

Single and Multiplexed Gene Editing in *Ustilago maydis* Using CRISPR-Cas9

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[Abstract] The smut fungus *Ustilago maydis* is an established model organism for elucidating how biotrophic pathogens colonize plants and how gene families contribute to virulence. Here we describe a step by step protocol for the generation of CRISPR plasmids for single and multiplexed gene editing in *U. maydis*. Furthermore, we describe the necessary steps required for generating edited clonal populations, losing the Cas9 containing plasmid, and for selecting the desired clones.

Keywords: CRISPR-Cas9, Multiplexing, Gene editing, *Ustilago maydis*, Smut fungi

[Background] The basidiomycete fungus *U. maydis* is a model organism that has enabled important discoveries in topics like DNA repair and homologous recombination, mating, sexual development, secondary metabolism, filamentous growth, RNA transport and virulence (Holliday, 2004; Steinberg, 2007; Bakkeren *et al.*, 2008; Holloman *et al.*, 2008; Lanver *et al.*, 2017; Niessing *et al.*, 2018). *U. maydis* is a biotrophic plant pathogen that causes smut disease on maize. As a typical member of the large group of smut fungi its sexual development is intimately coupled with its ability to colonize plants. The popularity of *U. maydis* as a model organism resides in its short pathogenic life cycle that is completed in less than two weeks, its accessibility for forward and reverse genetics that includes the establishment of self-replicating plasmids (Tsukuda *et al.*, 1988), the development of solopathogenic strains able to cause disease without mating and an available well-annotated genome (Kämper *et al.*, 2006). In *U. maydis*, homologous recombination-based genome manipulations are very efficient, but become tedious when several genes have to be modified because the number of selectable markers is limited (Schuster *et al.*, 2016). The most recent addition to the technical toolboxes for this organism has been the adaptation of the CRISPR-Cas9 genome editing technology (Schuster *et al.*, 2016) and its development for multiplexed editing (Schuster *et al.*, 2018). The CRISPR-Cas9 genome editing technology, developed from research in bacterial immune systems (Barrangou *et al.*, 2007), has revolutionized molecular biology. The system allows efficient modification of almost any given sequence (Jinek *et al.*, 2012) and has been established for a large number of organisms including filamentous fungi (Deng *et al.*, 2017; Shi *et al.*, 2017).

The CRISPR-Cas9 technology established for *U. maydis* is based on an all-in-one plasmid approach. A self-replicating plasmid (Tsukuda *et al.*, 1988) is used as a backbone to provide the *U. maydis* codon-

optimized Cas9 gene and the U6 promoter for sgRNA expression. This plasmid is unstable without selection and is rapidly lost (Figure 1 and Schuster *et al.* [2016]). The system proved very efficient for disruption of single genes reaching on average 70% of edited cells in progeny of one transformant (Schuster *et al.*, 2016). A multiplexing version of the system was generated by using tRNA promoter-based expression cassettes that enabled the expression of several sgRNAs from the same plasmid (Schuster *et al.*, 2018). This modification combined with elevated and longer expression of the Cas9 protein was used for the simultaneous disruption of five effector genes with 70% efficiency (Schuster *et al.*, 2018). The multiplexed version of CRISPR-Cas9 has also been successfully applied for elucidating the contribution of oligopeptide transporters to virulence (Lanver *et al.*, 2018).

Materials and Reagents

1. Consumables
 - a. Sterile pipette tip
 - b. Toothpick
 - c. 60 mm x 15 mm round Petri dishes
2. Competent cells

E. coli competent cells *e.g.*, One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific, catalog number: C404010)
3. Plasmids and double-stranded DNA
 - a. pCas9_sgRNA_0 (Addgene, catalog number: 70763 or available on request) (Figure 1A)
 - b. pMS73 (Addgene, catalog number: 110629 or available on request) (Figure 1B)
 - c. Double-stranded DNA fragments can be purchased *e.g.*, gBlocks (Integrated DNA Technologies) or GeneStrands (Eurofins Genomics)
4. Oligonucleotides 10 pmol/μl
 - a. oMS59: 5' ATTCGTGATTTACACCAAACACGC 3'
 - b. oMS49: 5' CCCCTCGTCTCGCGCCTCATTGGTCTGAATTG 3'
 - c. oSR224: 5' CTACACTCAGCACACGATGT 3'
5. Enzymes and buffers
 - a. Acc65I restriction endonuclease (New England Biolabs, catalog number: R0599S)
 - b. Gibson Assembly® master mix (New England Biolabs, catalog number: E2611S)
 - c. Phusion High-Fidelity DNA Polymerase (New England Biolabs, catalog number: M0530S) or BioMix Red (Bioline, catalog number: BIO-25006)
6. Kits
 - a. QIAprep Spin Miniprep Kit (QIAGEN, catalog number: 27104)
 - b. Wizard SV Gel and PCR Clean-Up system (Promega, catalog number: A9281)
 - c. Optional: Plate Seq Kit PCR (Eurofins genomics)
7. Antibiotics
 - a. Ampicillin 10 mg/ml stock in Milli-Q water (Carl Roth, catalog number: HP62.1)

