Robust Cre recombinase activity in the biotrophic smut fungus *Ustilago maydis* enables efficient conditional null mutants *in planta*

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**Abstract**

Site-specific recombinases have been used in higher eukaryotes, especially in animals, for a broad range of applications, including chromosomal translocations, large deletions, site-specific integration, and tissue-specific as well as conditional knock-outs. The application of site-specific recombination has also been demonstrated in simple eukaryotes like fungi and protozoa. However, its use in fungal research, especially in phytopathogenic fungi, has often been limited to “recycle” the marker genes used in transformation experiments. We show that Cre recombinase can be used for conditional gene deletions in the phytopathogenic fungus *Ustilago maydis*. Conditional gene knock-outs can be generated via the transcriptional control of the recombinase by *U. maydis* promoters specifically activated during the biotrophic phase of fungal growth, enabling gene deletions at defined developmental stages inside the plant tissue. Also, we show that a tamoxifen-activated Cre-recombinase allows the tight control necessary for the induced deletion of essential genes by the addition of tamoxifen. These tools will be helpful to address the function of genes under both axenic and *in planta* conditions for the *U. maydis*-maize pathosystem and should pave the way for similar approaches in other plant pathosystems.

**Keywords:** Cre recombinase; *Ustilago maydis*; fungal development

**Introduction**

Plant fungal diseases have led to high social and economic costs in food systems (Avery et al. 2019). Besides, fungal infections are rising due to climate change-associated global warming (Cavicchioli et al. 2019). Thus, new approaches in antifungal treatment are required (Kettles and Luna 2019), and it is desirable to identify novel biochemical and molecular targets in phytopathogenic fungi (Steinberg and Gurr 2020). An inherent caveat for identifying antifungal targets is that different approaches are likely to be necessary for different fungi due to the great diversity of phytopathogenic fungi concerning lifestyle and symptoms (Riquelme et al. 2018). However, despite such diversity, infection frequently involves differentiation processes that require the induction of morphogenetic programs and often the control of the cell cycle (Gow et al. 2002, Rooney and Klein 2002). How growth, morphogenesis, and cell cycle progression are coordinately regulated during development is an active research area in fungi (Perez-Martin and Di Pietro 2012). Several phytopathogenic fungal systems have been used to define the role of the cell cycle and morphogenetic regulators as virulence factors. The corn smut fungus *Ustilago maydis* represents an excellent model (Perez-Martin et al. 2006, Steinberg and Perez-Martin 2008). The virulence program of *U. maydis* is initiated by combining two compatible haploid cells to produce an infectious dikaryotic hypha. This process involves substantial morphological changes (budding of the haploid cells to hypha transition) as well as genetic changes (haploid to dikaryotic transition), advocating for accurate control of the cell cycle and morphogenesis during these transitions (Perez-Martin 2012).

A critical bottleneck when studying genes involved in the control of morphogenesis and cell cycle in pathogenic fungi is that the function of these genes is frequently crucial for the life cycle. Accordingly, the classical analysis of loss-of-function (LOF) mutants (typically obtained by deletion or inactivation of the gene) to describe the function of such genes is not possible. Often, the genes in question are essential and cannot be deleted at all, or, in the best of situations, gene deletions result in severe phenotypes causing accumulation of suppressor mutations.
Alternatively, one single gene may have distinct roles in successive steps during the infection process. However, as the deletion of the respective gene halts the infection process at the first step where the gene is required, studying this factor’s function in later stages is impossible.

One way to bypass these problems is to use conditional alleles, which allows the study of the effects of LOF mutants under re-strictive conditions. Of these, the use of temperature-sensitive alleles is a standard solution, but their construction, when possible, is usually laborious and time-consuming (Ben-Aroya et al. 2010). A more common and easy way to obtain conditional alleles in a gene of interest is to exchange their native promoters with regulatable promoters. This way, the expression of the gene of interest can be controlled (downregulated) through the experimental conditions.

In U. maydis, for many years, researchers took advantage of two regulatable promoters, namely Pnar1 and Pcrg1, which are controlled by nitrogen (induced in the presence of nitrate, repressed in the presence of ammonium and amino acids) and carbon (induced in the presence of arabinose, repressed in the presence of glucose) sources, respectively (Brachmann et al. 2001). These promoter systems have been used successfully to downregulation essential genes (Garcia-Muse et al. 2004; Mahlert et al. 2006; Castillo-Lluva et al. 2007). However, although helpful, there are several caveats to transcriptionally regulated conditional alleles: (1) the basal activity in the “off-state” may not be as tight as required, most likely because of effects from the chromatin environment where the regulatable unit is inserted; (2) the expression level under induced conditions may not relate to the native expression levels and might cause expression artifacts, and (3) as the activity of these promoters is dependent on nutritional cues that interfere with cellular metabolism, the study of regulatory factors linked to nutritional conditions is hampered. The last caveat is significantly limiting if the gene under study has to be downregulated during the pathogenic stage because when the fungus is growing inside the host plant, it is not possible to control the expression by changing the nutritional conditions. Alternatives such as the tetracycline-regulated TetR system allow the heterologous control of gene expression by adding effector molecules (doxycycline or tetracycline) without interference with the nutritional conditions (Berens and Hillen 2004). Although this system works for controlled expression in U. maydis, the level of repression is not tight enough to allow complete LOF conditional alleles (Zarnack et al. 2006).

