease symptoms were rated 8 days post-inoculation (dpi) and grouped into categories as shown in the figure. n = number of inoculated plants. Pictures of leaves were taken at 8 dpi and represent the most common infection symptom. Significance was calculated using the Mann–Whitney test. ***P < 0.001; n.s., not significant.

Overexpression of cib1 does not disturb pathogenic development in planta

To set up a system that allows for proper functioning of conditional promoters we constructed plasmids harbouring promoters of the mig1, mig2_1, mig2_2 or mig2_3 genes. 3′ sequences were followed by a SfiI restriction site for integration of the gene of interest, an FRT-HygR or an FRT-NatR resistance marker cassette and a 1 kb sequence harbouring the 3′ UTR for recombination and integration into the genomic locus of respective mig genes. It is important to note that neither the single nor the combined deletion of all mig genes negatively affected pathogenic development of U. maydis (Farfsing et al., 2005). To specifically increase cib1 levels in planta and address the effect of UPR hyperactivation on pathogenic development, we expressed cib1 under the control of the mig1 or the mig2_1 promoter. To this end, the mig1 or mig2_1 ORFs were replaced by cib1, followed by the removal of the resistance marker cassette in the U. maydis strain FB1Δcib1 (Heimel et al., 2010b) (see Fig. S1 for an overview of the approach). We first checked for leaky cib1 expression by testing ER stress resistance and filamentous growth of the generated strains. When spotted on solid medium supplemented with the ER stress-inducing drugs tunicamycin (TM) or dithiothreitol (DTT), the hypersusceptibility of the FB1Δcib1 progenitor strain was not suppressed, suggesting that Pmig1 and Pmig2_1 are not active in axenic culture (Fig. 2A). Consistently, filamentous growth of respective strain combinations was not affected in mating assays on charcoal-containing potato dextrose (PD-CC) solid medium (Fig. 2B), thus confirming the absence of leaky cib1 expression.

Mixtures of mating compatible strains FB1, FB2, FB1Δcib1, FB2Δcib1 and the derivatives FB1Δcib1Δmig1::cib1 and FB1Δcib1Δmig2_1::cib1 were used for plant infection studies (Fig. 2C). Pmig1 or Pmig2_1-mediated expression of cib1 did not affect pathogenicity when strains were combined with the compatible FB2 WT strain. By contrast, when FB1Δcib1Δmig1::cib1 or FB1Δcib1Δmig2_1::cib1 were combined with the compatible FB2Δcib1 deletion mutant, virulence was strongly increased compared to the nonpathogenic FB1Δcib1 × FB2Δcib1 control, although not to WT (FB1 × FB2) levels. This result suggests that the mechanisms to prevent UPR hyperactivation in planta are robust and efficient in U. maydis, thereby confirming the previous assumption that the UPR is specifically required during biotrophic development in planta (Heimel et al., 2010b, 2013).

Establishment of a system for in planta-specific gene depletion

We next aimed to establish a gene expression system that would allow us to examine gene functions during defined developmental stages in planta by using promoters that are specifically repressed during plant infection. To this end, we screened the publicly available RNA-sequencing (RNA-Seq) data set published by Lanver et al. (2018) and identified a total of four candidate genes that are expressed during axenic growth and early stages of pathogenic development before plant penetration, but strongly repressed shortly after plant penetration (1–2 dpi; UMAG_00050, UMAG_05690 and UMAG_12184) or at later stages during biotrophic growth in planta (UMAG_03597) (Lanver et al., 2018).