- b. Carboxin 5 mg/ml stock in methanol (Sigma-Aldrich, catalog number: 45371)
- 8. Reagents
 - a. Tryptone (BD Biosciences)
 - b. Yeast extract (BD Biosciences)
 - c. Sodium chloride (NaCl)
 - d. Peptone (BD Biosciences)
 - e. Sucrose (BD Biosciences)
 - f. Sorbitol (Sigma-Aldrich, catalog number: S1876)
 - g. Bacto agar (BD Biosciences)
 - h. Potato Dextrose Broth (BD Biosciences)
- 9. Media (see Recipes)
 - a. Yeast extract tryptone (dYT) liquid medium + 100 µg/ml ampicillin
 - b. YT agar plates + 100 µg/ml ampicillin
 - c. YEPS light liquid medium +/- 2 µg/ml carboxin
 - d. Two layered regeneration agar light (RegAgar) plates (Bottom layer (10 ml) with 4 µg/ml carboxin and top layer (10 ml) without carboxin (Prepare fresh))
 - e. Potato dextrose (PD) agar plates +/- 2 µg/ml carboxin

Note: All media need to be autoclaved. For plates, 20 ml of media should be used per Petri dish.

Equipment

Note: No equipment from specific manufacturers is required. Any equivalent device can be used.

1. Pipettes
2. Computer with internet access
3. Plate incubators (28 °C, 37 °C)
4. Shakers (28 °C, 37 °C)
5. ThermoMixer (e.g., Eppendorf, model: ThermoMixer® C, catalog number: 5382000015)
6. Thermocycler (e.g., Analytik, Jena, Biometra, catalog number: 846-2-070-301)
7. Tabletop centrifuge (e.g., Thermo Fisher Scientific, catalog number: 75008801)

Software

No specific software is required. Any appropriate alignment program like CloneManager 9 Professional Edition (Scientific & Educational Software) or CLC Main Workbench (QIAGEN) can be used.

Procedure

1. Select target sequences with E-CRISP (Heigwer *et al.*, 2014)

Note: Although several tools have been developed for sgRNA design, our experience is limited to the E-CRISP tool that proved to be very reliable.

- a. Select *U. maydis* as the organism in the E-CRISP webpage (<http://www.e-crisp.org/E-CRISP/>).
- b. Click on the 'Enter target sequence' box and insert target genomic DNA sequence in FASTA format.

Note: Use the coding sequence of the gene you want to inactivate and restrict your search to the most 5' end of the gene. In case you do not find a suitable sgRNA you can expand the input sequence towards the 3' end.

- c. Click on 'display advanced options' on the 'Start application' panel and make sure the 3' PAM is NGG.

Notes:

- i. *The E-CRISP tool offers to restrict the search of target sequences to those starting with a G. However, this restriction is not necessary when using our plasmid backbones, because our linearized plasmids retain a G overhang at the 3' end of the U6 promoter that serves as the first G preferred for initiating transcription at the U6 promoter.*
 - ii. *The E-CRISP tool gives you the option to design truncated target sequences (Fu *et al.*, 2014). We usually use target sequences of 20 nt but have been successful using target sequences as short as 18 nt.*
 - d. Click on 'Start sgRNA search'.
 - e. Select the target sequence with the highest score and most 5' in the gene. Double check that the target gene(s) correspond to the gene(s) you want to disrupt.
2. Design sgRNA expression cassettes following examples in Table 1 and insert them into pCas9_sgRNA_0 or pMS73 (Figure 1)

