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Genome editing in *Ustilago maydis* using the CRISPR–Cas system

Mariana Schuster, Gabriel Schweizer, Stefanie Reissmann, Regine Kahmann *

Max Planck Institute for Terrestrial Microbiology, Department of Organismic Interactions, Karl-von-Frisch-Strasse 10, 35043 Marburg, Germany

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ABSTRACT

This communication describes the establishment of the type II bacterial CRISPR–Cas9 system to efficiently disrupt target genes in the fungal maize pathogen *Ustilago maydis*. A single step transformation of a self-replicating plasmid constitutively expressing the *U. maydis* codon-optimized *cas9* gene and a suitable sgRNA under control of the *U. maydis* U6 snRNA promoter was sufficient to induce genome editing. On average 70% of the progeny of a single transformant were disrupted within the respective *b* gene. Without selection the self-replicating plasmid was lost rapidly allowing transient expression of the CRISPR–Cas9 system to minimize potential long-term negative effects of Cas9. This technology will be an important advance for the simultaneous disruption of functionally redundant genes and gene families to investigate their contribution to virulence of *U. maydis*.

1. Introduction

The basidiomycete fungus *Ustilago maydis* is the causative agent of corn smut disease. In the past two decades *U. maydis* has become one of the fungal model organisms for studying biotrophic plant-pathogen interactions. Biotrophic pathogens do not kill the plant but establish an intimate relationship in which the host needs to stay alive to provide the pathogen with nutrients. With 20.5 Mb and only about 6900 genes *U. maydis* has one of the smallest genomes of eukaryotic pathogens (Kämper et al., 2006). Pathogenic development is initiated when two haploid cells of different *a* and *b* mating type fuse and develop an infectious filamentous dikaryon. This process is controlled by the *a* locus encoded pheromone-receptor system and the products of the *b* locus encoding a divergently transcribed pair of homeodomain proteins designated bE and bW (Gillissen et al., 1992). bE and bW form an active homeodomain complex in non-allelic combinations triggering filamentation and pathogenic development (Kämper et al., 1995). The elucidation of this process has allowed the construction of so-called solopathogenic haploid strains, which can initiate the disease cycle without prior mating. In such strains the *b* locus is a hybrid locus of *bE* and *bW* genes from different alleles and in addition the *a1* locus is engineered to express the *a2* pheromone gene (Bolker et al., 1995b; Kämper et al., 2006). Such strains form filamentous colonies (Fuz^+ phenotype (Banuett and Herskowitz, 1989) on charcoal-containing solid media due to the presence of an active bE–bW complex and autocrine pheromone stimulation. The availability of such haploid solopathogenic strains has spurred reverse genetics approaches where the highly efficient homologous recombination system of *U. maydis* (Holloman et al., 2009) is used to delete individual genes (see examples in Kämper et al., 2006; Wahl et al., 2010; Schilling et al., 2014). The most widespread method for the generation of deletion mutants in *U. maydis* is a PCR-based system (Kämper, 2004). This method relies on the transformation of constructs where a marker gene is flanked by about one kb of homology regions on either side of the target gene. By homologous recombination the endogenous locus is then replaced by the marker gene. This technology represents a very effective way to produce deletion mutants in selected genomic regions or to modify genes. To generate random insertions in the *U. maydis* genome, restriction enzyme mediated integration (REMI) (Bolker et al., 1995a), transposon tagging (Ladendorf et al., 2003) and *Agrobacterium*-mediated transformation (Ji et al., 2010) methods have been established. However, REMI proved to result in many structural rearrangements (N. Rössel, K. Münch and R. Kahmann, Unpublished results) while Tc1 transposition and T-DNA insertion both showed a preference for non-coding regions (Ji et al., 2010; Ladendorf et al., 2003).

Presently, with genomic insights and the demonstration that about 300 novel, secreted proteins (the effectome) are the key players for virulence in *U. maydis* (Kämper et al., 2006; Schilling et al., 2014; Schirawski et al., 2010) there is an increasing need for techniques that address genetic redundancy as well as the function of genes that exist in gene families. For such studies *U. maydis* provides only a limited number of dominant selection markers (currently used markers include hygromycin, phleomycin,

* Corresponding author at: MPI für terrestrische Mikrobiologie, Abt. Organismische Interaktionen, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany.
E-mail address: kahmann@mpi-marburg.mpg.de (R. Kahmann).

