New strategies to find chromatin silencers in the pathogenic fungus *Ustilago maydis*

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ABSTRACT

Ustilago maydis is a smut fungus that infects maize, causing tumors, stunted growth and consequently reduced yields leading to economic losses [1]. A key aspect of the pathogenic development of *U. maydis* is the action of effectors, which are secreted virulence factors with principal roles in plant defense suppression and host's metabolism alterations. Many genes encoding effector proteins are grouped in silenced clusters in the genome highly induced during infection. It has been shown that introduction of resistance marker genes with high expression promoters in these clusters de-repress the surrounding region of the insertion point [2]. Consequently, it is suggested that these clusters are subjected to chromatin silencing. However, *U. maydis* lacks the canonical factors involved in chromatin silencing. The main purpose of this project is to find regulators that control the silencing state of these regions. To achieve this goal we are going to develop a strategy to perform a future screening in a *U. maydis* strain harboring an antibiotic resistant marker gene inside a silenced cluster.

RESULTS

1. Strain constructions

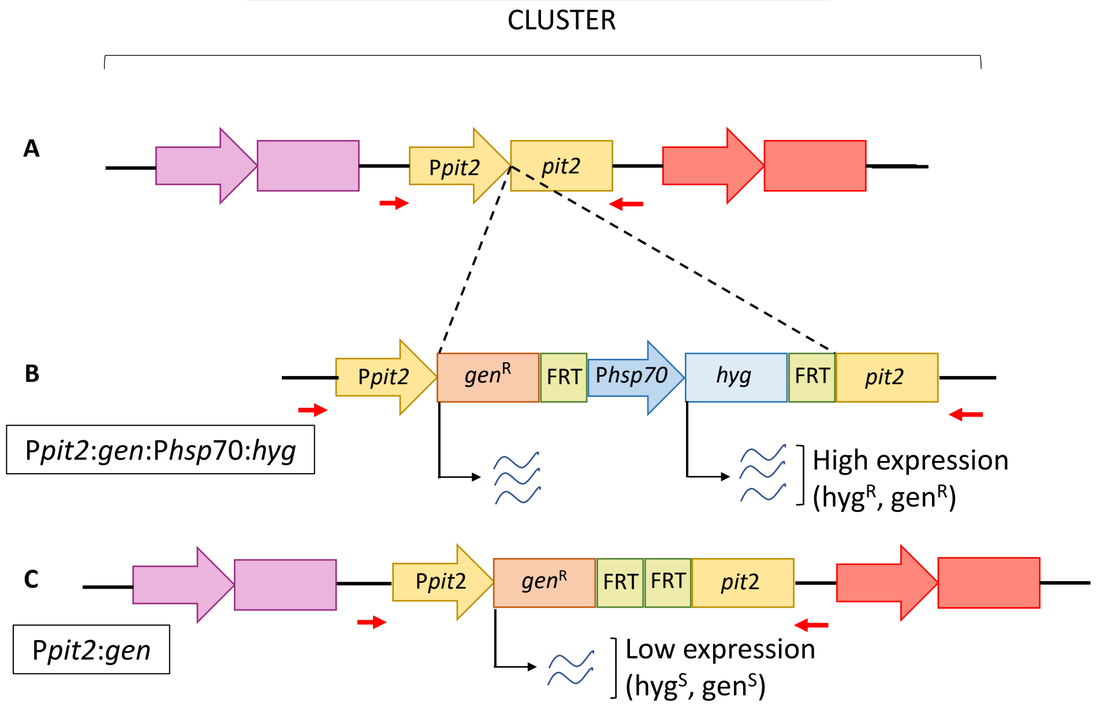

Figure 1. A. Cluster of effectors to which *pit2* belongs**. B.** Insertion of a resistance gene cassette with a high expressed promoter into the *pit2* ORF, resulting in de-repression of the surrounding zones and resistance to geneticin (gen) and hygromycin (hyg).
C. Hygromycin and its high expressed promoter are removed by flippase, resulting in this zone being silenced again and hygromicin and geneticin sensitive. We have introduced the same construction in the cluster to which *tin2* belongs. Changing geneticin resistance gene to nourseothricin (nat) resistance gene.