We focused on UMAG_12184 and UMAG_03597 for our current studies. Both genes are expressed in axenic culture and
at early stages of pathogenic development, but are strongly repressed at 2 dpi (UMAG_12184) or 4 dpi (UMAG_03597) (Fig. 3A), during and shortly after *U. maydis* has established a compatible biotrophic interaction with its host plant. To test if these genes are involved in virulence, we deleted the genes in the haploid, solopathogenic *U. maydis* strain SG200. SG200 expresses a compatible bE1/bW2-heterodimer, and is thus capable of forming filaments and infecting its host plant, *Z. mays*, without the need for a compatible mating partner (Kämper et al., 2006). Both deletion strains were not affected in virulence (Fig. 3B), demonstrating that these genes are dispensable for pathogenic development. In addition, neither ER or cell wall stress resistance, nor filamentous growth on PD-CC solid medium were strongly affected by either deletion, although filament formation was reduced in the UMAG_03597 deletion mutant (Fig. S2). However, since SG200∆UMAG_03597 showed full virulence, this phenotype does not impair the ability of the fungus to cause disease. Based on these results, the respective promoters were regarded as suitable candidates to be used for conditional gene expression.

**Cib1 is required throughout biotrophic development in planta**

The bZIP transcription factor Cib1 is the central regulator of the UPR in *U. maydis* and is required for coordinating pathogenic development, efficient secretion of effectors and plant defence suppression (Heimel et al., 2013; Pinter et al., 2019). Pathogenic development of cib1 deletion strains is blocked immediately after plant penetration, resulting in the complete absence of tumour formation (Heimel et al., 2013). To test if cib1 is only important directly after plant penetration (e.g. for release of the cell cycle block and establishment of the biotrophic interaction) or if it is also necessary at later stages of pathogenic development, we expressed cib1 under control of the UMAG_12184 and UMAG_03597 promoters (switch off at 2 and 4 dpi, respectively). To this end, we replaced UMAG_12184 or UMAG_03597 genes with the cib1 ORF in strain FB2∆cib1 (Heimel et al., 2010b), generating strains FB2∆cib1 ∆UMAG_12184::cib1 and FB2∆cib1 ∆UMAG_03597::cib1. Resistance cassettes used for selection of successful integration events were removed by FLP/FRT-mediated recombination (Khrenyk et al., 2010).

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**Fig. 3** Identification and testing of promoters for conditional gene expression. (A) Fragments per kilobase million (FPKMs) of the UMAG_12184 and UMAG_03597 genes up to 8 days post-inoculation (dpi). Six-day-old maize seedlings were injected with a mixture of compatible haploid strains FB1 and FB2 and plant material was harvested at the indicated time points. Raw data was extracted from Lanver et al. (2018). (B) Plant infection assay with the solopathogenic strain SG200 and derivatives. SG200, SG200ΔUMAG_12184 and SG200ΔUMAG_03597 were inoculated into 8-day-old maize seedlings. Disease symptoms were rated 8 dpi and grouped into categories as shown in the figure. n = number of inoculated plants. Significance was calculated using the Mann–Whitney test. n.s., not significant at α = 0.05.
The generated strains were tested for correct expression of cib1 under axenic conditions by ER stress assays using TM or DTT. Both mutants showed ER stress resistance similar to the WT (FB2) control, demonstrating that cib1 expression driven by either promoter is sufficient to suppress the ER-stress hypersensitivity of the FB2∆cib1 progenitor strain (Fig. 4A) (Heimel et al., 2013). Additionally, when compatible mixtures of WT (FB1 × FB2), Δcib1 derivatives (FB1Δcib1 × FB2Δcib1) or derivatives expressing cib1 under control of conditional promoters (FB1Δcib1 × FB2Δcib1ΔUMAG_12184::cib1 or FB1Δcib1 × FB2Δcib1ΔUMAG_03597::cib1) were spotted on PD-CC solid medium (Fig. 4B), all tested combinations developed white fuzzy colonies (Banuett and Herskowitz, 1989), indicating that mating is not affected in these strains.