Widely used alternatives to conditional expression are gene silencing approaches using RNA interference (RNAi) (Agrawal et al. 2003) or the use of drug-responsive degrons (Yesbolatova et al. 2019). However, in the case of U. maydis, RNA interference is not a choice of use because of the absence of crucial pathway components (Laurie et al. 2012). In the same way, although no degron system has been so far put to work in U. maydis, the widely used Auxin-Induced Degron (AID) system (Nishimura et al. 2009) cannot be adopted to U. maydis because of the intrinsic toxic activity of auxin-derived molecules in this fungus (Prusty et al. 2004).

Site-specific recombination (SSR) using phage- or yeast-derived recombinases has been a method of choice in many model systems for gene modification or deletion (Gorman and Bullock 2000). Two recombinases, Cre and FLP, have been most widely used. Both recognize short, 34 bp sequences, called lox and FRT, respectively, and mediate either excision or inversion of intervening sequences, depending on the relative orientation of the recognition motifs. Flanking the gene of interest with recombinase recognition sites in a direct orientation induces the deletion of the modified gene in the presence of the corresponding recombinase (Branda and Dymecki 2004). Regulated control of the activity of recombinases enables the efficient conditional deletion of the genes of interest. For that, ligand-regulated versions of Cre and FLP selectively activated by synthetic drugs provide valuable tools to control recombination timing. One widely used approach to achieve this is via the fusion of the respective recombinase to the ligand-binding domain (LBD) of a steroid hormone receptor (Picard 1994). These chimeric site-specific recombinases have been instrumental for controlled gene deletion in many experimental systems (Wirth et al. 2007). Also, the controlled expression of the recombinase genes by using tissue- or effector-specific promoters enables the gene deletion only in specific tissues or conditions (Orban et al. 1992).

In fungi, the use of SSR activity for selectable marker-removal systems has been reported in different organisms, such as Aspergillus nidulans (Forment et al. 2006), Candida albicans (Morschhauser et al. 1999), and Cryptococcus neoformans (Patel et al. 2010). In U. maydis, the FLP recombinase has been successfully established as a system for removing selectable marker genes (Khrunyk et al. 2010), with the excision ratio ranging from 30% to 60% of the cell population. However, this ratio is not sufficient to construct conditional alleles based on induced gene deletion, which requires excision ratios close to 100% of the population.

Here, we generated a codon-optimized version of Cre recombinase for U. maydis, capable of excising lox-flanked sequences from a genomic locus with an efficiency close to 100%. Using a chimeric Cre recombinase fused to a tamoxifen-responsive LBD, we show that Cre activity can be controlled in U. maydis by adding the drug to the culture medium, allowing a precise and timely controlled deletion of the gene of interest. As proof of principle, we have constructed a U. maydis strain with a conditional tor1-gene deletion. The Tor1 kinase is a conserved essential protein involved in transmitting nutritional conditions to cell machinery (Tatebe and Shiozaki 2017). We also show that the Cre activity can be controlled during the biotrophic stages of U. maydis by placing the recombinase gene under the control of a promoter that is only active when the fungus grows within the plant. As proof of principle, we describe the deletion of rbf1 in U. maydis cells after infection of the plant host. Rbf1 is a transcription factor essential for the very early stages of plant infection by U. maydis (Heimel et al. 2010). Strains deleted for rbf1 are unable to penetrate the plant cuticle; thus, it has not been possible to address the function of Rbf1 for subsequent stages of fungal development in planta.

**Material and methods**

**Strains and growth conditions**

**Ustilago maydis** strains are derived from FB1, FB2, and SG200 genetic backgrounds (Banuett and Herskowitz 1989; Kämper et al. 2006) and are listed in Supplementary Table S1. Cells were grown in rich medium (YPD), complete medium (CMD or CMA), or minimal medium (MMD) (Holliday 1974). Controlled expression of genes under the crg1 and nar1 promoters was performed as described previously (Brachmann et al. 2001).

**Ustilago maydis strain generation, and plasmid construction**

Transformation of U. maydis protoplasts with the desired constructs was performed as described previously (Tsukuda et al. 1988). The integration of a construct into the corresponding genomic region was verified in each case by diagnostic PCR and
subsequent Southern blot analysis. Ustilago maydis DNA isolation was performed as previously described (Tsukuda et al. 1988).

Plasmid pGEM-T easy (Promega), PCR-TOPO-TA (Thermo Fischer), and pET1.2 (Thermo Fisher) were used to subclone and fragmenting sequences generated by PCR. Details for plasmid constructions are described in Supplementary Materials and Methods.

RNA analysis

For qRT-PCR, total RNA was extracted with acidic phenol solution. After extraction, the RNA was cleaned using the High Pure RNA Isolation Kit (Roche Diagnostics GmbH). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), employing 1 μg total RNA per sample. PCR amplification was performed using the SsoAdvanced Universal SYBR Green Supermix (BioRad) in a CFX96 Real-Time PCR system (BioRad). Reaction conditions were as follows: 3 min 95°C followed by 40 cycles of 10 s 95°C/10 s 60°C/30 s 72°C.

Quantification of recombination events by quantitative PCR (qPCR)

For axenic cultures, U. maydis DNA isolation was performed as previously described (Tsukuda et al. 1988) from cell cultures at the indicated times. For qPCR, 1 μg of genomic DNA was used per reaction using specific primers and the SsoAdvanced Universal SYBR Green Supermix (BioRad) in a CFX96 Real-Time PCR system (BioRad). Reaction conditions were as follows: 3 min 95°C followed by 40 cycles of 10 s 95°C/10 s 60°C/30 s 72°C.