*Note: The CRISPR-Cas9 plasmids developed for genome editing in *U. maydis* confer ampicillin resistance in *E. coli* and carboxin resistance in *U. maydis*. These plasmids contain a *U. maydis* ARS element for autonomous replication (Tsukuda *et al.*, 1988). The plasmid backbones also contain the *U. maydis* U6 promoter just before an *Acc65I* site that is used for integration of the sgRNA expression cassette via Gibson Assembly® (Figure 1). Expression of the codon-optimized Cas9 is driven by the constitutive *otef* promoter in pCas9_sgRNA_0 (Figure 1A) or by the stronger *hsp70* promoter in pMS73 (Figure 1B).*

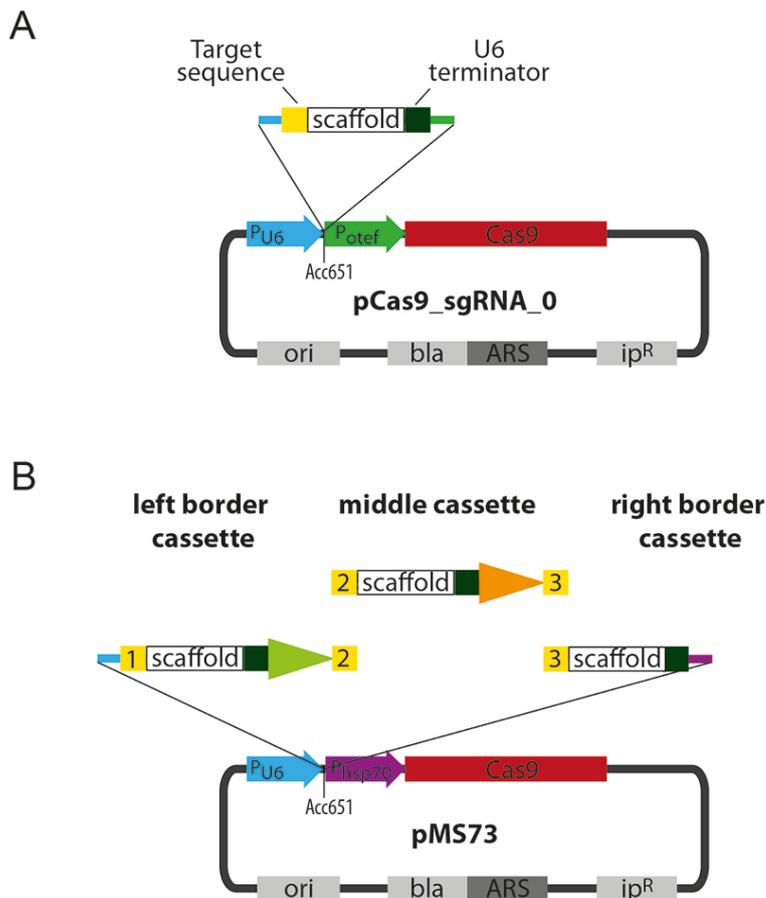


Figure 1. Scheme of the CRISPR-Cas9 plasmids and cloning strategy. A. Schematic representation of pCas9_sgRNA_0 and cloning strategy for the introduction of a single sgRNA. A user-defined targeting fragment can be cloned into the Acc65I-linearized pCas9_sgRNA_0 backbone via Gibson Assembly[®]. B. Schematic representation of pMS73 and cloning strategy for the introduction of two or more sgRNA expression cassettes. User-defined left and right border cassettes and a variable number of middle cassettes can be cloned into the Acc65I-linearized pMS73 backbone via Gibson Assembly[®]. The target sequences are used as Gibson overhangs and the number of middle cassettes can be modified depending on the number of target sequences that need to be inserted. ori: Origin of replication, bla: β -lactamase gene that confers resistance to ampicillin in *E. coli*, ARS: autonomously replicating sequence for replication in *U. maydis*, ip^R: *U. maydis* ip allele that confers resistance to carboxin. Numbers in yellow boxes indicate different targeting sequences. The scaffold sequence corresponds to the minimal scaffold required for Cas9 binding (Hsu *et al.*, 2013). tRNA promoters used to drive expression of different sgRNAs are indicated by differently colored arrowheads. Cyan line indicates the Gibson overhang to P_{U6}. Green line indicates the Gibson overhang to P_{otef}. Purple line indicates the Gibson overhang to P_{hsp70}.

- a. Introduce your target sequences in the appropriate template (Table 1).
- b. Purchase the designed sequences as double-stranded DNA fragments.