Next, we investigated the effect of plant-specific repression of cib1 in plant infection assays. When compatible mixtures of FB1Δcib1 × FB2 strains were used for inoculation of maize plants, symptom development was indistinguishable from the WT (FB1 × FB2) control (Fig. 4C), demonstrating that a single functional copy of cib1 is sufficient for full virulence of the fungus. However, when cib1 was expressed under the control of P_UMAG_12184 (FB1Δcib1 × FB2Δcib1ΔUMAG_12184::cib1), virulence was almost completely abolished and no tumours were formed, resembling the Δcib1 phenotype. By contrast, expression of cib1 under the control of P_UMAG_03597 (FB1Δcib1 × FB2Δcib1ΔUMAG_03597::cib1) was sufficient to trigger anthocyanin production and the formation of small tumours. This indicates that prolonged expression of cib1 is sufficient to overcome the

Fig. 4  Conditional cib1 expression restores endoplasmic reticulum (ER) stress resistance, but not pathogenicity. (A) ER stress assay of strains FB2 (WT), FB2Δcib1 and derivatives. Serial 10-fold dilutions were spotted on YNBG solid medium supplemented with tunicamycin (TM) (1 μg/ml) or dithiothreitol (DTT) (1 mM). Photographs were taken after 48 h of incubation at 28 °C. (B) Mating assay with FB1, FB1Δcib1 and FB2Δcib1 (ΔUMAG_12184::cib1 and FB2Δcib1 ΔUMAG_03597::cib1). Compatible mixtures of strains were spotted on potato dextrose solid medium supplemented with 1% charcoal (PD-CC). Photographs were taken after 24 h at 28 °C. White fuzzy colonies indicate the formation of filaments. (C) Plant infection assay with FB1 and FB2, FB1Δcib1 and FB2, FB2Δcib1 ΔUMAG_12184::cib1 and FB2Δcib1 ΔUMAG_03597::cib1. Eight-day-old maize seedlings were co-inoculated with compatible strain mixtures as indicated in the figure. Disease symptoms were rated 8 days post-inoculation (dpi) and grouped into categories as shown in the figure. n = number of inoculated plants. Pictures of leaves were taken at 8 dpi and represent the most common infection symptom. Significance was calculated using the Mann–Whitney test. ***P < 0.001.
developmental block of ∆cib1 strains and initiate pathogenic growth in planta.

To visualize fungal growth in planta and assess at which step biotrophic development of the fungus stopped, infected leaves were harvested at 2, 4 and 6 dpi and stained with Chlorazol Black E (Fig. 5A). Microscopic analysis revealed extensive proliferation and clamp cell formation when plants were inoculated with combinations of WT (FB1 × FB2) or FB1 × FB2∆cib1 strains. When cib1 was expressed under the control of $P_{UMAG_{12184}}$ until 2 dpi (FB1∆cib1 × FB2∆cib1∆UMAG_12184::cib1) infectious dikaryotic filaments penetrated the plant surface via appressoria at 2 dpi, but did not progress further in the plant at later stages (4 and 6 dpi). Consequently, clamp cell formation and extended fungal proliferation was not observed. By contrast, expression of cib1 under control of $P_{UMAG_{03597}}$:cib1 (FB2∆cib1∆UMAG_03597::cib1) enabled the...

![Image](image_url)

**Fig. 5** Analysis of fungal morphology and plant defence response of conditional cib1 mutant strains. (A) Fungal proliferation of compatible mixtures of FB1 and FB2, FB1∆cib1 and FB2, FB2∆cib1 ∆UMAG_12184::cib1 or FB2∆cib1 ∆UMAG_03597::cib1 investigated by Chlorazol Black E staining of infected leaf samples at 2, 4 and 6 days post-inoculation (dpi). Arrows point to clamp cells indicative of fungal proliferation in planta. Scale bar = 20 µm. (B) RT-qPCR analysis of PR1, PR3 and PR5 gene expression of infected maize leaves at 2, 4 and 6 dpi. Maize seedlings were inoculated with the indicated strains. GAPDH was used for normalization. Expression values represent the mean of two or three biological replicates with two technical duplicates each. Error bars represent the SEM. Statistical significance was calculated using the Student’s t-test. *P < 0.05.
fungus to overcome the cell cycle block and induce proliferation, as reflected by hyphal branching and the formation of clamp cells at 4 dpi. However, the subsequent colonization of host tissue by fungal hyphae at 6 dpi appeared strongly reduced in comparison to the controls (FB1 × FB2 and FB1Δcib1 × FB2) (Fig. 5A). This suggests that the reduced activity of P_{UMAG_03597} and the resulting decrease of cib1 levels at this stage prevents further progression of fungal hyphae inside the plant.