Plant infections, fuz+ assays

Pathogenic development of wild-type and mutant strains was assayed by plant infections of the maize (Zea mays) variety Early Golden Bantam as described before (Kamper et al. 2006). To determine the fuz+ phenotype, cell cultures from strains to be assayed were spread on charcoal-containing PD (Potato Dextrose) plates (Holliday 1974) and incubated at 22°C (Banuett and Herskowitz 1989).

Microscopy

Images were obtained using a Nikon Eclipse 90i fluorescence microscope with a Hamamatsu Orca-ER camera driven by Metamorph (Universal Imaging, Downingtown, PA, USA). Images were further processed with ImageJ software.

Quantification and statistical analysis

To determine the statistical significance of differences, a two-tailed Student t-test was used; for statistical analysis of plant infection assays, the Mann-Whitney U-test was used.

Results

Establishment of a Cre-mediated SSR system in Ustilago maydis

We first sought to analyze the capability of the Cre recombinase to excise a chromosomal region flanked by lox sites in direct orientation in U. maydis. To this end, we constructed an autonomously replicating plasmid, pPnar1:cre (Supplementary Figure S1A), carrying a carboxin-resistance marker and the coding sequence for Cre recombinase. To avoid the premature polyadenylation that frequently occurs in heterologously expressed genes in U. maydis (Zarnack et al. 2006), we adapted the coding sequence of the Cre-recombinase according to the optimal di-codon usage of U. maydis (Supplementary Figure S1B). The optimized cre gene was expressed under the control of the nitrate-inducible nar1 promoter (Brachmann et al. 2001). In this way, the Cre recombinase expression can be controlled by different growth media (repressed: YPD; induced: minimal medium with nitrate) to measure SSR efficiency in U. maydis cells (Supplementary Figure S1C).

To measure the Cre-mediated SSR in U. maydis, we constructed a reporter-cassette (GFPlox) consisting of the GFP gene and, as a selectable marker, the hygromycin phosphotransferase (HygR) gene, flanked by lox sites in direct orientation. The cassette was used to replace the b-locus in wild-type F81 cells (mating type a1/b1) by homologous recombination (Figure 1A). The two homeodomain proteins encoded by the b-locus (b6 and bW) are the critical regulators for initiating the pathogenic stage but are not required during the saprophytic stage in axenic culture (Kahmann et al. 1995). The resulting reporter strain cells (Ab1::GFPlox) showed hygromycin resistance and pan-cellular GFP expression (Figure 1, B and C). In the presence of a functional Cre recombinase, SSR between lox sites should excise the entire cassette. Thus, analysis of the percentage of reporter cells losing GFP fluorescence and resistance to hygromycin should give a measurement for the efficiency of the Cre-mediated excision reaction at the cellular level.

Subsequently, the Ab1::GFPlox strain was transformed either with plasmid pPnar1: cre or, as a control, a plasmid lacking the Cre-recombinase (pCM54). To prevent cre expression during strain construction, the transformed protoplasts were grown on a regeneration-rich medium (YPD amended with sorbitol), a condition that represses the transcription of the nar1 promoter. Independent colonies from transformations were re-isolated in YPD medium (to maintain the repression of the cre gene) supplemented with carboxin (for plasmid selection). Fifty independent transformants were tested, always under Pnar1 repression conditions generated Cre-protein levels sufficient to excise the entire cassette. A g of genomic DNA was used per PCR amplification followed by 40 cycles of 10 s 95°C/20 s 60°C/40 s 72°C.

For qPCR assays of plant-infected fungal genomic DNA, infected plant tissue (100–300 mg) was harvested seven days post-inoculation, shock frozen with liquid nitrogen, and ground to a fine powder using a mortar. Genomic DNA was isolated from ground powder as described previously (Hoffman and Winston 1987). qPCR was performed using MESA GREEN qPCR MasterMix (Eurogentec) in a CFX96 Real-Time PCR system (BioRad). Cycling conditions were as follows: 7 min 95°C followed by 40 cycles of 10 s 95°C/10 s 60°C/30 s 72°C.

To determine the statistical significance of differences, a two-tailed Student t-test was used; for statistical analysis of plant infection assays, the Mann-Whitney U-test was used.
cells harboring pPnar1::cre grown under noninducing conditions, while more than 90% of the cells harboring pCM54 showed a GFP signal (Figure 1C). PCR analysis on DNA isolated from two independent transformants showing GFP fluorescence and hygromycin sensitivity revealed a 459 bp fragment indicative of the GFPlox cassette excision (Figure 1D). Sequencing of the PCR product revealed the DNA-sequence expected for the footprint left by the correct Cre-mediated excision of the cassette (Figure 1D).

These results strongly suggested that Cre recombination is functional in U. maydis and that even the low expression from the noninduced nar1 promoter is sufficient to excise the reporter cassette in the majority of the cell population. To further support this conclusion, we used qPCR to quantify the level of Cre-mediated recombination in transformants grown under repressive conditions; as expected, the levels of SSR were close to 100% (Supplementary Figure S3).

