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CRISPR-Cas9 Editing of Nitrate Transporter Gene, um03849, in Ustilago maydis

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CRISPR-Cas9 editing of nitrate transporter gene, *um03849*, in *Ustilago maydis*

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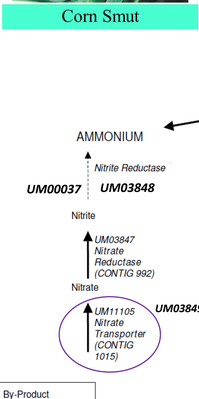
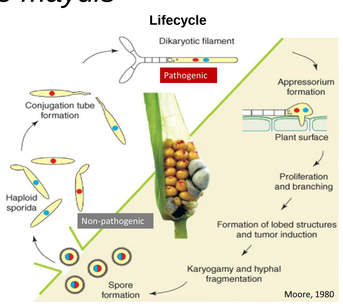
Abstract

Ustilago maydis, the basidiomycete smut-fungus, can infect and cause tumors in corn plants. For this, mating between compatible haploid cells is important. The mating and subsequent dimorphic transition in *U. maydis* require starvation for nutrients such as nitrogen, in addition to pheromone-receptor interactions between compatible partners. In this research, the CRISPR-Cas9 gene-editing technique was used to create INDEL mutations (sequence insertion or deletion) in the nitrate transporter gene, *um03849*, in *U. maydis*. The gene was edited in mating compatible haploid strains 1/2 and 2/9. The phenotypes were characterized for the *um03849* mutants as to growth ability, mating efficiency and pathogenesis. DNA sequence analysis confirmed isolates with 3 bp-deletion, 19 bp-deletion and 2 bp-substitution in the 1/2 mating strain, while a 3 bp-deletion and a 66 bp-insertion were found in independent isolates of the 2/9 strain. The matting assay results showed that any forms of mutation in *um03849* in *U. maydis* didn't affect mating with its compatible partner, as assessed by "fuzz" on charcoal media. However, the growth of mutated 1/2 strains was affected when grown in a medium with nitrate or nitrite as a source of nitrogen. With respect to host plant pathogenesis, the 1/2 strain with 2 bp substitution crossed with 2/9 WT strain showed dramatically reduced infection. Base substitution in the 1/2 strain resulted in arginine being substituted for lysine. Thus, this study suggests that the nitrate transporter affects the growth and pathogenesis of *U. maydis* on its host plant in a manner dependent on the 1/2 background.

Background

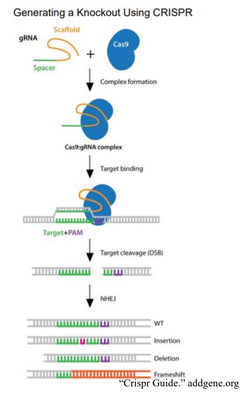
Ustilago maydis





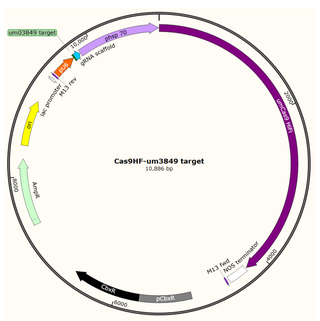
***U. maydis* must undergo a dimorphic switch from a yeast-like form to a filamentous form in order for infection to occur. This switch requires signals from both the mating partners and the environment cues such as nitrogen starvation.**

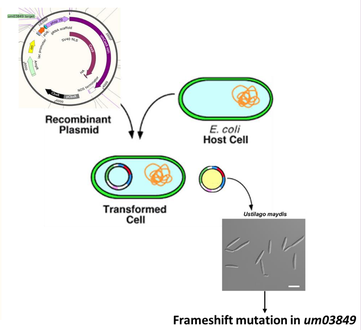
The pathway for the metabolism of nitrate in *Ustilago maydis*. The nitrate transporter gene, *um03849*, was selected for this study to understand nitrogen assimilation in *U. maydis*.



Schematic diagram showing basic mechanism of CRISPR-Cas9 gene editing technique.

Method





CRISPR-Cas9 gene editing technique was used to create INDEL mutations (sequence insertion or deletion) in the *um03849* gene in *U. maydis*. The plasmid expressing Cas9 as well as a 20 bp guide RNA (target) for *um03849* gene was cloned and transformed into competent *E. coli* cells. The successful cloning was identified by sequencing. The isolated plasmid was then used to transform compatible *U. maydis* mating partners, 1/2 and 2/9 strains.