Previous studies revealed that plants inoculated with Δcib1 mutant strains show increased plant defence reactions as demonstrated by elevated expression of pathogenesis-related (PR) gene expression at 2 dpi (Heimel et al., 2013). It is conceivable that this observation is connected to the requirement of a functional UPR for efficient secretion and processing of effectors (Hampel et al., 2016; Lo Presti et al., 2015b; Pinter et al., 2019). To investigate if Cib1 is also required for plant defence suppression at later stages, we determined expression levels of PR genes PR1, PR3 and PR5 at 2, 4 and 6 dpi in plants inoculated with strains conditionally expressing cib1. All three PR genes are markers for salicylic acid (SA)-related defence responses that are typically suppressed by biotrophic plant pathogens like U. maydis (Glazebrook, 2005). Consistent with the results obtained in infection studies, P_{UMAG_12184}-driven expression of cib1 resulted in increased expression of PR3 and PR5 genes at 2 dpi, whereas expression of PR1 was not induced (Fig. 5B). By contrast, when cib1 was expressed under the control of P_{UMAG_03597}, expression of all three PR genes was induced at 6 dpi. These observations are consistent with the expected activity of the P_{UMAG_12184} and P_{UMAG_03597} promoters that are repressed at 2 and 4 dpi, respectively. Hence, our data indicate that cib1 expression under control of the promoter of UMAg_12184 is not sufficient to establish a compatible biotrophic interaction in planta leading to a block in pathogenic development. By contrast, when cib1 is expressed for an extended time (from promoter P_{UMAG_03597}), a compatible interaction appears to be established, allowing further proliferation. This suggests that cib1 is required for plant defence suppression not only at the onset (2 dpi), but also during later (4 and 6 dpi) stages of biotrophic development in planta.

**DISCUSSION**

Analysis of gene function typically involves the generation of gene deletion and overexpression strains. To test for functions related to the virulence of plant pathogenic fungi, deletion strains are inoculated into the host plant and scored for development of disease symptoms (Dean et al., 2012). However, the analysis of virulence factors that are essential for pathogenic development relies on the description of the first phenotype that is observed, i.e. the stage when pathogenic development is blocked. Hence, potential functions of these factors that might also be important at later stages of pathogenic development remain to be determined. To date, suitable tools to address this problem are restricted to the introduction of a gatekeeper mutation in kinases that can be chemically inhibited by non-hydrolysable adenosine triphosphate (ATP) analogues. However, this strategy is only suitable for the analysis of kinase functions and requires extensive controls to exclude potential side-effects of the chemical treatment (Sakulkoo et al., 2018).

In this study, we report a conditional gene expression system for U. maydis that enables the study of gene functions at different stages of pathogenic development in the plant. We identified suitable promoters that are active during axenic growth and repressed during pathogenic growth in planta. We demonstrate that promoters (e.g. P_{ mig2_1} or P_{pitt2}), previously used for plant-specific gene expression, are active during axenic growth and produce considerable amounts of transcripts (up to 800-fold induced expression for pit2) when integrated into the ip locus or when resistance marker cassettes are located in their vicinity. Proper promoter function required the maintenance of the genomic environment by ‘in locus’ integration (as demonstrated for the mig2_1 or pit2 genes) and removal of the resistance marker cassette. RNA-Seq data suggest that during axenic growth the hsp70 promoter used for the hygR cassette is less active than the gapdh promoter used for the natR marker (Lanver et al., 2018). It thus appears possible that the degree of deregulation by integration of different resistance cassettes might be related to the expression levels of resistance marker genes.

