Novel Technique of Achieving sgRNA Efficiency in Mitigating Gene Flow of Transgenic Populus Tremula using CRISPR Cas9

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TABLE OF CONTENTS

ABSTRACT 1

INTRODUCTION 2

METHODS 9

RESULTS 12

DISCUSSION 16

CONCLUSION 18

REFERENCES 19

LIST OF TABLES

Table 1: Mutagenesis Rate of Genes in Conducted Trials 12

Table 2: Comparison of Mutagenesis Rates with Other Techniques 15

Table 3: Target Site Rates of CRISPR Cas9 17

LIST OF FIGURES

Figure 1: Benefits of a Sterile Poplar 5

Figure 2: CRISPR Cas9 Two part System 6

Figure 3: AGAMOUS and LEAFY target sites 7

Figure 4: CRISPR Cas9 Vectors 10

Figure 7: *AGAMOUS 1* results 13

Figure 8: *AGAMOUS 2* results 14

Figure 9: *LEAFY* target site 1 results 14

Figure 10: *LEAFY* target site 2 result 15

ABSTRACT

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas9 is a new and exciting interference technique in biotechnology that could help resolve one of the biggest controversies of our time – the long standing genetically modified organism (GMO) controversy.

My research revolved around a well-known fast growing tree species "poplar"- commonly known as cottonwood. Poplars are widely grown for timber production along wet river banks predominately in the Pacific Northwest, where their exceptional growth rate provides a large crop of wood within just 10–30 years. Poplar is a good candidate for genetic engineering because it is relatively easier to modify. Hence using poplar I will be able to understand and continue research in a shorter timespan thus helping us with our ultimate need to create a safe and nutritional way to produce food to feed the large growing population of the world.

However anti-GMO groups do no approve of a Genetically Modified (GM) poplar being released into the wild because they fear that it will reproduce with natural poplars. In order to facilitate gene flow containment of a GM poplar, an effort was made to develop a sexually sterile poplar clone. For achieving this goal, I chose to target two essential flowering genes, the poplar *AGAMOUS* and *LEAFY* genes. When mutated in *Arabidopsis,* both these genes led to sterile plants with non-functional reproductive organs. I targeted two sites simultaneously in two *AGAMOUS* genes and in the single *LEAFY* gene found in poplar.

I wanted to know how efficient the single guide ribonucleic acid's (sgRNA's) for CRISPR were at generating double stranded breaks in conjunction with the Cas9 protein when targeting two sites. I found that the sgRNA's had a 100% mutagenesis rate in the *AGAMOUS 1* and *AGAMOUS 2* genes and a 93% mutagenesis rate in the *LEAFY* gene. This led to an overall incredible efficiency of 97% using the CRISPR Cas9 system.

I concluded that CRISPR works with an incredible efficiency when targeting both sites in the *AGAMOUS* and *LEAFY* genes thus helping develop a tool for scientists to continue working on genetic research without interfering with the natural population.

INTRODUCTION

Background:

One of the greatest problems we face is our obligation to satisfy the global demand for food as our population continues to grow (Conforti, 2009). It is imperative that we find ways to confront this growing problem, and one branch of science may just provide the answer.

Biotechnology is a rapidly growing field that is constantly finding new ways to approach problems that we face every day. It has been extremely impactful in the fields of agriculture and medicine. Using it, scientists can develop ways to harvest more food from less land and make sure that the food has more nutritional value (Wieczorek, 2003). This technology has been especially important because of the current conditions we face with overpopulation in the world. However, research in this field is prevented due to the infamous GMO controversy.

For years, the topic of genetically modified organisms (GMO's) has been long debated (Abou-Gabal, 2016). Proponents of the science say that it is only being used to make people's lives better. For example, they argue that GMO's have the potential to terminate world hunger (Robbins, 2011). Meanwhile, opponents insist that GMO's could have lasting effects on our health that we are currently unaware of (Burke, 2015).

Methods of gene modification, such as indirect modification, have been used for centuries to enhance crops to better suit our needs. Still, these methods take large amounts of time, even decades, to produce the desired results. Because of the current conditions we face, these methods are no longer an option. Direct modification is more precise and accurate than previous methods, however, because these organisms DNA are being modified directly, people run into moral and ethical complications.

It is important to consider both sides of this controversy and find a way to promote coexistence between both sides. As technology continues to grow, it is crucial that we also find ways to appease both sides of this dispute by mitigating the flow of transgenic organisms into natural environments.

Why Poplar:

Populous, commonly known as "cottonwood" in the Pacific Northwest is an extremely popular tree species found along riverbanks and streams. Of the variety of poplar plants, *populus tremuloides* is a deciduous tree native to North America (Crampton, 2003). Also referred to as aspen, is it the most widely distributed tree in North America. Aspen is especially familiar in Oregon's upper Cascades and eastward. They can be found near the edges of mountain lakes, riverbanks and meadows (Elbert et al, 1971).

