Disruption of Mating-type Switching in Industrial Strains of *Saccharomyces cerevisiae* Strains by CRISPR/Cas9

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Abstract

Yeast, *Saccharomyces cerevisiae*, is an important organism used to make a multitude of products for human consumption including bread, beer, and wine. There are different strains of yeast that exhibit a variety of flavor profiles and ethanol content during alcohol fermentation due to their differing genetics. To exhibit new characteristics and flavor profiles in alcoholic beverages, genetic changes to existing yeast strains must occur. I plan to use cell fusion of two strains to make a novel hybrid strain. For successful cell fusion, both strains must sporulate to produce stable haploids that cannot switch their mating type. I am interested in using English cider for cell fusion; however, it has the ability to switch its mating rendering impossible to use. I plan to solve this problem by using CRISPR/Cas9 technology to introduce a mutation into the gene that allows the mating type switch to occur. Once a mutant is identified, cell fusion experiments of English cider with banana sake yeast will create novel yeast hybrids. These hybrids can be used to make new alcoholic beverages that exhibit good flavor profiles while also having a high ethanol content.

Introduction

Yeasts are a widely used organism that makes the creation of alcoholic drinks easy and affordable. Companies that brew alcoholic drinks using yeast are always looking for ways to improve their products by creating new flavors with different levels of ethanol content. The dream of any consumer would be a drink with high ethanol content with a pleasant taste. The creation of novel yeast strains will change the future of the alcohol industry. To make new yeast strains, genetic changes must occur. Several technologies currently being used to cause genetic changes include mutation, spheroplast (protoplast) fusion, electrofusion and transformation, and cell fusion (hybridization)[1]. Cell fusion imitates the natural yeast life cycle therefore making it the most natural technique.

Yeast cells can be found in three different cell types: α-haploid, a-haploid, and α/a diploid. The α/a diploid cells cannot mate with each other, however, they can sporulate into four haploid cells through meiosis producing two α-haploids and two a-haploids. The two opposing haploid mating types can mate through a process called cell fusion in order to become one α/a-diploid cell[2]. To fuse two different yeast strains, the haploids must be isolated into their respective mating types. Homothallic strains have the ability to switch their mating type while in haploid form, which allows them to mate with the other cells with which they were isolated. This instability of haploids mating type makes homothallic strains impossible to use for cell fusion with another yeast strain's haploid.

Two requirements must be met if any yeast strain can be used for cell fusion: 1. the yeast must sporulate into haploids and 2. those haploids must exhibit mating type stability (that is, no mating-type switch). Several industrial strains have been tested and characterized in our lab for those two requirements (Table 1). Six strains successfully sporulated and only two of those six could switch mating type. The strain that I will be using is the English cider strain, which is able to sporulate stable haploids, but the haploids switch mating types thus cannot be used for fusion with other haploids. This mating type switch is

Yeast strain	Sporulation	Mating-type switch
English cider	yes	yes
Chardonnay wine	yes	no
Banana sake	yes	no
sake	yes	yes
Sweet mead	yes	no
American wheat	no	N/A
Irish ale	no	N/A
Italian red	no	N/A
Octoberfest	no	N/A
Abbey IV ale	yes	no

Table 1: Testing of sporulation and mating-type switch of yeast strains.

possible because of a site-specific endonuclease

Position	209	238	279	369	413	417	485	486	492	506	527	565	667	668	789	836	1009	1184	1214	1377	1424	1635	1710	1740	1741	1759
Wild-type *HO*	G	C	C	G	C	T	T	T	T	T	T	A	G	G	A	A	G	A	T	C	A	C	C	C	G	T
English cider	G	C	C	G	C	T	T	T	T	T	T	A	G	G	A	A	G	A	T	C	A	C	C	C	G	T
Mutant *ho*	G	T	A	A	C	T	T	T	T	T	T	G	A	G	A	A	A	A	C	T	T	T	T	T	G	T
Banana sake	G	C	A	A	C	T	T	C	T	T	T	G	G	G	G	A	A	A	T	C	T	C	A	T	G	T

Figure 2: DNA sequence positions of wild-type *HO* gene compared to mutant *ho*. English cider strain exhibits *HO* genotype, whereas Banana sake exhibits *ho* genotype.

expressed from the *HO* gene[3]. In one study, others have isolated many *ho*$^{-}$ mutants from nature and characterized their mating type switch[4]. There were a few common sites of mutation in the *HO* gene in heterothallic cells found in nature that seem to inactivate the endonuclease and thus mating type switch[4]. The main goal of my project is to use CRISPR/Cas9 technology to precisely mutate A->T at the 1424th position of the *HO* gene to create a heterothallic English cider strain.