**Figure 1** Cre-mediated recombination is highly efficient in U. maydis. (A) Scheme of the reporter cassette for in vivo Cre activity in U. maydis. The reporter cassette (GFPlox) harboring the hygromycin marker gene and a constitutively expressed eGFP gene were integrated to replace the b-locus via homologous recombination. Upon Cre-mediated site-specific recombination, GFP fluorescence and the Hygromycin resistance will be lost. (B) Serial 10-fold dilutions of cultures from the strains indicated, spotted on YPD medium plates with or without hygromycin. Plates were incubated for 3 days at 28 °C. (C) Microscopic images of strains incubated for 8 h in liquid YPD medium. Merged images of DIC and GFP channels are shown. Bar: 15 µm. (D) Analytic PCR to identify recombination events. The upper panel shows a schematic of the b-locus after excision of the lox-cassette, the primers’ position, and the resulting PCR product. The middle panel shows a representative agarose gel with the result of PCR amplification from DNA extracted from the following strain cultures: 1 control; 2 Δb1::GFPlox; 3 Δb1::GFPlox/pCbxR #1; 4 Δb1::GFPlox/pCbxR #2; 5 Δb1::GFPlox/pPnar1:cre #1; 6 Δb1::GFPlox/pPnar1:cre #2. Bottom panel: partial sequence of the PCR product shown in lane 5, encompassing the recombination site. Sequences corresponding to the recombined loxP site and the Sfi sites used for the cassette construction are indicated.

**Design of a tamoxifen-responsive SSR system in Ustilago maydis**

The high efficiency shown by the Cre recombinase in U. maydis should facilitate a conditional gene deletion system for this fungus. However, first, it would be required to reduce the basal activity of the expressed Cre-recombinase under noninducing conditions. For that, we took advantage of the well-described mammalian fusion of the Cre recombinase with the LBD of the tamoxifen-responsive mouse estrogen receptor (Cre-ER7) (Feil et al. 1996).

A U. maydis di-codon optimized gene of the Cre-ER7 recombinase was synthesized and cloned under the control of the crg1 promoter, repressed when cells are grown in glucose, and induced in arabinose-containing media (Brachmann et al. 2001). The construct with the Cre-ER7 was integrated as a single copy into the ip locus (encoding a protein subunit of succinate dehydrogenase), a site frequently used to insert ectopic copies of genes in U. maydis (Broomfield and Hargreaves 1992; Loubradou et al. 2001) (Figure 2A). Analysis of mRNA levels by qRT-PCR revealed that in a strain harboring the Cre-ER7 construct, only background levels of cre-ER7 expression were detectable when cells were grown in glucose, while an approximately 200-fold induction was observed in arabinose-containing medium (Figure 2B). We did not observe any detrimental effects in growth for cells expressing Cre-ER7.
For targeted, conditional deletion of a particular gene or chromosomal region, we constructed two different cassettes integrated via homologous recombination at both sides of the gene or chromosomal region of interest. The 5'-cassette is composed of the promoter of the \textit{cdk1} gene \cite{encoding the essential mitotic cyclin-dependent kinase of \textit{U. maydis}, which is active in a broad range of axenic conditions \cite{Garcia-Muse et al. 2004}], cloned upstream of a \textit{lox} site, followed by a G418/Geneticin-resistance marker. The 3'-cassette consists of a \textit{lox} site upstream of a promoter-less NLS-GFP-encoding open reading frame and a Nourseothricine-resistance marker. Once integrated at their respective sites (5' upstream and 3' downstream regions of the gene of interest), the intervening region's excision in the presence of Cre recombinase activity will result in the fusion of the \textit{cdk1} promoter to the NLS-GFP coding sequence. Hence, cells in which the DNA region of interest is deleted can be detected by the presence of a nuclear green fluorescence signal (Figure 2C).

Controlled excision of \textit{rbf1} using a chimeric \textit{cre} recombinase responsive to tamoxifen

As a proof of principle for our design, we have chosen the \textit{rbf1} gene, encoding a transcription factor required for filamentous growth and pathogenic development \cite{Heimel et al. 2010}. Filamentous growth and onset of the pathogenic stage in \textit{U. maydis} are initiated by the fusion of two cells with compatible a- and b-mating types. The cell fusion requirement for filamentous growth and pathogenicity can be bypassed in haploid strains that harbor a compatible combination of the \textit{a}-locus derived pheromone and receptor genes and a compatible combination of the two transcription factors bE and bW that are encoded by the \textit{b}-locus. \textit{SG200 (pra1 mfa2 bE1/bW2)} is one of these so-called solopathogenic strains \cite{Bo¨ lker et al. 1995; Kämper et al. 2006}. The formation of hyphae in \textit{SG200} can be easily scored by plating cells on charcoal-containing PD plates \cite{Banuett and Herskowitz 1989}, where \textit{SG200} wild-type cells form white, fuzzy colonies (\textit{fuz}+\ phenotype). \textit{SG200} cells deleted for \textit{rbf1}, however, are unable to form filaments and produce pale creamy colonies (\textit{fuz}− phenotype).

To score the Cre-lox mediated deletion of \textit{rbf1} in \textit{SG200}, we first integrated the respective 5’ and 3’ lox sites-containing cassettes flanking the \textit{rbf1} locus to generate the \textit{rbf1}\textsuperscript{lox} allele (Figure 3A). The 5’ region of \textit{rbf1} is unusually large, encompassing about 4.4 kb of the intergenic region to the neighboring gene, \textit{UMAG_10390}, which suggests a complex regulation. To avoid the disruption of this regulation, we inserted the 5’ lox-cassette 70 bp
downstream of the transcriptional stop of UMGAG_10390; by that, most of the 5' region from rbf1 remains unaltered. The 3' cassette was inserted 65 bp downstream of the stop codon of rbf1. SG200 cells carrying the lox-flanked version of rbf1 (SG200_rbf1lox) showed no difference compared to the SG200 wild-type strain concerning growth and ability to produce the fuz+ phenotype (Supplementary Figure S4, A and B).