Poplar is a good target for further work in biotechnology because of its high economic value and fruit - cotton is grown commercially in over 50 countries with varying climates (Genoscope, 2008). Furthermore, poplar can be considered a good candidate for genetic engineering research because:

- It is a model tree species that is relatively easier to genetically engineer by comparison to other trees because of its known genomic structure , rapid growth and vegetative propagation capacities (Marchadier and Sigaud, 2004)

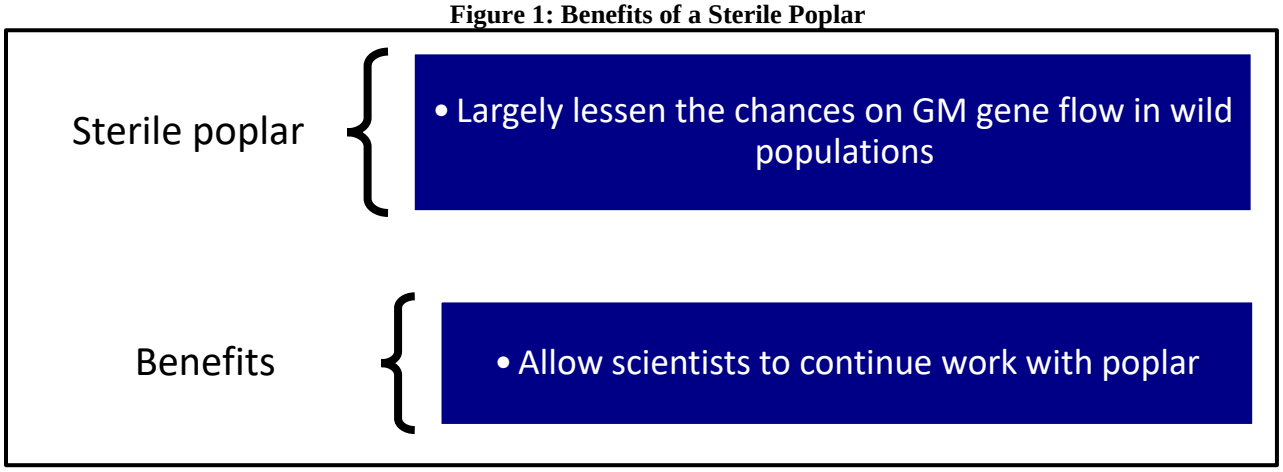
- Poplar has a high economic value as it can be used as a biofuel or for paper and wood
- And it is a quickly growing tree which allows scientists to observe the outcomes of their work in a reasonable amount of time

Opponents of GMO's have an issue with a genetically engineered poplar. This is because poplar reproduces via wind dispersal. This means that seeds from cottonwood can be carried long distances by the wind (Schopmeyer, 1974). If a transgenic poplar was to be put into the wild, gene flow becomes an issue. Gene flow is the transgenes of an organism being released into the wild. This would allow a transgenic poplar to reproduce with a natural poplar and possibly create a hybrid (Snow, 2002).

A sexually sterile poplar could promote coexistence because it would largely lessen the likelihood of a genetically modified (GM) poplar actually being able to reproduce with a natural one. This would allow scientists to continue their work using poplar and relieve anti-GMO groups of the worry of gene flow (Gressel and Valverde, 2009).

In order to stop the gene flow of a transgenic poplar from dispersing into the wild via wind, genes that are responsible for flowering in poplar must be targeted. If these genes were knocked out, then the likelihood of a GM poplar's gene flow being released would be largely lessened. However, the knock-out rate of these genes would have to very high. In order for this to happen, a highly accurate technology would have to be used.

Figure 1: Benefits of a Sterile Poplar



Techniques:

Currently, there are four common techniques for genetic modification that are used:

- CRISPR Cas9 – two part system that creates specific cuts in the targeted DNA
- RNAi - RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules (Satyajit et al, 2014)
- TALEN's (Transcription Activator-Like Effector Nucleases) - artificial restriction enzymes able to cut DNA only where they encounter a specific sequence of nucleotides
- ZFN's (Zinc Finger Nucleases) - engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations

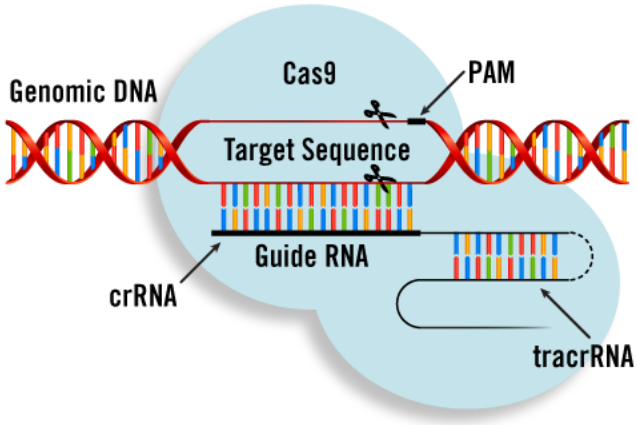
CRISPR:

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas9 technology is a relatively new technique that is shattering the scientific community. It was named the "Breakthrough of the Year" in 2015 by Science Magazine, and since has helped make incredible leaps in the field of biotechnology (*Science*, 2015). It can cleanly cut a targeted section of DNA to

induce insertions or deletions in an organism's genetic code (Ann et al, 2013). It has proven to be more accurate and efficient than past methods such as zinc finger nucleases, ZFN's and RNA interference, RNAi. Additionally, CRISPR Cas9 is cheaper than previous methods, allowing even small labs to use this technology (Xie and Yang, 2013). CRISPR Cas9 is essentially a two part system (Sander and Joung, 2014):

1. The **single guide RNA (sgRNA)** – locates the targeted section of DNA and directs the Cas9 protein to make the cuts
2. The **Cas9 protein** cleanly cuts the targeted section in the DNA by enveloping itself around the double helix and then snipping it like a scissor

Figure 2: CRISPR Cas9 Two part System



<u>Genetic Sequence in Poplars</u>:

In order to make the poplar plant sterile the genes that were responsible for flowering were targeted. In poplar, there are two of them, *AGAMOUS* and *LEAFY* (Colby, 2014). By targeting one of these genes it would be possible to break the flowering chain thus preventing seeds from producing or dispersing via wind. As seen in Figure 3, both *AGAMOUS* and *LEAFY* have 2 target

sites. However, the *AGAMOUS* also experience a double cloning, meaning that there are 4 target sites in the *AGAMOUS* gene.

Figure 3: AGAMOUS and LEAFY target sites

AGAMOUS:
…ATGGAATATCAAAATGAATCCCTTGAGAGCTCCCCCCTGAGGAAGCTAGGAA**GGGGAAAGGTGGAGATCAAG**CGGATCGAGAACACCACCAATC**GCCAAGTCACTTTCTGCAAA**AGGCGCAGTGGTTTGCTCAAGAAAGCCTACGAATTATCTGTTCTTTGCGATGCTGAG…

LEAFY:
…GACATGCACCAGTGAAA**GATCACAGAGAGAGAGACAA**GGGGGCAGATAGATATGGATCCGGAGGCTTTCACGGCGAGTTTGTTCAAATGGGACACGAGAGCAATGGTGCCACATCCTAACCGTCTGCTTGAAATGGT**GCCCCCGCCTCAGCAGCCAC**CGGCTGCGGCGTTTGCTGTAAGGCCAAGGGAGCTATG…

Gene Sequencing Location: **Site 1** and **Site 2**

Hypothesis:

- **By using CRISPR Cas9 will have a 70 – 80% accuracy when targeting two sites in poplar**
    - When used in other plants such as *Arabidopsis* and rice 70 - 80% of the desired target sites were edited with deletion, insertion, substitution, and inversion, displaying high editing efficiency (Gang et al, 2015).

Background Research:

- **Which gene will facilitate flowering prevention in poplar?**

  Candidates:
    - *AGAMOUS 1*
    - *AGAMOUS 2*
    - *LEAFY*

Current molecular strategies for the gene containment of transgenic crops have focused on maternal inheritance and male sterility. Other containment strategies have included:

- Apomixis – the vegetative propagation and asexual seed formation
- Cleistogamy – self-fertilization without opening of the flower
- Gene incompatibility
- Fruit-specific excision of transgenes
- Transgenic mitigation though compromise fitness in the hybrid

However, no single strategy has proved broadly applicable to all crop species and most effective approaches are at best a combination of techniques mentioned (Daniell, 2002).

In efforts to suppress specific transgenes to mitigate floral development, RNAi technique was used (Di et al, 2015). Using a gain- or loss-of-function approach, RNAi has had a 52% mutagenesis rate in *Arabidopsis* when targeting the *AGAMOUS* gene (Travella, 2006).
CRISPR Cas9 work to target mutagenesis in populous in the first generation was conducted, resulting in a 51.7% estimated efficiency Four single guide RNA's were developed to target the phytoene desaturase gene 8 (Liang et al, 2015).

Presently there is no relevant data about using CRISPR Cas9 poplar while targeting the *AGAMOUS* and *LEAFY* genes. Neither is there research on which gene is more efficient when using the CRISPR Cas9 technique. This opens a promising gateway for further investigation of my research.