Note: An alternative to purchasing the sgRNA expression cassettes is their generation by PCR amplification from a template. You can use double-stranded DNA fragments as templates for such a PCR.

Table 1. Templates for generating double-stranded DNA fragments containing the target sequences of interest

No.	Description	Template	To be used
1*	Overhang to pU6 TARGETSEQUENCE Scaffold** Terminator Overhang to P _{Oief}	CAAAATTCCATTCTACAACG TARGETSEQUENCE ENCEGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAA AAGTGGCACCGAGTCGGTGC TTTTGT ACCCGTACCGAGCTCGAC	When inserting only one target sequence in pCas9_sgRNA_0.
2	Overhang for pU6 TARGETSEQUENCE Scaffold Terminator Overhang to P _{hsp70}	CAAAATTCCATTCTACAACG TARGETSEQUENCE ENCEGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAA AAGTGGCACCGAGTCGGTGC TTTTGT ACCTGCCAAGAGTCGAACGCCG	When inserting only one target sequence in pMS73.
3	Overhang for pU6 TARGETSEQUENCE1 Scaffold Terminator P _{iRNA} Leu ^{TAA} TARGETSEQUENCE2	CAAAATTCCATTCTACAACG TARGETSEQUENCE1 ENCE1GTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAA AAAGTGGCACCGAGTCGGTGC TTTTGT CGATGTGTGCACAAATCATCGTGCTAGAC GCCAAGCGAAGCGGAAAGTTTGAGACC CGATTATAATCGGTATTGAGCAATTCTGTCT TCCAAACTTCATGACCAGTAGGCAAGTGG TTAAGCCGCGAGCTTTAAGCCCGATGTAG GCCCGATCTCAGCCCTTCGTTAACTCTCG TTTGTCGCGGGTTCGAATCCCGTCTGGTC AAG TARGETSEQUENCE2	When inserting two or more target sequences in pMS73. Example shown is for left border cassette.
4	TARGETSEQUENCE2 Scaffold Terminator P _{iRNA} Gly ^{GCC} TARGETSEQUENCE3	G TARGETSEQUENCE2GTTTTAGAGCTAGA AATAGCAAGTTAAAATAAGGCTAGTCCGTT ATCAACTTGAAAAAGTGGCACCGAGTCGG TGC TTTTGT ACCTCAGACCAAGCGTGAAC AGGCGATGCTTGTGACATAGAAATGGAAC GGTCATAATATTACATATAATTACAGTGTAAC AAGATATATGCACCTGAAAGCATTGGTAGT GTAGTGGTATCACGGGACGTTGCCAGCCC GCCTTATTCAAGGCTTAACCACGAATTCGT CCCGACCGGGGTTTCGATTCCCCGTTCGAT GCA G TARGETSEQUENCE3	When inserting more than 2 target sequences in pMS73. Example shown is for a middle cassette.

Table 1. Continued

5	<p>TARGETSEQUENCE3Scaffo</p> <p>Id Terminator P_{tRNA}Tyr^{GTA} TAR</p> <p>GETSEQUENCE4</p>	<p>GTARGETSEQUENCE3GTTTTAGAGCTAGA AATAGCAAGTTAAAATAAGGCTAGTCCGTT ATCAACTTGAAAAAGTGGCACCGAGTCGG TGCTTTTTTTGAAGACTCAACCAACTCACA CAGATCCAGCTTGATGATTATACCAGAAAT AATAGTACATATTATAGACGCAATCTTGTTG CTCCTTGCCAATCTAAAACCCGGACATAGC TCAGTTGGAAGTAGCGATGGTTTGTAATTG GAAACCCTAATCCGGTATTAACGGAAAAGC ACCATAGGTCGCCGGTTCGATTCCGGCTG TCCGGAGTARGETSEQUENCE4</p>	<p>When inserting more than 2 target sequences in pMS73. Example shown is for a different middle cassette than the one depicted in (4).</p>
6	<p>TARGETSEQUENCE4Scaffo</p> <p>Id Terminator P_{tRNA}Gly^{TCC} LAS</p> <p>TTARGETSEQUENCE</p>	<p>GTARGETSEQUENCE4GTTTTAGAGCTAGA AATAGCAAGTTAAAATAAGGCTAGTCCGTT ATCAACTTGAAAAAGTGGCACCGAGTCGG TGCTTTTTTTGCCCGTGCTGATTCCGCCA GACAGCAAAGTGGACTTAGTTTGTGAAAA CAATATACTATAGATAATATGTGCAACTGATA TCGAAGATACATATCAGTAAGCATTGGTAG TGTAGTGGTATCACGGGACGTTGCCATCC CGCCTTCAGTGAAGGCTTCATCACGAATT CGTCCGACCGGGGTTTCGATTCCCGGTC AATGCAGLASTTARGETSEQUENCE</p>	<p>When inserting more than 2 target sequences in pMS73. Example shown is for a different middle cassette than the one depicted in (4 or 5).</p>
7	<p>LASTTARGETSEQUENCE</p> <p>Scaffold Terminator</p> <p>Overhang for P_{hsp70}</p>	<p>GLASTTARGETSEQUENCEGTTTTAGAGCT AGAAATAGCAAGTTAAAATAAGGCTAGTCC GTTATCAACTTGAAAAAGTGGCACCGAGT CGGTGCTTTTTTTGTACCTGCCAAGAGTC GAACGCCG</p>	<p>When inserting two or more target sequences in pMS73. Example shown is for right border cassette.</p>