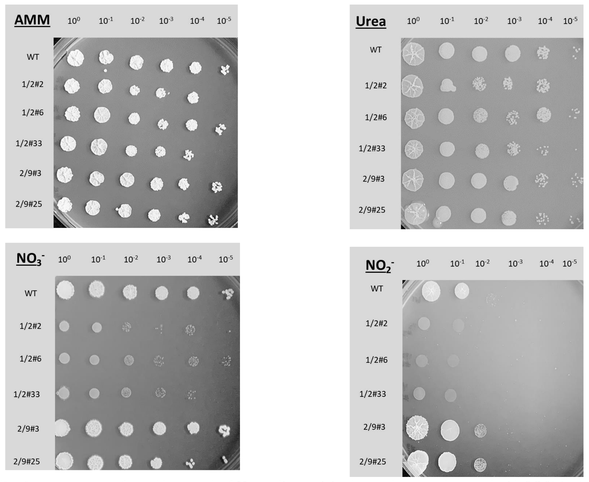
Results

Sequencing and Alignment

WT	CGACGGGCTGATTGACGGGAAAGCCAATCAGAAGAAGGGTCGAGAACATGAGAGCGA	
2/9_Cas3	CGACGGGCTGATTGACGGGAAAGCCAATCAG---AAGGGTCGAGAACATGAGAGCGA	3 bp deletion
1/2_Cas33	CGACGGGCTGATTGACGGGAAAGC----------GAACATGAGAGCGA	19 bp deletion
1/2_Cas6	CGACGGGCTGATTGACGGGAAAGCCAATCAG---AAGGGTCGAGAACATGAGAGCGA	3 bp deletion
1/2_Cas2	CGACGGGCTGATTGACGGGAAAGCCAATCAGCCGAAGGGTCGAGAACATGAGAGCGA	2 bp substitution (Lysine to Arginine)

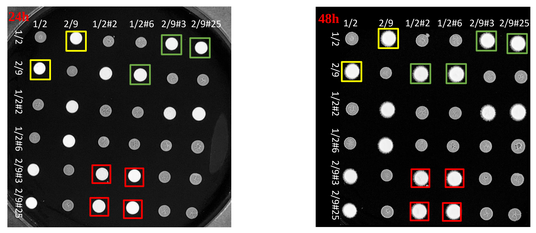
Sequencing: The colonies obtained from the transformation of Cas9+target plasmid into 1/2 and 2/9 mating strains were sub-cultured to eliminate the plasmids bearing Cas9. Such putative transformed strains now lacking Cas9 were then sequenced at the targeted region of the gene. The yellow highlight in the figure shows the 20 bp *um03849* target sequence in WT strain. From the sequencing and alignment along with the WT nucleotides, it was confirmed that a 3 bp deletion, 19 bp deletion and 2 bp substitution (shown by purple box) in the 1/2 mating strain were obtained. Similarly, there was a 3 bp deletion and 66 bp insertion in the 2/9 mating strain, shown by "–" in the figure.

Nitrogen Utilization



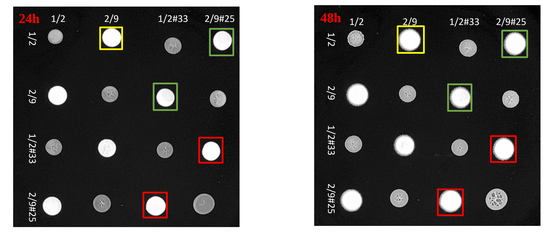
Nitrogen utilization assay was performed by growth of *U. maydis* on minimal agar plates supplemented with 0.3 % (wt/vol) of either ammonium sulphate (AMM), urea, sodium nitrate ($NO3^-$), or sodium nitrite ($NO2^-$). Strains grown in rich medium up to exponential stage were collected, washed, and diluted 10 fold, up to 10^{-5} fold dilutions. 10 µl of diluted and undiluted *U. maydis* strains were spotted in different nitrogen medium and grown for 4 days at 28° C. The growth of mutated strains was compared to the WT. The mutant 1/2 strains were affected when grown in a medium with nitrate or nitrite as a source of nitrogen while 2/9 strains grew at the same rate as the WT.