Research Gap:

Currently, there has been no research in the use of CRISPR Cas9 biotechnology to knockout target genes that could prevent flowering in poplar plants using the *AGAMOUS* and

LEAFY genes. Since CRISPR Cas9 has proved to be one of the most efficient gene editing techniques it is important to consider this tool when doing this research.

Research Problem:

I will address this gap in knowledge by designing an experiment to:

a. Evaluate how efficient CRISPR Cas9 is when targeting the specified genes in poplar and concluding if it is a viable tool for this research.

b. Compare which of the two genes is most efficient when using this new tool

c. Determine whether targeting both target sites in CRISPR Cas9 is efficient and what are the results of doing this.

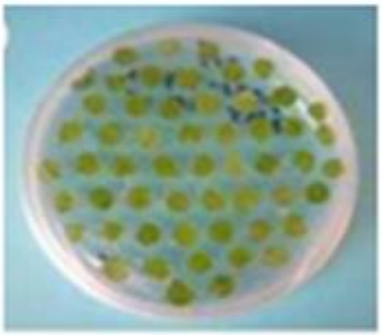
METHODS

Part 1 – Development and Insertion of CRISPR Cas9 Constructs:

Two CRISPR – Cas9 vectors were developed to target and test regions in the *AGAMOUS* and *LEAFY* genes:

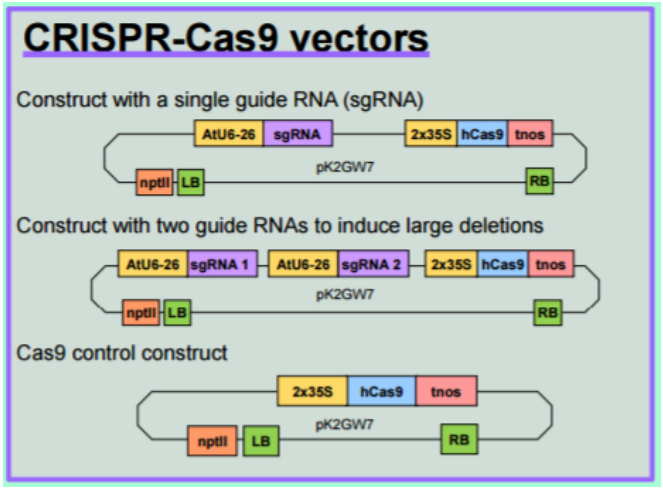
- A construct with one single guide RNA (sgRNA) for smaller deletions
- A construct with two sgRNA's to induce larger deletions

After building the constructs, the poplar tissue was transformed using *Agrobacterium* with the specified constructs in them. Then, using a series of media with the necessary sustenance for the leaf tissue to grow, the tissue was grown into plantlets that apparently had the CRISPR constructs within them.



Poplar tissue in *Agrobacterium*

Figure 4: CRISPR Cas9 Vectors



Part 2 – DNA isolation and PCR:

In order to ensure that mutagenesis occurred in the plants and to test the effectiveness of the constructs, DNA isolation was performed on each individual plantlet. Small leafs from plantlets were harvested into centrifuge tubes. In them, 200ul of extraction buffer and 50 ul of 5% N-lauroylsarcosine were inserted. These samples were grinded with a tissue drill and incubated for 1 hour at 60ºC. The tubes were centrifuged at max rpm for 20 minutes at room temperature. Then the supernatant was transferred and mixed with 150 ul M NH_4OA and 300 ul isopropanol and inverted approximately 20 times. The supernatant was discarded and the pellets were left to dry. Once completely dry, they were then re-suspended with 200 ul 10mM Tris.

Once the DNA was extracted, the target regions for the *AGAMOUS* and *LEAFY* genes were amplified using the Sanger Polymerase Chain Reaction (PCR) technique and a thermocycler. Promoters AG1seq-F_1 and Promoters AG1seq-R_3 were used to identify target regions in *AGAMOUS 1*. Similar promoters were used for *AGAMOUS 2* and *LEAFY*. After the thermocycler had finished 30 repeats, the samples were loaded into an agarose gel and read under a UV light.

The results of these amplifications were then read on a gel electrophoresis. If clean bands were observed like that of figure 6, then gel purification and sequencing could be conducted.