*Colors in Table 1 correspond to those in Figure 1.

**The scaffold sequence corresponds to the minimal scaffold required for Cas9 binding (Hsu *et al.*, 2013).

Note: The sequences labeled as tRNA promoter contain both the DNA sequence of the tRNA ending up in the mature tRNA and 100 bp upstream from the part encoding the mature tRNA.

3. Cloning of the sgRNA expression cassettes into the CRISPR plasmids via Gibson Assembly[®] (Gibson *et al.*, 2010)
 - a. Linearize pCas9_sgRNA_0 or pMS73 with Acc65I.
 - b. Clone the sgRNA expression cassettes into the backbone via Gibson Assembly[®] according to the manufacturer's protocol.

Note, we have used Gibson Assembly[®] Master Mix (Instruction Manual, NEB #E2611S/L 10/50 reactions, Version 4.0).
 - c. Transform part of the Gibson Assembly[®] reaction into competent *E. coli* cells according to

the manufacturer's protocol and select for growth on YT plates containing 100 µg/ml ampicillin. Incubate the selection plates overnight at 37 °C.

Note: We have used the heat shock E. coli transformation protocol ([Bacterial Transformation Workflow—4 Main Steps](#)) as given at Thermo Fischer website.

- d. Pick some colonies to check for the correct insertion of sgRNA expression cassettes. Use a sterile pipette tip or toothpick to inoculate a single colony into 3 ml of dYT medium with 100 µg/ml ampicillin. Incubate with shaking at 37 °C overnight.
- e. Isolate the plasmid DNA from bacterial cultures by using e.g., a QIAprep spin miniprep kit according to the manufacturer's instructions.
- f. Test for plasmids with inserts by restriction analysis and sequencing.

Notes:

- i. *For assessing the successful insertion of only one cassette, it is normally sufficient to test plasmids from two transformants or go directly for sequencing. If more than one cassette is to be inserted, it is usually sufficient to analyze plasmids from six transformants by restriction analysis first and follow candidates up by sequencing. When restriction analyses are performed, enzymes should be chosen which cleave outside the cassette(s) and allow to estimate the number of cassettes inserted.*
- ii. *For plasmid sequencing use the U6-Fwd primer oMS59: ATTCGTGATTTACACCAAACACGC and the reverse primer Potef-Rv oMS49: CCCCTCGTCTCGCGCCTCATTGGTCTGAATTG for pCas9_sgRNA_0 derived plasmids or oMS59: ATTCGTGATTTACACCAAACACGC and P_{hsp70}-Rv oSR224: CTACACTCAGCACACGATGT for pMS73-derived plasmids.*

4. Transformation of the CRISPR-Cas9 plasmid in *U. maydis* protoplasts
 - a. Transform 50 µl of *U. maydis* protoplasts with no more than 500 ng of the pCas9_sgRNA_0 derived or pMS73 derived plasmids.
 - b. Plate the transformation mixture on a freshly prepared two-layered RegAgar plate containing 4 µg/ml carboxin in the bottom layer and no carboxin in the top layer and incubate four days at 28 °C until transformants are clearly visible.

Note: Transformation of the CRISPR-Cas9 constructs into U. maydis follows the protocol developed by Schulz et al. (1990).