The strain we have chosen to mutate is English cider because it retains good flavors during fermentation, can sporulate, and is homothallic. The English cider strain has the wild-type *HO* genotype (Figure 2), which is why it's haploids can switch-mating types. Banana sake exhibits high ethanol content and is a possible candidate for cell fusion.

Methods

The CRISPR/Cas9 protocol used was developed by Cold Springs Harbor Laboratory[7]. I first designed a primer set to create pCAS-gRNA plasmid that would express a guide RNA which locates the exact position in the genome that we want to mutate. I also constructed the repair DNA by designing another primer set and PCR reactions. This repair DNA is needed when the yeast double-strand break repair system introduces the A1424T mutation after the Cas9 enzyme cuts the DNA where the guide RNA binds. The purified pCAS-gRNA and repair DNA were transformed into competent English cider yeast and screened for mutants. The plasmid had an antibacterial resistance gene for kanamycin. We used YPD+ Kanamycin plates to screen for cells that had successfully taken-up and expressed the plasmid. Possible candidates were grown in YPD to increase the cell count, then Quiagen DNA miniprep kit was used to lyse and extract the DNA from the cells. PCR was performed to amplify the *HO* gene and some candidates were sent to Eton Biosciences Inc. for sequencing.

Results

Previous members of my lab analyzed sequences to find that Banana sake is heterothallic and has the mutant *ho* genotype with the A1424T mutation that we are targeting for English cider (Figure 2). A study that tested the genotypes of several yeast strains found in nature found several common mutations in the *HO* gene in heterothallic strains. One of the most common mutations to the gene found in all but one strain tested was an adenine to thymine at 1424th position (CAT → CTT, His → Leu). According to Meiron *et al*[5], the A1424T mutation causes a loss of function of the *HO* endonuclease that inhibits its binding to DNA. This mutation has also been shown to decrease the mating type switching ability, but did

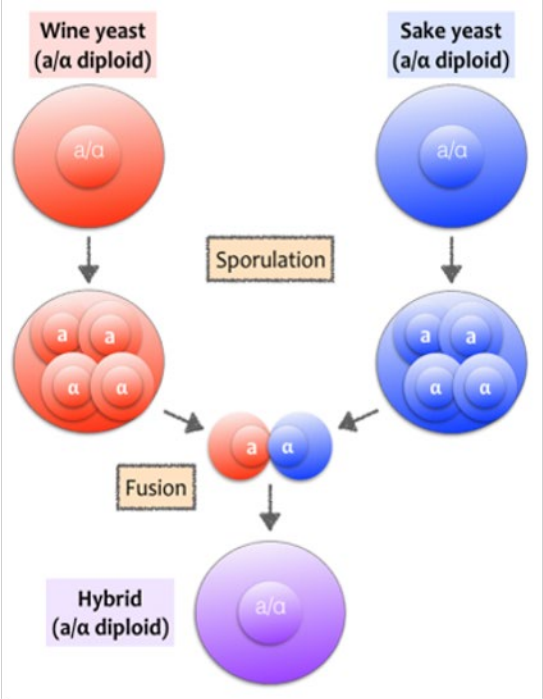

Figure 1: Cell fusion of two yeast strains creating a hybrid diploid.

not eradicate it completely[6]. For these reasons, the A1424T is the target mutation in English cider strain. If this mutation gives a heterothallic phenotype in the English cider yeast, it would add to the overall understanding of the yeast genome. It would also provide an easy solution to creating more natural yeast hybrids from other naturally homothallic strains.

and change possible variables within the protocol that may be producing low yields. CRISPR/Cas9 is a new technology that has not yet been perfected. With repetition and trouble-shooting, I could design a CRISPR protocol that works best with the English cider strain.