Figure 5: Unclean bands

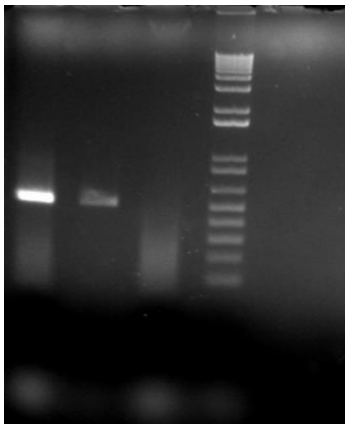
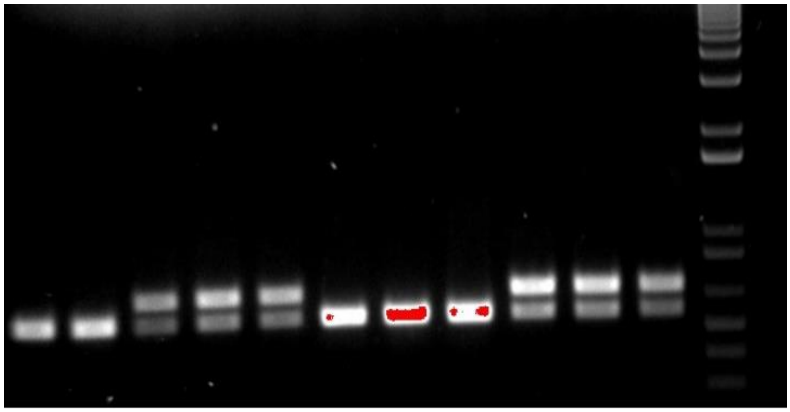


Figure 6: Clean Bands



Part 3 – Purification, Sequencing and Analysis:

Clear bands from the gel electrophoresis were identified under a UV light and cut using a razor blade. Only clean candidates were chosen for further steps. Clean candidates were defined as easy to see with no contamination and thick bands. Figure 6 shows a set of clean candidates while Figure 5 shows poor candidates that were not considered for further testing.

Gel purification was conducted in accordance to the Qiagen QIAquick PCR Purification Kit with no modifications. Samples were then sent to be sequenced at a DNA sequencing center. Once sequenced, the targeted regions of the plantlets' DNA was aligned using DNA alignment software (MEGA) to determine the mutation type, allele frequency and overall effectiveness of insertion technique used.

Safety:

Proper safety protocols included wearing lab coats, rubber gloves, and safety goggles whenever in the laboratory and whenever handling chemicals. Chemical waste and biohazards

were disposed of in a proper manner. All growing mediums and glassware were autoclaved before experimentation.

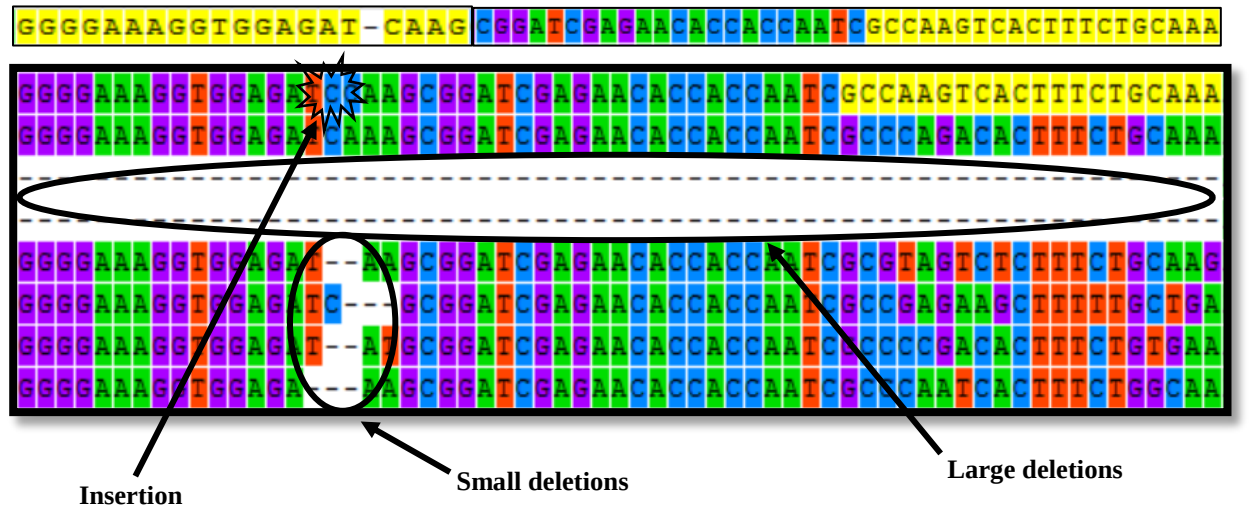
RESULTS

Table 1: Mutagenesis Rate of Genes in Conducted Trials

AGAMOUS 1		*AGAMOUS 2*		*LEAFY*	
Independent events	8	Independent events	8	Independent events	15
Non-mutants	0	Non-mutants	0	Non-mutants	1
Homozygous	3	Homozygous	6	Homozygous	6
Heterozygous	5	Heterozygous	6	Heterozygous	9
Mutation efficiency	100%	Mutation efficiency	100%	Mutation Efficiency	93%

As seen in table 1, *AGAMOUS* had an overall mutation frequency of 100%. Of these 16 independent trials, a total of 5 were a homozygous mutation and 11 were a heterozygous mutation. None of these events were non-mutant.