Note: See Bösch et al. (2016) for a detailed description of the protocol for the generation of protoplasts and the polyethylene glycol (PEG)-mediated transformation.

5. Optional: Transfer single colonies from the transformation plate to 3 ml liquid YEPSL medium containing carboxin (2 µg/ml) to increase CRISPR-Cas9 efficiency.
 - a. Incubate the culture and shake it at 200 rpm and 28 °C overnight.
 - b. Dilute the cultures 1:100 into fresh selection medium and allow them to grow until saturation (approximately 20 h). Repeat three times.

Note: CRISPR-Cas9-mediated genome editing in U. maydis is very efficient (Schuster et al., 2016). This step is only necessary when three sgRNAs or more are used simultaneously or a

chosen sgRNA works inefficiently.

6. Isolation of colonies with a defined editing event
 - a. Streak out 10-12 transformants (in case of single gene disruption), or more (in case of multiple gene disruptions) from the original transformation plate (Step 4b) on PD plates without carboxin, or in the case of incubation in liquid medium (Step 5b), spot 1 μ l of cells on a PD plate without carboxin and streak them out .
 - b. Incubate the plate for two days at 28 °C.
 - c. Generate a master plate to store colonies to be tested. To do this, pick single colonies with a flat toothpick and transfer to a new PD agar plate. Spread the transferred cells to obtain a larger patch in which cells grow up to form a colony and arrange these using a grid pattern for easier later identification. Incubate overnight at 28 °C (Figure 2).

Notes:

- i. *This step is of crucial importance because only in the absence of carboxin will the Cas9-containing plasmid be lost. Fungal colonies and cultures grown under selection pressure are heterogeneous populations: a colony (or culture grown with carboxin selection) will contain cells with independent editing events depending on the generation in which editing occurred - except when the CRISPR event took place in the initially transformed cell.*
- ii. *At this step, you can confirm the loss of the CRISPR-Cas9 plasmid (see Step 8) by replicating the single colonies on a PD-carboxin plate in parallel to generate the master plate and selecting those colonies from the master plate which are unable to grow on the carboxin plate.*

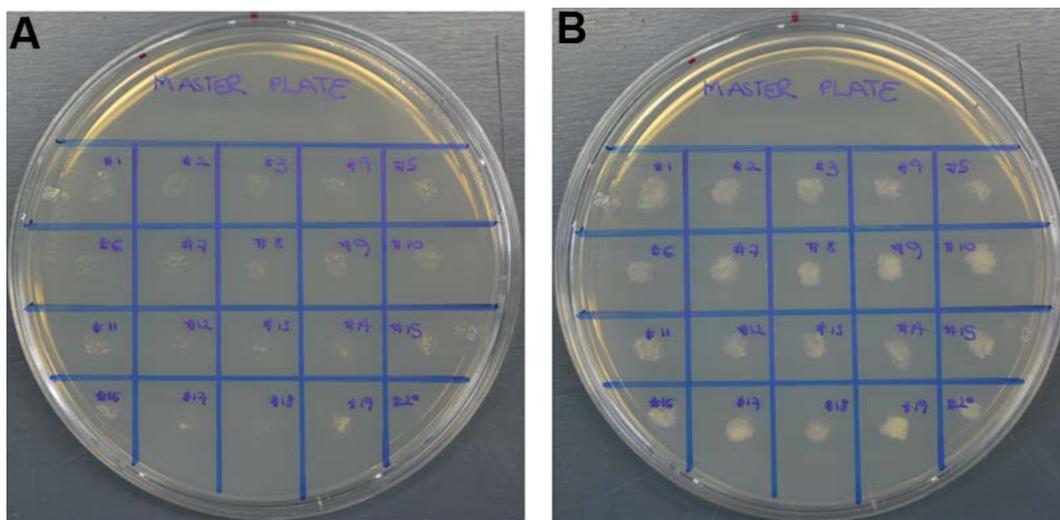


Figure 2. Example of a master plate containing colonies to be tested. Generation of a master plate is advisable to facilitate the screening process. Single colonies derived from individual *U. maydis* transformants were transferred to a new PD plate following a grid pattern and labeled with numbers for identification. Transfers were made with flat toothpicks, cells were placed in the center of a grid square and slightly spread to increase the area in which cells can grow up and form a colony. A. Master plate before incubation; B. Master plate after incubation

overnight at 28 °C.