Similar to the mig2 gene cluster, the virulence factors pit1 and pit2 are part of a gene cluster that is specifically up-regulated in planta (Basse et al., 2002; Doehlemann et al., 2011). Interestingly, gene expression of the majority of effector gene clusters, including mig2 and pit clusters, is induced in strains deleted for the histone deacetylase hda1 (Reichmann et al., 2002; Treutlein, 2007), suggesting that these clusters are subject to epigenetic regulation. It remains to be investigated if this effect is restricted to clustered effector genes or accounts for the regulation of non-clustered effectors as well. Chromatin-based regulation of effector genes appears to be a common feature in plant pathogenic fungi (Soyer et al., 2014). It is well established that the RNA polymerase II complex closely interacts with histone-modifying enzymes, including the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex and histone acetyltransferases (Wittschieben et al., 1999, 2000). This complex is supposed to function as a chromatin snowplough leading to increased accessibility of the genomic neighbourhood (Barton and Crowe, 2001). Hence, although the underlying molecular details remain to be addressed, it is tempting to speculate that high expression of the sdh2 gene (ip locus) or of highly expressed resistance marker genes might affect the chromatin structure and thus derepress silent promoters in their vicinity by providing access for the transcriptional machinery to normally silenced, inaccessible genomic regions.
The conditional overexpression of cib1 transcript using the mig1 or mig2_1 (max. FPKMs 2393 at 4 dpi) promoter did not result in alterations of disease symptoms. As the mig1 promoter is highly active in planta (max. FPKMs: 10200; cib1-promoter 551 FPKMs at 4 dpi) (Basse et al., 2000; Lanver et al., 2018), it is especially remarkable that high levels of cib1 transcript are not detrimental for fungal proliferation in planta. This suggests that U. maydis has established effective control mechanisms to prevent UPR hyperactivation, one of which is based on the functional modification of the UPR by the Cib1–Cip1 interaction, providing ER stress hyper-resistance of Clp1-expressing strains (Heimel et al., 2013; Pinter et al., 2019). A potential second mechanism might be reminiscent of UPR regulation in higher eukaryotes and involve the unspliced cib1 transcript or the encoded Cib1 protein (Heimel et al., 2013). In higher eukaryotes, the U-isofrm of the Hac1-like UPR regulator XBP1 functions as a repressor of the UPR (Yoshida et al., 2006). If a similar mode of action would potentially counteract increased cib1 transcript levels, then expression of the unspliced cib1 transcript itself would be subject to Cib1-dependent gene regulation.

The increasing body of transcriptomic data provides a highly valuable treasure box to identify promoters with desired expression dynamics. In theory, this enables establishment of tailor-made expression systems to address gene specific functions in a sophisticated manner. However, our attempt to identify promoters that are active during axenic growth, but strongly repressed at different stages of pathogenic development in planta, revealed only a low number of candidates. Moreover, we observed that it is desirable for correct promoter function to maintain the genomic context. Using Cib1, an essential virulence factor in U. maydis, we carried out a proof-of-principle analysis demonstrating that a functional UPR is not only required directly after penetration of the leaf surface (Heimel et al., 2010b, 2013), but also at later stages of pathogenic development. The increased expression of PR genes correlates with repression of promoter activity and thus reduced cib1 transcript levels. This strongly suggests that continuous suppression of the SA-related plant defence depends on sustained UPR activity. This is consistent with the observation that not only early but also late effectors require the UPR for efficient secretion and/or processing (Hampel et al., 2016; Lo Presti et al., 2015b; Pinter et al., 2019). Although our system is applicable for a wide range of genes, a potential limitation is met when examining stage-specific functions of genes with dynamic expression patterns. One way to enable these studies would be the stage-specific expression of site-specific recombinases, such as cyclization recombinase (CRE) or FLP (Sadowski, 1995; Sauer and Henderson, 1988; Sternberg and Hamilton, 1981), as established for a variety of model systems including numerous fungi (Krhrunyk et al., 2010; Kopke et al., 2010; Kück and Hoff, 2010; Mizutani et al., 2012; Twaruschek et al., 2018; Zhang et al., 2013). In this way, loxP or FRT flanked genes could be targeted for genomic deletion in a stage- or development-specific manner, while maintaining their dynamic expression pattern.