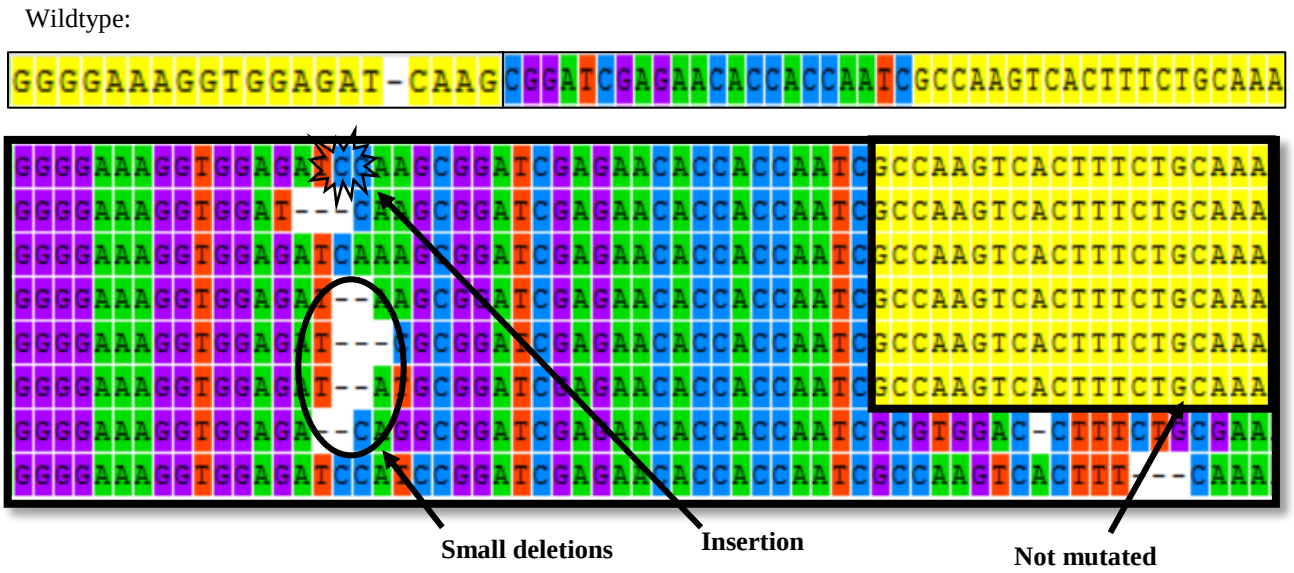
Figure 7: *AGAMOUS 1* results



AGAMOUS 1 gene induced two large deletions, 4 small deletions and two insertions, which can be observed in figure 7. One event was not mutated in target site 1 and then experienced an insertion in target site 2. In the *AGAMOUS 1* gene, CRISPR Cas9 had an overall mutation frequency of 100%. It also had a mutation frequency of 87.5% when targeting two sites at the same time.

AGAMOUS 2 contained 7 small deletions and 1 insertion. No large deletions occurred. This can be seen in figure 8. 6 events in target site 1 were not mutated and then changed in target site 2. In the *AGAMOUS 2* gene, CRISPR Cas9 had an overall mutation frequency of 100%. However, when targeting two sites simultaneously, it had a mutation frequency of only 25%.

Figure 8: *AGAMOUS 2* results



As seen in table 1, the *LEAFY* gene had an overall mutation efficiency of 93%. Of the 15 independent trials tested, 6 were homozygous mutations and 9 were heterozygous mutations. One event was also not mutated. In *LEAFY*, large mutations occurred in between target site 1 and 2. All mutated trials had a mutation in both target sites. One event also had a small deletion and insertion which can be observed in figure 9.