3. Phenotypic demonstration of strain constructs

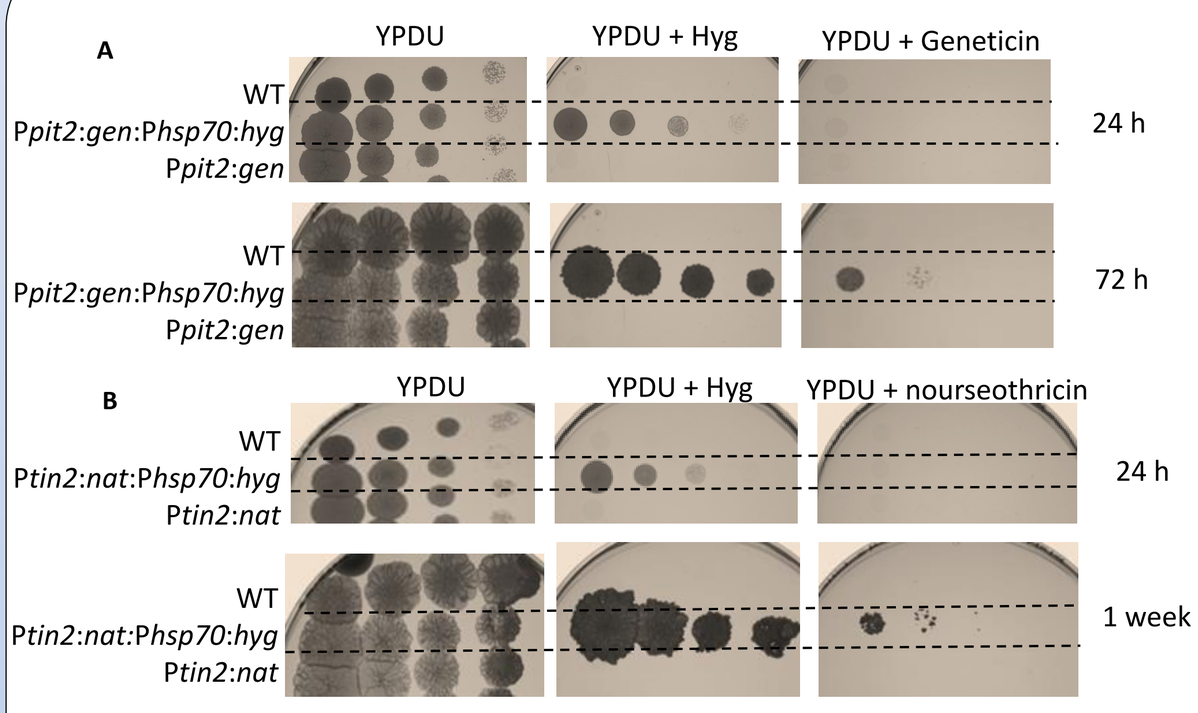

Figure 2. Drop assay of serial dilutions on different plates. **A. PIT2 CLUSTER.** wt only grows on YPDU plate because it hasn't resistance to hygromycin and geneticin. Due to the insertion of the cassette that de-represses this and surrounding regions, P*pit2*:*gen*:P*hsp70*:*hyg* strain grows in both hygromycin and geneticin. P*pit2*:*gen* strain has lost the resistance gene cassette, so has the same phenotype as wt. **B. TIN 2 CLUSTER.** wt only grows on YPDU plate. Due to the insertion of the cassette that de-represses this and surrounding regions, P*tin2*:*nat*:P*hsp70*:*hyg* strain grows in both hygromycin and nourseothricin. P*tin2*:*nat* strain has lost hygromycin resistance, and has the same phenotype as wt.