To induce the recombination, we integrated the Cre-ER T recombinase driven by the arabinose inducible crg1 promoter into the ip-locus of SG200_rbf1lox. The resulting strain (SG200_rbf1loxPcrg1: creERT) was checked for their ability to excise the rbf1lox gene after growth for 8 h under four different conditions: (i) glucose without ligand (CMD, repression of crg1 promoter); (ii) glucose plus 1 mM tamoxifen (CMD + TMX, repression of crg1 promoter but activation of Cre recombinase); (iii) arabinose without ligand (CMA, activation of crg1 promoter); (iv) arabinose plus 1 mM tamoxifen (CMA + TMX, activation of crg1 promoter and activation of Cre recombinase). Tamoxifen has been described as toxic in fungi at the millimolar range (Dolan et al. 2009); however, the 1 mM concentration used in our experiments did not influence the growth rate of U. maydis (Supplementary Figure S5).

We assessed the SSR of rbf1lox by PCR analysis on genomic DNA extracted from cells grown in liquid culture, using a pair of primers flanking the lox sites (Figure 3A). The primers were located at a distance of 8.9 kbp before recombination, and Cre-ER T-mediated recombination should reduce the distance to 0.35 kbp. In the presence of glucose and absence of ligand for the Cre-ER T recombinase, no PCR product reminiscent for the recombination event was obtained, indicating that the rbf1lox allele was stable. However, under conditions that lead to the expression of the Cre-ER T recombinase (growth in arabinose), activation of the Cre-ER T recombinase (addition of tamoxifen), or both, the 0.35 kb DNA fragment could be detected (Figure 3B). Sequencing of the PCR fragment showed the expected DNA footprint left by the Cre-mediated excision of the gene, which couples the Pcdk1 promoter to the NLS-GFP gene (Figure 3C). Moreover, plating the cells on charcoal-containing PD plates and observing the presence of colonies with fuz+ phenotype supported the PCR results: while growth in glucose without tamoxifen resulted in a cell population producing mostly fuz+ colonies, the other three conditions led to the appearance mostly of fuz- colonies (Figure 3D).

In order to quantify the efficiency of Cre-mediated recombination, we addressed the frequency of cells with Cre-induced rbf1lox excision under different conditions (activation with tamoxifen, induction of cre expression, and both) during a 6-h time-course experiment. To this end, we analyzed in parallel the emergence of cells with nuclear GFP signals (Figure 4, A and B), the ratio of fuzzy vs pale colonies on PD-charcoal plates (Figure 4, C and D), and the fraction of recombined versus nonrecombined rbf1 alleles in the population using qPCR analysis (Figure 5).
tamoxifen to cultures growing in glucose resulted in gene deletions in about 75% of the cells after two hours. Strikingly, 6 h after tamoxifen addition, \textit{rbf1lox} was found to be excised in all cells observed. Growing cells in the presence of arabinose alone (Cre overexpression) resulted in the \textit{rbf1lox} excision in about half of the population during the time analyzed. The addition of tamoxifen to cells growing in arabinose improved the excision process at shorter induction times. However, after 6 h in the presence of tamoxifen, no differences were noted between cells grown either in glucose or arabinose.

These results indicate that the weak expression of \textit{cre-ERT} due to the basal level of the \textit{crg1} promoter in the glucose-containing medium resulted in efficient and tamoxifen-dependent gene excision in \textit{U. maydis}. In addition, we observed that under conditions where \textit{cre-ERT} is expressed at high levels, the fusion protein is active without the addition of tamoxifen. In mammalian cells, \textit{Cre-ERT} without its ligand tamoxifen is retained in the cytoplasm by interaction with cytoplasmic chaperones (Picard 1994). It seems that this retention mechanism is functional in \textit{U. maydis} but that it can be titrated out by high levels of \textit{Cre-ERT} recombinase (as it occurs upon induced expression by growing cells in arabinose). This observation implies that for efficient control by tamoxifen of the SSR process in \textit{U. maydis}, the \textit{cre-ERT} expression level should be kept at low levels.

In summary, our results illustrate that the regulation of \textit{Cre-ERT} on both the transcriptional level and by the addition of tamoxifen to cultures can be used to generate conditional gene deletions in \textit{U. maydis}.

Addressing the feasibility of Cre-mediated conditional strains: the essential Tor1 kinase