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nourseothricin, carboxin and geneticin resistance, see http://www.mikrobiologie.hhu.de/ustilago-community.html#c51403 for references) for the generation of strains carrying multiple mutations at defined sites. RNA interference (RNAi) induced downregulation of gene families cannot be applied in *U. maydis* due to the lack of a functional RNAi system in this organism (Kämper et al., 2006). The establishment of the FLP-dependent recombination technique for marker recycling currently represents the only way to introduce multiple mutations into the *U. maydis* genome without running into the problem of using up all available selection markers. However, the technique to recycle the selection marker is cumbersome, requires the generation of PCR-based replacement constructs for each gene and requires multiple cycles of retransformation, followed by growth cycles to induce marker loss (Khrunyk et al., 2010).

The CRISPR–Cas9 system originally discovered in bacteria and archaea as a defense system against phage and plasmids (Barrangou et al., 2007) has been successfully modified for a wide array of genome editing applications in prokaryotes and eukaryotes (Cong et al., 2013; Doudna and Charpentier, 2014; Jiang et al., 2013; Jinek et al., 2012; Nissim et al., 2014; Sander and Joung, 2014). This modified two component system consists of Cas9 nuclease that is guided to a specific target by a single guide RNA (sgRNA) that introduces a double stranded break at the desired site in the genome. The break is then repaired by the non-homologous end joining (NHEJ) pathway often resulting in short deletions and substitutions that may lead to frameshifts and generation of premature stop codons. Alternatively, a DNA repair template (donor DNA) is simultaneously provided with the sgRNA, to activate the homology directed pathway (HDR) leading to the desired alterations in the genome (Doudna and Charpentier, 2014).

CRISPR–Cas9 based technologies have emerged as an efficient and clean way to generate mutations in several organisms (Sander and Joung, 2014) including the yeasts *Saccharomyces cerevisiae* (DiCarlo et al., 2013), *Schizosaccharomyces pombe* (Jacobs et al., 2014), Kluyveromyces lactis (Horwitz et al., 2015), the filamentous ascomycete fungi *Trichoderma reesei* (Liu et al., 2015), *Magnaporthe oryzae* (Arazoef et al., 2015), and *Neurospora crassa* (Matsu-ura et al., 2015). The CRISPR–Cas9 system has been shown to be highly efficient in targeting multiple unrelated genes when using more than one sgRNA in most of the organisms where the technique has been established, including yeasts and the filamentous fungi *T. reesei* (Cong et al., 2013; Niu et al., 2014) and some *Aspergillus* species (Nødvig et al., 2015). This has allowed the inactivation of gene families using a few sgRNAs targeted to conserved regions of up to two genes in *Candida albicans* (Vyas et al., 2015) and even larger gene families in *Trypanosoma cruzi* (Peng et al., 2015).

Successful application of the CRISPR–Cas9 system requires the heterologous expression of the *cas9* gene from *Streptococcus pyogenes* fused to a nuclear localization signal and simultaneously expression of the sgRNA molecule. Alternatively, the mRNA of the *cas9* gene or the purified protein can be transfected into the cells (Kim et al., 2014). The sgRNA has to be expressed in such a way that it can fold into the specific secondary structure required for interaction with Cas9 inside the nucleus. This implies that typical mRNA processing events like splicing, capping and poly A tail addition have to be prevented. Several approaches have been established for the expression of active sgRNA molecules. Most *in vivo* systems make use of RNA polymerase III promoters since RNA polymerase II promoters are often not suitable for sgRNA expression. Exceptions are the 35S CAMV promoter which allowed sgRNA expression in wheat and sweet orange (Jia and Wang, 2014; Upadhyay et al., 2013) and the Tprc promoter which was successfully used for sgRNA expression in *M. oryzae* (Arazoef et al., 2015). Strategies for expression of sgRNA from conventional RNA polymerase II promoters include usage of RNA-triple-helix structures, introns, and ribozymes to release the sgRNA from polymerase II transcripts (Jacobs et al., 2014; Nissim et al., 2014). In cell culture systems, as well as in several organisms including the fungus *T. reesei*, sgRNAs were introduced by transformation and in these cases the sgRNAs were generated by *in vitro* transcription by T7 or T3 RNA polymerase based systems (Liu et al., 2015).