Effects on Mating

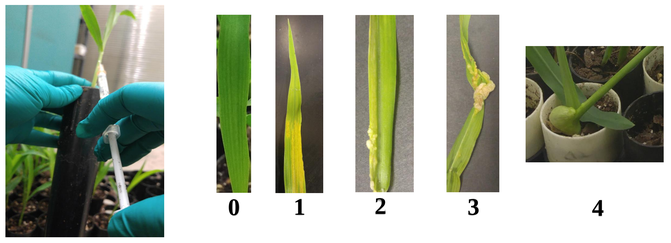


Mating assay was performed on PDA charcoal medium for WT and *um03849* mutant strains. Equal mixtures of haploid strains of opposite mating type background were grown to exponential phase, mixed with their mating partner and spotted onto PDA plates containing activated charcoal. The strains were grown for 24 hrs and 48 hrs. A positive mating reaction produced a white "fuzz" phenotype of aerial hyphae production. Yellow boxes indicate the positive "fuzz" reaction between WT mating strains. Spots in diagonal are the haploid strains, negative control. Green boxes indicate the "fuzz" reaction between opposite mating of mutated *um03849* strains to WT strains. Red boxes indicate the "fuzz" reaction between opposite mating *um03849* mutants.

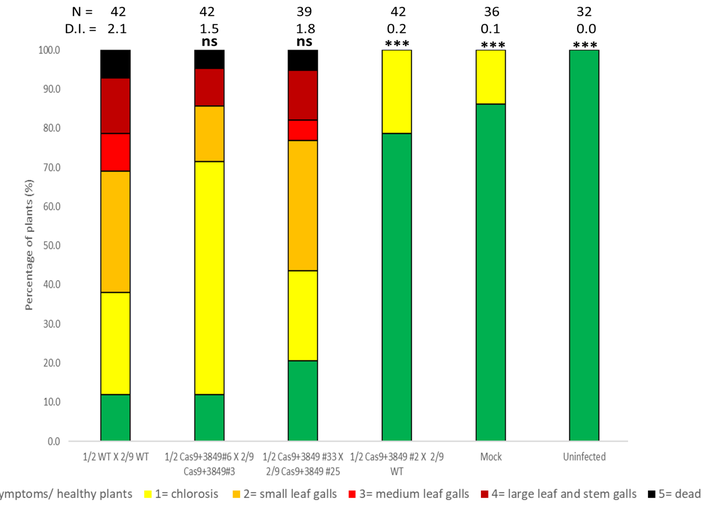
Effects on Mating: Strains Later Used for Infections



Plant Pathogenesis



Pathogenesis assay: *U. maydis* strains were grown to the exponential phase, concentrated to OD_{600} = 3, washed twice with sterile distilled water, and injected into 7 day old maize (*Zea mays*) seedlings (Early Golden Bantam). Disease symptoms and gall formation were quantified 14 days post infection. A disease index (DI; (Gold et al., 1997)) from 0 to 5, was used to rate virulence of each infection, with: 0 = no symptoms/ healthy plants, 1= chlorosis and / or anthocyanin production, 2 = small leaf galls, 3 = small leaf and stem galls, 4 = large galls and 5 = plant death



Pathogenicity assay: The graph displays the percentage of plants with specific symptoms of infection. The X axis indicates the paired background that was injected into maize plant. On the top of graph, N indicates number of plants injected and D.I. means disease index. The disease symptoms were categorized from 0 to 5 as depicted above the figure. The data was analyzed using the Kruskal-Wallis test followed by post-hoc comparison. An asterisk (*) indicates significant difference ($p < 0.05$) compared to those where both partners were wild-type strains (1/2 WT x 2/9 WT). A significant reduction (shown by ***, $p < 0.0001$) of plant pathogenesis was seen in the cross between 1/2 Cas9+3849 #2 and 2/9 WT. While there was non-significant (shown by **ns**, $p>0.05$) pathogenicity by other mutated mating type strains to the maize plants.

Conclusions

- **The growth of *um03849* mutated strains showed similar growth as compared to WT in media with different nitrogen sources, except mutated 1/2 strains when grown in a medium with nitrate or nitrite as a source of nitrogen, where growth was affected.**
- **None of the mutations in *um03849* gene in *U. maydis* affected mating with its compatible partner, as assessed by "fuzz" on charcoal media.**
- **The mutant in 1/2 strain with a 2 bp substitution (1/2Cas9+3849#2) when crossed with the 2/9 WT strain showed drastic reduction in pathogenesis. The 2 bp substitution in the 1/2 strain resulted in arginine being substituted for lysine. Sokalingam et al., 2012 have mentioned that mutagenesis of lysine to arginine adversely affect the protein folding and decrease the productivity of the functional form of the variant.**
- **This study suggests that the nitrate transporter affects the growth and pathogenesis of *U. maydis* on its host plant in a manner dependent on the 1/2 background.**

Acknowledgements

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