2. Genotypic demonstration of strain constructs

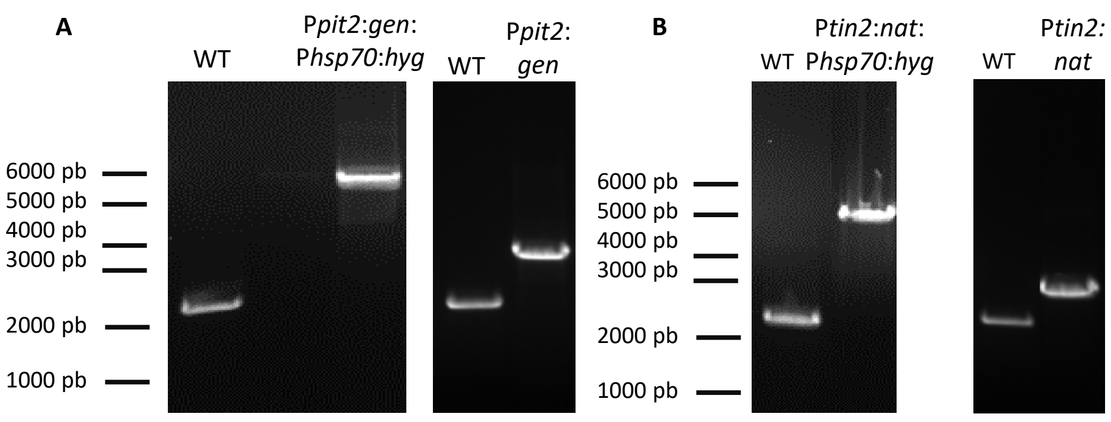

Figure 3. Results corresponding to PCRs using the primers showed in red arrows in the figure 1. **A. PIT 2 CLUSTER.** The total size of *pit2* gene in the WT of *U. maydis* is 2076 pb, and the expected size of P*pit2*:*gen*:P*hsp70*:*hyg* fragment is 6143 pb. When the hygromycin is removed, the expected size of P*pit2*:*gen* fragment is 3500 pb. **B. TIN2 CLUSTER**. The total size of the *tin2* gene in the WT of *U. maydis* is 2060 pb, and the expected size of the P*tin2*:*nat*:P*hsp70*:*hyg* fragment is 5687 pb. When the hygromycin is removed, the expected size of P*tin2*:*nat* fragment is 2800 pb.

4. Optimization for future mutagenesis

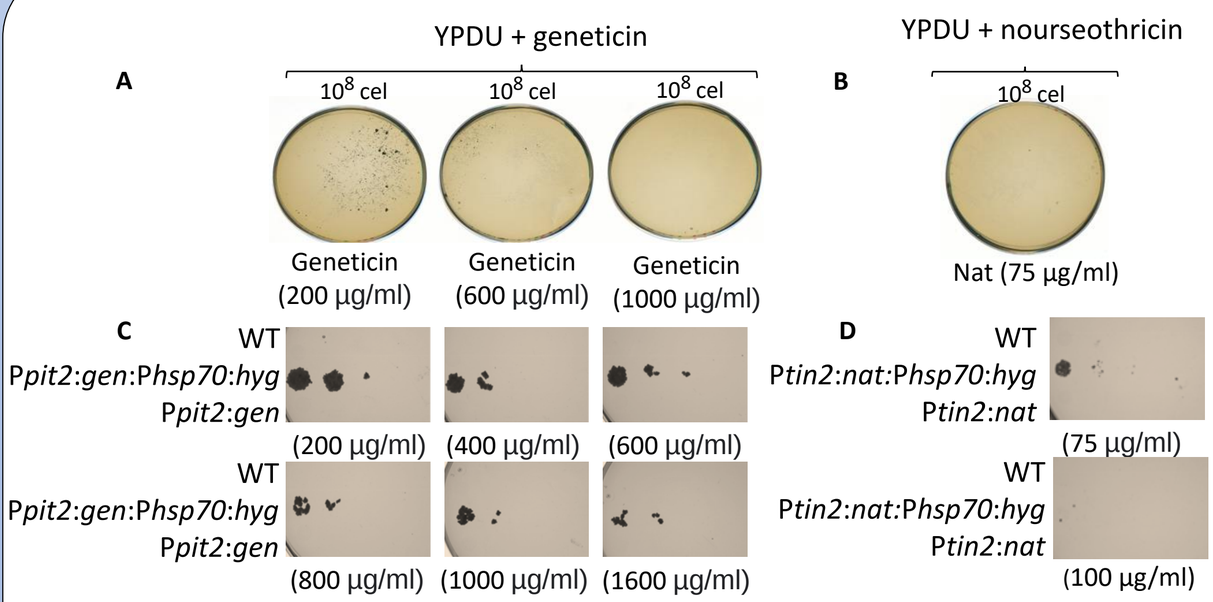

Figure 4. We looked for the generation of spontaneous mutants at different drugs concentrations. This figure shows the optimal drug concentration where no spontaneous mutants grow, 75 µg/ml for nourseothricin and 1000 µg/ml for geneticin. **A**. We plated 10^8 cells of P*pit2*:*gen* mutant on YPDU + geneticin plates. **B**. We made the same assay as in figure A but with P*tin2*:*nat* mutant in YPDU + nourseothricin plate. **C.** It demonstrates the different antibiotic concentration at which P*pit2*:*gen*:P*hsp70*:*hyg* can grow. **D**. Same assay as figure C but with P*tin2*:*nat*:P*hsp70*:*hyg*

CONCLUSIONS

During this work, we were able to successfully obtain the strains P*pit2*:*gen* and P*tin2*:*nat*, which have the antibiotic resistance in the silenced effector cluster. We observed that these strains do not produce spontaneous mutants at certain drug concentrations, but they can grow at those concentrations when the resistance gene is de-repressed, indicating that this strains are ready to perform mutagenesis in the hopes of discovering chromatin silencers.

REFERENCES

[1] Mendgen, K. & Hahn, M. (2002). Plant infection and the establishment of fungal biotrophy. Trends Plant Sci. 7, 352–356.

[2] Schmitz, L., Kronstad, J. W., & Heimel, K. (2020). Conditional gene expression reveals stage-specific functions of the unfolded protein response in the Ustilago maydis–maize pathosystem. Molecular plant pathology, 21(2), 258-271