We focused on the TOR kinase to test Cre-mediated deletion as a helpful method to generate conditional alleles for essential genes in \textit{U. maydis}. This protein is the central component of the wide-conserved TORC1 complex, which plays primordial roles during cell growth, activating anabolic pathways and repressing catabolic pathways (De Virgilio and Loewith 2006). We have chosen this kinase because it has been described to be essential in all studied organisms. Since the activity of the TORC1 complex is strongly modulated by nutritional conditions, \textit{tor1} represents the typical case in which the use of promoters that are responsive to nutritional conditions (as the \textit{nar1} and \textit{crg1} promoters used in \textit{U. maydis}) to construct a conditional allele is not advisable. Moreover, this protein has been proposed as a target for antimicrobial drugs (Shertz and Cardenas 2011). The gene encoding TOR kinase has undergone a duplication event in several fungal species (Shertz et al. 2010), but in the case of \textit{U. maydis}, there is a single gene encoding this kinase (UMAG_03216, from here \textit{tor1}), which so far has been uncharacterized (van Dam et al. 2011).
We constructed a lox-flanked allele of \( \text{tor1}^{\text{lox}} \), integrating the 5' and 3' cassettes described above into the \( \text{tor1} \) locus via homologous recombination (FB1 background). The intergenic region between the coding sequence of \( \text{tor1} \) and the preceding gene (UMAG_10643) is about 0.9 kbp. We inserted the 5' cassette at 816 bp upstream of the translation start site of \( \text{tor1} \), 86 bp downstream of the transcriptional end of UMAG_10643. The 3' cassette was inserted 80 bp downstream of the stop codon of \( \text{tor1} \) (Figure 6A). We also inserted the \( \text{cre-ERT} \) gene under the control of the \( \text{crg1} \) promoter in a single copy into the \( \text{ip} \)-locus of the \( \text{tor1}^{\text{lox}} \) strain.

As shown above, for the deletion of the \( \text{rbf1} \) gene, it appears that the basal expression of the \( \text{crg1} \) promoter in the YPD medium was already sufficient to drive levels of Cre-ERT sufficient to promote efficient SSR events (assessed by qPCR) at high frequency when tamoxifen was added in the medium (Supplementary Figure S6). Therefore, we analyzed the growth on solid YPD medium under restrictive or permissive conditions for
gene excision (absence or tamoxifen presence, respectively). We found a drastic effect on colony formation when cells were grown under conditions to induce the \textit{tor1}lox excision (Figure 6B). When the \textit{tor1}lox \textit{Cre-ERT} strain was grown in liquid culture for 6 h in the presence of tamoxifen (inducing the Cre-mediated recombination), we observed the presence of cells showing nuclear GFP fluorescence in more than 95% of the population (Figure 6C).

Interestingly, cells with \textit{tor1} deletions (positive for GFP nuclear fluorescence) were swollen in the central region (Figure 6D), most likely as a consequence of vacuole enlargement, which was described as a typical response to TORC1 inhibition in other fungi (Michaillat et al. 2012). In agreement with these observations, we have found a dramatic decrease of \textit{tor1} mRNA levels in the \textit{tor1}lox \textit{P\textsubscript{org1}cre-ERT} cells grown under conditions that promote Cre recombinase activation (Figure 6E). We also sought to correlate the observed effects on growth and cell morphology with the Tor1 activity. For that, we used the transcriptional level of genes encoding ribosomal proteins as an indirect readout for Tor1 activity, since in all organisms studied so far, this process is controlled by Tor1 kinase (Gonzalez and Rallis 2017). Transcription of genes for ribosomal proteins, which is positively controlled by Tor1 kinase, was analyzed by qRT-PCR analysis on two different ribosomal genes, \textit{rpl43} and \textit{s21b}. In concordance, we observed that upon 6 h of incubation under conditions that induce the gene deletion, mRNA levels for \textit{rpl43} and \textit{s21b} decreased dramatically in the \textit{tor1}lox \textit{P\textsubscript{org1}cre-ERT} strain (Figure 6F).

These results support the applicability of the Cre-induced deletion system as an alternative and feasible method to generate conditional gene deletions in essential genes in \textit{U. maydis}.

**Figure 6** Conditional deletion of \textit{tor1}. (A) Scheme of the \textit{tor1}lox allele. (B) Serial tenfold dilutions of cultures from strains (FB1 background) carrying the indicated alleles, spotted on solid YPD medium supplemented or not with 1 \textmu M tamoxifen. Plates were incubated for 3 days at 28°C. (C) Fraction of cells showing nuclear GFP fluorescence of liquid cultures of \textit{tor1}lox \textit{P\textsubscript{org1}cre-ERT} cells incubated for 6 h in YPD medium supplemented or not with 1 \textmu M tamoxifen (TMX). (D) Microscopic images of liquid cultures of \textit{tor1}lox \textit{P\textsubscript{org1}cre-ERT} cells incubated for 6 h in YPD medium supplemented or not with 1 \textmu M tamoxifen. DIC and GFP channel images are shown. Bar: 20 \textmu m. (E) qRT-PCR of \textit{tor1} expression for the indicated strains. RNA was isolated after 6 h of growth in the indicated media. As an internal control, the expression of \textit{tub1} (encoding Tubulin) was used. Values refer to the expression of \textit{tor1} in the control strain (\textit{tor1}lox) grown in YPD. Each column represents the mean value of three independent biological replicates (**\textit{P} < 0.01, ns not significative). (F) qRT-PCR of the ribosomal genes \textit{rpl43} and \textit{s12B} expression in \textit{tor1}lox \textit{P\textsubscript{org1}cre-ERT} cells. RNA was isolated after 6 h of growth in the media indicated. As an internal control, the expression of \textit{tub1} (encoding Tubulin) was used. Values are referred to the expression of each ribosomal gene expressed in the control (YPD) where Cre is not activated. Each column represents the mean value of three independent biological replicates (**\textit{P} < 0.01).
One paradigmatic case is the above-mentioned Rbf1 protein. This transcriptional regulator is required to initiate pathogenic development, and deletion of rf1 results in avirulent cells because they cannot invade the plant tissue (Heimel et al. 2010). However, because of its crucial role in the initial stage of a plant infection, it is unknown whether Rbf1 is required at later stages after plant penetration when the fungus is proliferating within the plant tissue. Hence, Cre recombinase controlled by promoters only active at specific steps during the infection process (i.e., after plant penetration) would help address this question. Therefore, we set up a system to use the conditional recombinase system to delete the rf1 gene after plant penetration.