In summary, we established a conditional expression system that allows plant-specific functions of genes of interest to be addressed in the U. maydis–maize pathosystem. The generation of constructs to be integrated into the genome is facilitated by an efficient one-step cloning procedure. Plasmids for conditional induction or repression of genes during biotrophic development in planta are cross-compatible and harbour identical SfiI restriction sites for easy exchange of genes. Since the constructs can either be integrated into the genome of solopathogenic or compatible haploid strains, future studies using combinations of conditionally expressed constructs will allow the consideration of even more sophisticated scientific questions, such as the relevance of post-translational modifications or enzymatic activity of a protein for biotrophic growth of U. maydis.

**EXPERIMENTAL PROCEDURES**

**Strains and growth conditions**

*Escherichia coli* TOP10 was used for cloning and amplification of plasmid DNA. *Ustilago maydis* cells were grown at 28 °C in yeast extract peptone sucrose (YES) light medium (Tsukuda et al., 1988), complete medium (CM) (Holliday, 1974) or yeast nitrogen base (YNB) medium (Freitag et al., 2011; Mahlert et al., 2006). Mating assays were performed as described before (Brachmann et al., 2001). ER-stress assays were carried out on YNB solid medium containing the indicated concentrations of DTT or TM (Sigma-Aldrich, Darmstadt, Germany). Sensitivity to Calcofluor White or Congo Red was tested by drop-assay on YNB solid medium containing the indicated concentration of the respective compound. Filamentous growth assays were carried out using PD medium supplemented with 1% charcoal (PD-CC) (Holliday, 1974). Strains used in this study are listed in Table S1.

**DNA and RNA procedures**

Molecular methods followed described protocols (Sambrook et al., 1989). For gene deletions, a PCR-based approach was used (Kämper, 2004). Isolation of genomic DNA from *U. maydis* and transformation procedures were performed according to Schulz et al. (1990). Homologous integration was performed using linearized plasmid DNA or PCR-amplified DNA. Integration was verified by Southern hybridization. Total RNA was extracted from exponentially growing cells in axenic culture using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany). RNA integrity was checked by agarose gel electrophoresis. Residual DNA was removed from total RNA samples using the TURBO DNA-free Kit (Ambion, Darmstadt, Germany). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). Primers used in this study are listed in Table S2.
Quantitative RT-PCR

Quantitative reverse transcription PCR (RT-qPCR) analysis was performed as described (Hampel et al., 2016). For all RT-qPCR experiments, three independent biological replicates and two technical replicates were analysed using the MESA GREEN qPCR MasterMix plus for SYBR Assay with fluorescein (Eurogentech, Cologne, Germany). RT-qPCR was performed using the CFX Connect Real-Time PCR Detection System and analysed with the CFX Manager Maestro Software (Bio-Rad).

Plasmid construction

For gene deletions, a PCR-based approach and the SfiI insertion cassette system were used (Brachmann et al., 2004; Kämper, 2004). For construction of plasmids for conditional gene expression, 0.5–1 kb flanking regions of chosen genes (UMAG_03597, UMAG_12184, mig1, mig2_1) were PCR amplified from genomic DNA, adding a SfiI restriction site to the 5′ of the left border (LB) and a BamHI (for UMAG_12184, mig1 and mig2_1) or KpnI (for UMAG_03597) restriction site to the 3′ end of the right border (RB). The gene of interest (GOI; cib1 or cib1s) was PCR amplified from genomic DNA or from plasmid P_{cib1-cib1}s, respectively, adding SfiI restriction sites to the 5′ and 3′ ends. The HygR cassette was amplified from plasmid pUMa1442 adding a BamHI (for UMAG_12184) or KpnI restriction site (for UMAG_03597) to the 3′ end and a SfiI restriction site to the 5′ end. The resulting DNA fragments were ligated to obtain LB-GOI-HygR-RB or LB-GOI-NatR-RB and integrated into the pCR2.1 TOPO vector (Invitrogen) or the pjet1.2 vector (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions to generate plasmids pCR2.1 P_{UMAG_12184-cib1(NatR)}, pCR2.1 P_{UMAG_03597-cib1(HygR)}, pjet1.2 P_{mig2_1-cib1}(NatR) and pjet1.2 P_{mig1-cib1}(NatR).