In this work we report the establishment of the CRISPR–Cas9 system for generating targeted alterations in the genome of the basidiomycete fungus *U. maydis*.

2. Material and methods

2.1. Strains and growth conditions

For cloning purposes the *Escherichia coli* strain Top10 (Invitrogen) was used and grown in yeast extract, tryptone (YT) medium. The haploid solopathogenic *U. maydis* strain SG200 (*a1 mfa2 bE1 bW2*) has been described previously (Kämper et al., 2006). *U. maydis* strains were grown in YEPSL liquid medium (Brachmann et al., 2001) or on solid potato dextrose (PD, Difco) plates at 28 °C. PD plates containing 1% activated charcoal were used for filament induction (Holliday, 1974; Muller et al., 2003).

2.2. Plasmid and strain construction

To generate pMS6, where *NLS-cas9-HA-NLS* is under the control of the constitutive *otef* promoter (Spellig et al., 1996), we obtained an *U. maydis* codon-optimized allele of the *S. pyogenes cas9* gene (Eurofins, Ebersberg, Germany). The synthetic gene included a 5′ GAL4p nuclear localization signal (NLS) from *S. cerevisiae* (PPKKKRKVE) and a N-terminal HA tag (YPYDVPDYA) flanked by SGG linkers followed by the nucleoplasmin NLS (KRPAATAKAGQAKKKK) (Supplementary Fig. S1). The 4234 bp fragment containing *NLS-cas9-HA-NLS* was cloned into the BamHI and NotI sites of the plasmid p123 (Aichinger et al., 2003). The *cas9* gene, including the regulatory sequences was excised from pMS6 as a 5423 bp HindIII/EcoRI fragment and ligated into the corresponding restriction enzyme sites of pNEBUC (Weinzierl et al., 2002) yielding pMS7. pNEBUC is an autonomously replicating *U. maydis* plasmid conferring carboxin resistance. The *U. maydis* U6 promoter (P_{U6}) (Supplementary Fig. S2) was amplified from SG200 genomic DNA adding 5' HindIII and 3′ Acc65I restriction sites using primers oMS1: 5′TACGCCAAGCTTTAATACGTTCGTTCCG3′ and oMS2: 5′GGTACGGGTACCGTTGTAGAATGGAATTTTG3′. The 826 bp PCR product was digested with HindIII and Acc65I and inserted into the corresponding restriction enzyme sites of pMS7 to yield pCas9_sgRNA_0.

The plasmids pCas9_sgRNA_bW2 and pCas9_sgRNA_bE1 were generated by insertion of the respective sgRNA fragment followed by the human U6 gene terminator (Mali et al., 2013) into the Acc65I linearized pCas9_gDNA_0 using the Gibson assembly strategy (Gibson et al., 2010). To this end a double stranded DNA fragment was synthesized (gBlock, IDT, Coralville, USA) that was composed of the 20 bp region upstream of the Acc65I site in pCas9_gDNA_0 (including the first G of the Acc65I site) followed by the respective target sequence, the guide RNA scaffold (Mali et al., 2013), the U6 terminator (TTTTTT), a 34 nucleotide 'stuffer' sequence (Mali et al., 2013) and the 20 bp region downstream of the Acc65I site in pCas9_gDNA_0 (including the Acc65I site lacking the first G) (Supplementary Fig. S3). The *bW2* (TCCAGGATTCGGACTGCTT) and *bE1* (AAAGAAAAAGTTGGAGAC) target sequences were designed using the E-CRISP tool (Heigwer et al., 2014). The respective double stranded DNA fragments were combined with an Acc65I-linearized pCas9_sgRNA_0 vector according to the instructions provided by the Gibson Assembly Cloning Kit (NEB,