7. Colony PCR-based screen for CRISPR-Cas9 induced mutations

- a. Design primers enabling the amplification of a PCR product of about 500 bp containing the target site.
- b. With a sterile pipet tip or a toothpick, pick a small amount of a colony from the master plate (Step 6c) and place it in a well of a 96-well plate containing 20 µl of 20 mM NaOH.
- c. Shake the plate for 30 min at room temperature at 800 rpm using *e.g.*, a ThermoMixer.
- d. Set up a 20 µl PCR reaction using a high fidelity polymerase *e.g.*, Phusion DNA Polymerase or BioMix Red as suggested by the manufacturer. Use 1 µl of the lysed cells as DNA template.

Note: In case of multiplexing, a separate PCR for each target site needs to be performed. If you have many colonies and/or sites to test, we recommend using kits like the Plate Seq Kit PCR. In this case, perform the PCR reaction in this step directly in the 96-well-PCR-plate provided in the kit.

- e. Amplify the PCR products in a Thermocycler following the polymerase manufacturer's instructions.
- f. Sequence the PCR fragment either after cleanup with *e.g.*, the Wizard SV Gel and PCR cleanup System or *e.g.*, in the Plate Seq Kit from Eurofins.

8. Loss of the CRISPR-Cas9 plasmid

- a. If you have not screened for the loss of the plasmid before (see note after Step 6), pick some cells with a toothpick from the master plate and streak out on a PD plate containing carboxin. Incubate for two days at 28 °C. Go back to the master plate and use colonies that were unable to grow on the carboxin plate, these have lost the plasmid.

Note: We observe retention of the plasmid in colonies on the master plate in about 5% of the cases.

- b. Growth on the carboxin plate means that some cells still contain the plasmid. If this is the case and it is this colony that contains the desired mutation, inoculate cells from this strain from the master plate in 3 ml YESPSL medium without carboxin and grow overnight at 28 °C.
- c. Take cells from this overnight culture and spread on a PD plate without carboxin to obtain single colonies. Incubate for two days at 28 °C.
- d. Replica-plate single colonies on both, a PD plate with carboxin and a PD plate without carboxin, and incubate overnight at 28 °C. Select colonies that have lost the plasmid *i.e.*, are unable to grow on the plate containing carboxin.
- e. If necessary, repeat the loss of plasmid procedure until there is no growth on selection plates.

Note: Resequence the final clone to verify that you have obtained the desired mutation.

Data analysis

The sequencing data should be analyzed by alignment against the wild-type sequence using software like CloneManager or CLC Main Workbench. Frameshifts mutations are generated when the number of nucleotides deleted or inserted cannot be divided by three. Alternatively, indels that generate a premature stop codon can be used. It is mandatory to inspect the sequencing chromatograms carefully and check for the presence of overlapping peaks. Overlapping peaks are a sign of genetic heterogeneity in the sequenced sample (Figure 3).