Figure 9: *LEAFY* target site 1 results

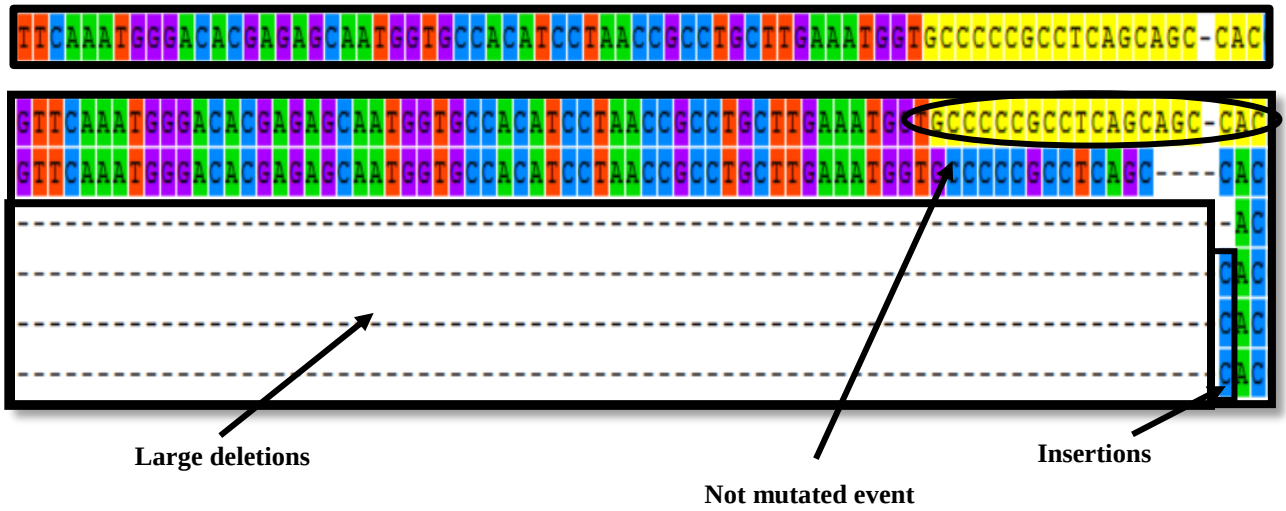
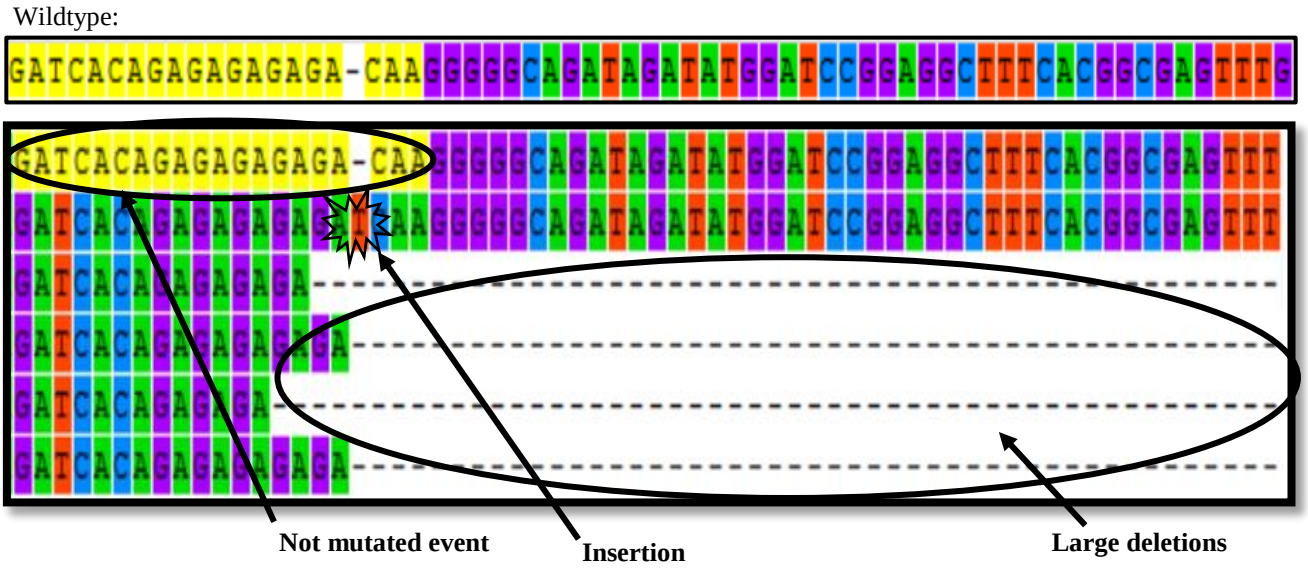


Figure 10: *LEAFY* target site 2 result



CRISPR Cas9 had an overall mutagenesis rate of 97%. In *LEAFY* it had an efficiency of 93% and in *AGAMOUS 1* and *AGAMOUS 2* it had an efficiency of 100%. When targeting two sites at the same time, the technique has an efficiency of 77%. Below is a table of the overall mutagenesis rates of common gene editing techniques used.

Table 2: Comparison of Mutagenesis Rates with Other Techniques

Technique	**Gene**	**Plant**	**Mutagenesis Rate**
CRISPR-Cas9	*AGAMOUS*	Poplar	**97%**
RNAi[1]	*AGAMOUS*	Arabidopsis	52%
Transcription activator-like effector nucleases (TALENs)[2]	Not specified	Not specified	10-50%
Zinc-finger nucleases (ZFNs)[3]	*LEAFY* and *AGAMOUS*	Poplar	2%

Gaj et al, 2013[1,] Shijia et al, 2012[2], Travella et al, 2006[3]

DISCUSSION

The development of efficient and reliable ways to make precise, targeted changes to the genome of living cells is a long-standing goal for biomedical researchers. (Cong L., et al. (2013) *Science*). CRISPR Cas9 is an efficient technique for genetic engineering. My results proved that CRISPR Cas9 can efficiently mutate target sites in both the *AGAMOUS* and *LEAFY* genes. Because it has such a high mutagenesis rate, it can seriously be considered as a technique to be used when trying to prevent floral development in poplar or other plants (Lin Hui et al, 2016).