Since we noticed that the cdk1 promoter (which will drive the NLS-GFP expression once the Cre-mediated recombination occurs) is only weakly expressed in planta, we exchanged in the 5’ lox cassette the cdk1 promoter with the hxt1 promoter (hexose transporter 1), which allows high and constitutive expression levels throughout the plant infection process (Schuler et al. 2015). The hxt1 promoter should ensure the strong expression of the GFP reporter once the recombination takes place and should allow the microscopic detection of recombination events in fungal hyphae growing inside the plant tissue. We then integrated the rf1lox-2 allele, carrying the modified promoter, in SG200 as described above for rf1lox (Supplementary Figure S7A). Plant infection experiments with 7-day old maize plants revealed no significant difference in virulence between SG200 and SG200rf1lox-2 (Supplementary Figure S7B), indicating that the allele rf1lox-2 does not interfere infection cycle.

As we mentioned above, Rbf1 is required to initiate pathogenic development, and deletion of rf1 results in avirulent strains that do not form appressoria and cannot invade the plant tissue (Heimel et al. 2010). Thus, we had to ensure that the Cre-recombinase is only expressed at stages post penetration. Otherwise, rf1 would be deleted prematurely, resulting in cells that are unable to penetrate the plant. To provide recombinase activity at the precise time during infection, we employed the mig2-1 promoter, induced early during pathogenic development once the infective hyphae penetrated the plant surface. The mig2-1 gene is not required for plant infection or during the biotrophic stage (Basse et al. 2002). Thus, we exchanged the native mig2-1 open reading frame with the sequence encoding Cre (without the tamoxifen-dependent regulatory domain) via homologous recombination. Subsequently, the hygromycin resistance cassette used for selection was removed via an FLP/FRT-based recombination system to ensure the proper functioning of the mig2-1 promoter as described previously (Schmitz et al. 2020) (Supplementary Figure S8).

We next combined the rf1lox-2 allele and Pmig2-1: cre fusion to generate the strain SG200rf1lox-2 Pmig2-1: cre. When grown in axenic culture without antibiotic selective pressure, we have observed a minor fraction of cells (around 4% of the population, Supplementary Figure S9) with a nuclear-localized GFP signal. Apparently, the basal activity of the mig2-1 promoter under axenic conditions is sufficient to induce the recombination at a low rate. To ensure that plants were inoculated with cells harboring the nonrecombined rf1lox-2 allele, cells were grown in a medium with G418/Geneticin, the G418-resistance cassette was used to integrate the 5’lox cassette and will be lost after Cre-mediated recombination of the rf1 gene (see Figure 2C). Thus, the antibiotic’s selective pressure ensures the maintenance of the rf1 cassette until the strains are used for plant infection.

The SG200rf1lox-2 Pmig2-1: cre strain was then used to inoculate plants. In two consecutive experiments with two independent clones, virulence was either unchanged (Figure 7A, experiment II) or slightly attenuated (Figure 7A, experiment I) compared to infections with control strain SG200rf1lox-2. The observed reduction in virulence is most probably accounted for by the small percentage of cells that lost the rf1 gene, which is required for the

![Figure 7](image-url)
infection, already before plant infection: one day post-infection, a minor fraction of cells with a nuclear GFP signal (indicative for the recombination event) could be observed on the plant surface (Supplementary Figure S10).

Most importantly, 5 days post infection, we observed hyphae growing inside the plant, with a well-defined nuclear GFP signal (Figure 7B) indicating the deletion of the rbf1 gene within the plant tissue. We also observed, less frequently, hyphae that did not show a nuclear-localized GFP signal, indicating that the rbf1 gene was still retained in a fraction of the cells. To quantify the frequency of Cre-mediated excision of the rbf1 gene in planta, total DNA from infected plants was analyzed by qPCR with two sets of primers that distinguish the recombined and the nonrecombined rbf1 allele (Supplementary Figure S11). The results indicated that most of the hyphae growing in planta had lost the rbf1 gene (Figure 7C), only a minor fraction (around 5%) of the cells still retained the rbf1 gene. Sequence analysis of a PCR-product spanning the recombination site revealed the expected fusion of the hxt1 promoter to the GFP gene (Supplementary Figure S11).

The hyphae that have lost the rbf1 gene were indistinguishable from cells lacking the nuclear GFP signal (that have retained rbf1). Both classes showed the typical symptoms of proliferating biotrophic hyphae, such as the formation of clamp cells. Thus, although Rbf1 appears to be essential for the plant penetration itself, it appears that the gene is not required for the proliferation and growth of hyphae within the plant tissue after plant penetration.

In conclusion, our results support the notion that the planta-induced expression of Cre is sufficient to generate conditional gene deletions during the biotrophic stage in U. maydis.