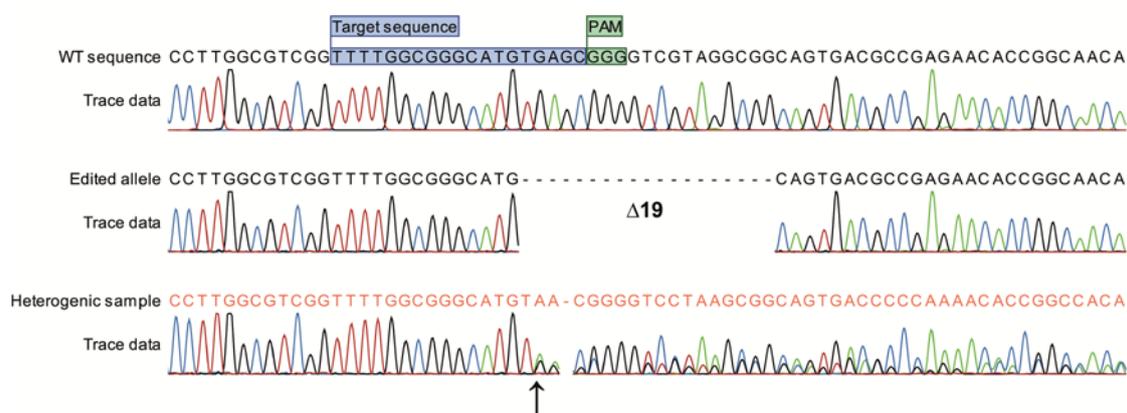


Figure 3. Representative sequencing results. Alignment of nucleotide sequences and chromatograms of a wild-type (WT) allele, a corresponding edited allele lacking 19 bp and a heterogenic sample from the same experiment. Note that a sequence is provided (dark orange) by the sequencing company for the heterogenic sample taking into account the highest peaks and incorrectly suggesting sequence homogeneity. Heterogeneity can only be recognized by careful analysis of the chromatogram which in this case shows that the sequence up to the arrow (predicted Cas9 cleavage site) is homogeneous and becomes heterogeneous from this point onwards. Target sequence and PAM sequence are depicted as colored boxes in the wild-type sequence. The alignment was generated with CLC Main Workbench.

Notes

1. The CRISPR-Cas9 system in *U. maydis* has proven to be highly efficient for the disruption of single genes where up to 100% of the tested transformants contained edited cells (Schuster *et al.*, 2016). For sgRNA with lower editing efficiency, the efficiency could be improved by higher expression of the Cas9 protein using a stronger promoter (*i.e.*, P_{hsp70} instead of P_{otef}) and by extending exposure to Cas9 by maintaining the transformed cells in liquid culture under conditions selecting for the Cas9 expressing plasmid (Schuster *et al.*, 2018). Using longer Cas9 exposure times, we have been able to improve editing using numerous sgRNAs. We have rarely been confronted with target sequences that either do not work at all or that give very low gene editing efficiency. In such cases, changing the target sequence provides an efficient solution.

2. Two independently edited strains have been analyzed for off-target mutations and no off-targets were found via whole genome sequencing (Schuster *et al.*, 2016). Since off-targets will be specific for each sgRNA, we recommend generating three independent mutants in a given gene or group of genes. If these mutants all show the same phenotypic alteration and this phenotype can be complemented by introducing the wild-type gene or genes, you have convincing evidence that the phenotype is caused by the generated mutation(s).
3. The genomic location of genes to be edited should be taken into account when designing a multiplexing experiment. Two or more simultaneous CRISPR events in the same chromosome could lead to undesired chromosomal deletions or rearrangements. In such cases, sequential editing should be performed.

Recipes

1. Yeast extract tryptone (dYT) medium
 This medium consists of:
 1.6% (w/v) tryptone (BD Biosciences)
 1% (w/v) yeast extract (BD Biosciences)
 0.5% (w/v) NaCl in double distilled water
Note: Autoclave and store at room temperature before use.
2. Yeast extract tryptone (YT) agar medium
 This medium consists of:
 0.8% (w/v) tryptone (BD Biosciences)
 0.5% (w/v) yeast extract (BD Biosciences)
 0.5% (w/v) NaCl
 1.3% Bacto agar (BD Biosciences) in double distilled water
Note: Autoclave and immediately pour into Petri dishes (20 ml per Petri dish).
3. YEPSL liquid medium
 This medium consists of:
 0.4% (w/v) yeast extract (BD Biosciences)
 0.4% (w/v) peptone (BD Biosciences)
 2% (w/v) sucrose (BD Biosciences) in double distilled water
Note: Autoclave and store at room temperature before use.
4. Regeneration agar light (RegAgar)
 This medium consists of:
 1% (w/v) yeast extract (BD Biosciences)
 0.4% (w/v) peptone (BD Biosciences)
 0.4% (w/v) sucrose (Roth)
 18.22% (w/v) sorbitol (Sigma)
 1.5% Bacto agar (BD Biosciences) in double distilled water

Notes:

- a. Autoclave and store at room temperature before use.
 - b. During *U. maydis* transformation, the agar is melted in a microwave oven, mixed well and cooled down to 50-60 °C. Carboxin (4 µg/ml final concentration) is added and 10 ml of this supplemented RegAgar is poured into a Petri dish. Once this is solidified, an additional 10 ml of molten RegAgar without carboxin is poured as a top layer into the same Petri dish. This second layer should be poured about 25-30 min before plating the transformation mixture (see transformation protocol by Bösch *et al.*, 2016).
5. Potato dextrose (PD) agar medium
- This medium consists of:
- 2.4% Potato Dextrose Broth (BD Biosciences)
 - 2% Bacto agar (BD Biosciences) in double distilled water
- Note: Autoclave and immediately pour into Petri dishes (20 ml per Petri dish).*

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