As shown in table 2, CRISPR Cas9 has a high mutagenesis compared to other techniques including:

- Transcription activator-like effector nucleases (TALENs)
- Zinc Finger Nucleases (ZFN's)
- A popular technique commonly used before the discovery of CRISPR Cas9 – RNAi.

Additionally, compared to RNAi, CRISPR Cas9 has less off target effects (Chuang and Meyerowitz, 2000). Before the discovery of CRISPR Cas9**,** RNAi became a laboratory staple enabling inexpensive and high-throughput interrogation of gene function **(**Elbashir et al, 2002 *Methods)*. However it provided only temporary inhibition of gene function and unpredictable off-target effects **(**Alic et al, 2012 *PLoS One).*

CRISPR Cas9 is very efficient in targeting two target sites at the same time, as seen in table 3. This is important because previous methods could only target one site at a time, thus providing no opportunity for comparison with other techniques.

Table 3: Target Site Rates of CRISPR Cas9

Technique	Target Sites	Efficiency
CRISPR Cas9	2	85%

My results concluded that CRISPR Cas9 was a better candidate in the *AGAMOUS* genes as opposed to the *LEAFY* gene. *AGAMOUS* had a 100% mutagenesis rate in both of its genes while *LEAFY* had a 93% mutagenesis rate. By using the gene that is more efficient with CRISPR Cas9, it will ensure that there will be a lessened likelihood of non-mutants to occur. This is prominent information because it will help determine what genes to target when preforming sterility trials in the field. Further trials will need to be conducted to confirm these results.

My results can help mitigate a large portion of the poplar GMO controversy. By preventing flowering development in transgenic poplar plants, the probability that the poplar's gene flow will be released into the wild will be severely reduced. My results have proved that CRISPR Cas9 is an efficient technique in poplar plants and should be used when knocking out *AGAMOUS* and *LEAFY* genes.

Furthermore, I have concluded that the *AGAMOUS* gene is the best target for these trials because it has a higher mutagenesis rate than *LEAFY* does while using CRISPR Cas9, and that it is best to target two sites simultaneously because it yields high mutation rates.

My results show relatively higher mutagenesis rates than other CRISPR Cas9 experiments conducted. Also, previous published literature has never focused on targeting the *LEAFY* and *AGAMOUS* genes while using the CRISPR Cas9 technique. My results are easily distinguishable from other works because I have determined the single guide RNA's (sgRNA's) for CRISPR were very efficient at generating double stranded breaks in conjunction with the Cas9 protein when

targeting two sites in these genes. I also determined which of these genes were better to use when using this technique.

Some argue that by modifying a transgenic tree more, we are only adding to the GMO crisis. However, by mitigating the gene flow of these plants, it would allow poplars to be planted on commercial fields or other controlled areas and not spread to other areas. As our population grows, we will continue to use new techniques of genetic modification to better suit or needs. With this, we will need to continue our research to keep this technology in a controlled environment as to not interfere with our natural vegetation. By using a clean, efficient system such as CRISPR Cas9, we can ensure that the chances of gene flow spreading past their controlled areas will be severely reduced.

CONCLUSION

My research concluded that CRISPR Cas9 is an efficient system that delivers an incredibly high mutagenesis rate in the *AGAMOUS* and *LEAFY* genes. This technique worked at 97% efficiency when targeting two sites in the same gene. It also shows a promising future in the field of genetic engineering because of how efficient it is. Targeting both sites in *AGAMOUS* gene proved helpful because target site 1 was not mutated as frequently as target site 2, but given the high mutation efficiency of the sgRNA targeting target site 2, the overall mutation efficiency for both *AGAMOUS* genes was high and when both sites are targeted, a large deletion can occur. I determined that the *AGAMOUS* gene was more efficient than the *LEAFY*.

- CRISPR is an extremely promising and accurate technology
- Targeting both target sites yields high mutation rates

- In genes *AGAMOUS 1* and *2*, site 2 was more efficiently mutated than site 1
- *AGAMOUS* gene was more efficiently mutated than the *LEAFY* gene
- Large deletions between the two CRISPR regions can occur when targeting two sites

Further work includes more testing of events to confirm mutagenesis rates of each gene and overall efficiency of CRISPR Cas9. This can be done by following the same procedure that was done during this experimentation. Once confirmed, plants must be chosen for field trials. Once trials are conducted, phenotypes should be observed, especially signs of floral development.

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