Discussion

In higher eukaryotic systems, the combination of the Cre-lox system with the regulative properties of steroid receptors has been successfully exploited to obtain conditional knock-out by simple administration of hormone (Logie and Stewart 1995). Previously, it has been shown that the hormone-binding domain of the human estrogen receptor can function as an autonomously regulatory domain in Saccharomyces cerevisiae (Louvion et al. 1993). Here, we show that tamoxifen-induced regulation is also functional in U. maydis. We have used the tamoxifen-binding domain to control Cre activity (Cre-ER(T) (Feil et al. 1996) and successfully induced conditional gene deletions in U. maydis. Using this system, we demonstrated the conditional knock-out of two genes in U. maydis cells grown in axenic culture: rbf1, encoding a transcription factor required for the onset of pathogenic development, and tor1. As tor1 encodes the essential Tor1 kinase, the tight control of the recombination system is essential, as premature recombination events would lead to inviable cells.

We have found that the expression level of Cre-ERT was crucial for the feasibility of the system. High expression of Cre-ERT (use of arabinose-inducible crg1-promoter under inducing conditions) was shown to release the tamoxifen-dependent regulation of Cre-ERT. This is most likely due to the saturation of the cytoplasmic chaperones interacting with Cre-ERT when no tamoxifen is bound to the hormone-binding domain fused to Cre (Picard 1994). However, the basal expression level of the crg1-promoter under noninducing conditions was sufficient to drive the Cre-mediated recombination, allowing the tight and controllable induction of the recombination reaction in a tamoxifen-dependent manner. The transfer of this system to other fungi should consider the use of constitutive promoters able to produce a low level of the Cre recombinase under their control (or alternatively, regulatable promoters in restrictive conditions, as we reported here for U. maydis).

The direct response of the Cre-recombinase system to the addition of tamoxifen is of advantage to the use of regulatable promoter systems to downregulate a gene of interest. Nutrient-dependent conditional promoters (like Pmig2 or Pmig1) usually require washing steps to change the medium to the “repressing” conditions. Often, these washing steps affect the physiological responses understudy, particularly in cell cycle-controlled processes (Castillo-Lluva et al. 2004; Castillo-Lluva and Pérez-Martín 2005). Also, altering the nutritional conditions to control the regulatable promoter systems may directly interfere with the function of the gene to be downregulated. For instance, this is the case for the tor1 gene since the activity of the Tor1 kinase is directly controlled by nutritional signals. On the disadvantages side, it is fair to mention that the construction of conditional alleles flanked by lox sites requires additional genetic manipulation steps (at least three, one for each cassette to be inserted), compared to the construction of promoter-controlled conditional genes (which involves only a single step).

In this study, we have chosen the use of the LBD to control the activity of Cre. However, the high efficiency shown by the Cre recombinase in U. maydis opens the door for the application of alternative ways to regulate the activity of Cre in vivo, like the use of optogenetics in the form of a light-controlled Cre system (Hochein et al. 2018).

We have also shown the feasibility of the Cre-lox system for conditional gene knock-outs at specific developmental stages during the life cycle of U. maydis. To induce the expression of the Cre recombinase after plant penetration of the fungus, we took advantage of the recently established method to generate promoter-gene fusions via homologous recombination events into the native genomic context of a regulated promoter and the subsequent marker recycling using the FLP-FRT recombinase system (Schmitz et al. 2020). By this, the disturbance of the chromatin structure that can affect the function of a promoter is kept to a minimum. We used this system to link the mig2_1 promoter to the cre-recombinase gene. As mig2_1 has no essential function during pathogenic development and its expression is induced at the stage of plant penetration, it is an ideal candidate to mediate the stage-specific expression of transgenes.

We have used the in planta-induced Cre-lox system to address the putative role of Rbf1 when the fungus is growing in the plant tissue. The mig2_1 promoter-driven cre-expression within the plant was sufficient to induce Cre1-mediated deletion of the rbf1lox_2 gene, as shown by the presence of cells with GFP expression (indicative for the recombination event) growing inside the plant tissue. Especially in this context, the reconstitution of GFP expression after recombination is essential to visualize the deletion events on a cellular level and to distinguish cells retaining rbf1 from Δrbf1 cells. We also observed cells without GFP expression, suggesting that the Pmig2_1-mediated expression level may differ between individual cells, eventually dependent on environmental cues of the surrounding plant tissue. However, as indicated by qPCR, most of the cells have lost the rbf1 gene, emphasizing the feasibility of the system to address the function of genes specifically at a stage post-plant infection. A small fraction of cells with GFP signals were also present on the plant surface, indicating that the expression of mig2_1 is not tightly restricted to the stage after plant penetration. The Rbf1 regulator is essential to initiate pathogenic development; its gene expression is highly induced when the infectious hyphae are formed.
and it is maintained on the plant surface and the initial stage of infection. However, at later stages during pathogenic development, expression decreases to levels that are not detectable by RNA-Seq analysis (Lanver et al. 2018). In line with this expression profile, our results indicate that the function of Rbf1 might be indeed restricted to the transition phase between saprophytic growth and pathogenic development. After plant penetration, hyphae deleted for rbf1 can proliferate within the plant tissue, showing that the gene is not required to develop U. maydis hyphae in planta.

By the successful stage-specific deletion of a gene essential for plant infection, we were able to prove the suitability of the Cre-lox recombination system to induce conditional gene deletions at defined developmental stages in U. maydis. This tool will be handy to address the role of the fungal cell cycle regulators once the fungus is growing inside the plant. These studies were so far precluded by the primary role of cell cycle regulators during the early stages of the infective process (Perez-Martin 2012).

In summary, we expect that the system described in this work will be instrumental in addressing the function of fungal genes essential for pathogenic development in the context of tissue-specificity and development.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplementary material is available at GENETICS online.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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