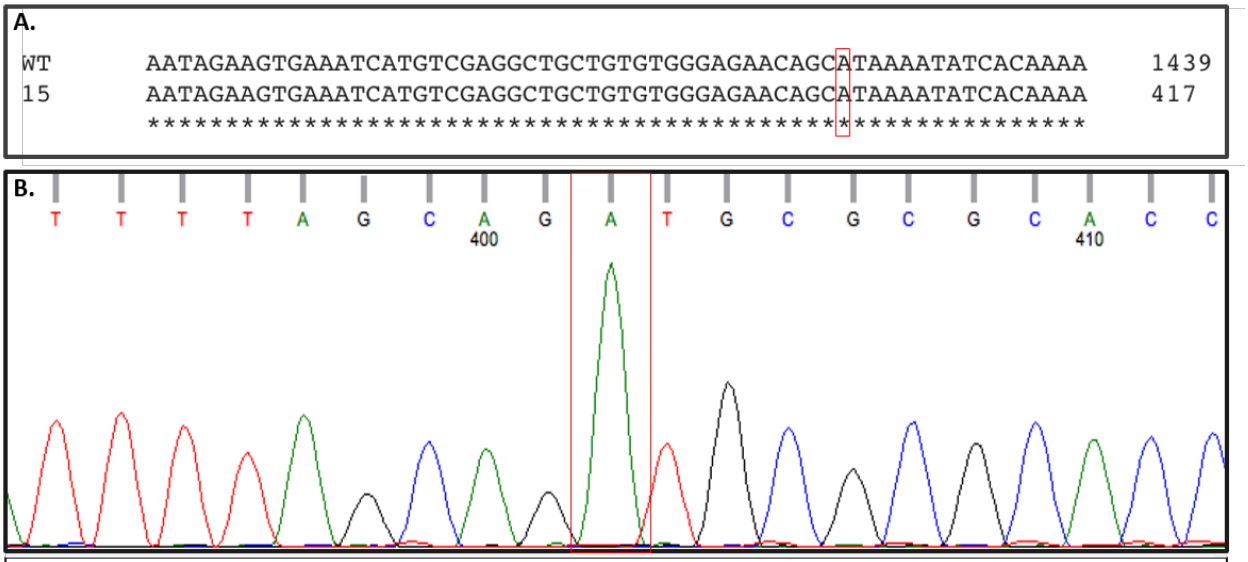

Figure 3: **A.** Wild-type sequence compared to the *HO* gene sequence for candidate #15. The 1424th position is boxed in red. The asterisks indicate a match in the two sequences. **B.** Absorbance spectrum data corresponding to the sequence readout. The 1424th position is boxed in red.

A total of 50 candidates were isolated after screening for expression of the pCAS plasmid. I could not predict the mutation success rate, so I wanted a plethora of candidates to analyze to increase the chances of finding a successful mutant. So far, ten candidates (#1-5, #11-15) had their *HO* gene genotyped for the A1424T mutation. All 10 of those candidates came back negative for the mutation and showed the wild type genotype. In figure 3.A, you can see the wild type genotype compared against the sequencing data from Candidate #15. At the 1424 position, there is still an A rather than a T. The figure shows only the section of the *HO* gene that the target mutation was located and not the entire gene sequence. The absorbance data in figure 3.B shows a strong peak for adenine at the 1424 position to confirm it was an accurate read. The remaining candidates still need to be sequenced to see if any mutations were successfully integrated into their genome.

Discussion

Within the 40 remaining candidates, It is possible that none of the 40 candidates have integrated the mutation. In this case, I would repeat the transformation experiments with the pCAS plasmid

A more desirable outcome would be one of the candidates having a successful mutation. A Successful mutation would give insight to the yeast genome, the functionality of the *HO* gene, and the importance of the sight specific mutation made If a mutation is found, the original colony of that candidate will be streaked on multiple YPD plates to increase the number of cells to be used for later experiments. They will then be tested for the two requirements for cell fusion, which are sporulation and haploid mating-type stability. If they are positive for both requirements, then that mutant candidate can be used for cell fusion experiments with Banana sake strain to create a new hybrid strain. The hybrid strain could be used to create new products as it may exhibit characteristics of both the English cider and Banana sake strain during fermentation. Though CRISPR products cannot legally be sold yet, this has the potential to transform the way companies and consumers think about alcohol production and the safety associated with introducing a single nucleotide targeted mutation.

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