For construction of the P_{mig2_1-cib1}s construct for ip locus integration, the vectors pMig2_1-clp1 and pRU11-cib1s were cut with NdeI and EcoRI. The resulting 2.0 kb cib1 fragment of pRU11-cib1s (Heimel et al., 2013) and the 5.2 kb backbone of Mig2_1-clp1 were ligated to obtain plasmid P_{mig2_1-cib1}s. Plasmids generated in this study are listed in Table S3.

Plant infections

The haploid, solopathogenic strain SG200 and its derivatives or FB1 and FB2 and their respective derivatives were grown to an OD600 of 0.6–0.8 in YEPS light medium, adjusted to an OD600 of 1.0 in water and mixed 1:1 with a compatible mating partner. The resulting suspension was used to inoculate 8-day-old maize seedlings of the variety Early Golden Bantam. Plants were grown in a CLF Plant Climatics GroBank (Wertingen, Germany) with a 14 h (28 °C) day and 10 h (22 °C) night cycle. Symptoms were scored according to disease rating criteria reported by Kämper et al. (2006). Three independent clones were used for each plant infection experiment and the average scores for each symptom are shown in the respective diagrams. Photographs were taken of infected leaves and show the most common infection symptoms for the respective mutant.

Chlorazole Black E staining and microscopy

Infected leaf tissue was harvested at 2, 4 and 6 dpi and kept in 100% ethanol until further processing. Chlorazole Black E staining was performed as described in Brachmann et al. (2001). Microscopic analysis was performed using an Axio Imager.M2 equipped with an AxioCam MRm camera (ZEISS, Jena, Germany). All images were processed using ImageJ.

Quantification of U. maydis gene expression in planta and PR gene expression

Infected leaf tissue was harvested at the indicated time points. Samples of five infected maize seedlings were pooled per replicate, frozen in liquid nitrogen and ground to powder by mortar and pestle according to Lanver et al. (2018). Total RNA was extracted using TRIzol reagent (Invitrogen) and used for RT-qPCR analysis as described above. For expression analysis of U. maydis genes, elf2b expression levels were used for normalization. Expression of PR1, PR3 and PR5 from Z. mays was determined and normalized to GAPDH expression levels.

Statistical analysis

Statistical significance was calculated using Student’s t-test. The statistical significance of plant infection phenotypes was calculated using the Mann–Whitney test as described previously (Freitag et al., 2011). Results were considered significant when P < 0.05.

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DATA AVAILABILITY STATEMENT

Sequence data from this article can be found in the National Center for Biotechnology Information database at https://www.ncbi.nlm.nih.gov/genbank/ under the following accession numbers: UMAG_12184, XP_011388913.1; UMAG_03597, XP_011390022.1;
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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1** Strategy for strain generation for conditional gene expression. (1) The gene of interest (GOI) is deleted from its native genomic locus. (2) The GOI is integrated into the genomic locus of the conditionally expressed gene, thereby replacing the native gene. (3) The resistance marker (here, NatR) is removed using the FLP/FRT recombination system.

**Fig. S2** ∆UMAG_12184 and ∆UMAG_03597 strains do not show increased sensitivity to cell wall or endoplasmic reticulum (ER) stresses. Cell wall and ER stress assays, and tests for filamentous growth of strains SG200, SG200Δcib1, SG200ΔUMAG_12184 and SG200ΔUMAG_03597. Serial 10-fold dilutions were spotted on YNB-Glucose solid medium supplemented with Congo Red (100 µg/mL) or Calcofluor White (50 µM) to induce cell wall stress, and on YNBG solid medium supplemented with tunicamycin (TM) (1 µg/mL) or dithiothreitol (DTT) (1 mM) to induce ER stress. Cells were spotted on charcoal-containing potato dextrose solid medium to induce filamentous growth. Pictures were taken after 48 h of incubation at 28 °C.

**Table S1** Strains used in this study
**Table S2** Primers used in this study
**Table S3** Plasmid used in this study

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