Characterizing the Function of Gene Editing Components in Tropical Maize

Kailee M. Ishikawa

Department of Molecular Cell Biology, University of Hawai‘i at Mānoa

Honors Thesis Proposal

Dr. Michael Muszynski

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Abstract

Gene editing technology has revolutionized our ability to make precise genetic changes to fix diseases and improve traits, but the efficiency of this technology has not been tested in important grain crops like tropical maize. My project aims to use various molecular techniques to analyze the inheritance and function of different gene editing components transformed into tropical maize. I will analyze tropical maize plants that were genetically transformed with different molecular elements including but not limited to gene editing reagents, herbicide resistance genes, antibiotic resistance genes, and colored fluorescent proteins. I will analyze two types of transformed maize plants: individual first transgenic (T1) generation plants and second transgenic (T2) generation families that are the progeny of T1 plants. My objectives are to determine if all the genetic components that were used for the initial transformation segregate in the T1 generation and if those components are functional. My objectives for the T2 families are to determine the inheritance pattern of the genetic components in the T2 progeny and if those components are still functional in that generation. To test for the presence and inheritance of specific genetic elements, I will extract total genomic DNA, use the genomic DNA as a template to amplify each genetic element using the polymerase chain reaction (PCR), and analyze the amplification products using agarose gel electrophoresis. My functional assays will include testing the plants for herbicide or antibiotic resistance and visualizing colored fluorescence in leaf tissue. Through observational and molecular analysis, I expect to determine the inheritance and function of the different elements used for gene editing in tropical maize.

Keywords: tropical maize, gene editing, transgenic plants, maize transformation, inheritance and functionality, DNA extraction, PCR, agarose gel electrophoresis

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Specific Aims

Through my project, I will better understand the use of new genetic modification technologies, such as genetic transformation and gene editing, on tropical maize. I will conduct functional assays on T1 and T2 transgenic generation plants to test the functionality of their genetic transformation components, which are herbicide resistance, antibiotic resistance, and the presence of colored fluorescence. I will also utilize molecular techniques such as DNA extraction, PCR, and agarose gel electrophoresis to analyze the inheritance of the different gene editing components. My results will then be used to support how molecular biology can be utilized to improve crop production.

Significance

Maize is a globally important staple cereal crop. In fact, maize, rice, and wheat provide 90% of the caloric intake for the majority of the human population (Su et al., 2017). Yet, population increases and environmental stressors influenced by climate change are straining our crop production systems. New tools are required that can speed the development of more sustainable and productive crops that are more stress resistant. Gene editing is one such tool that has the potential to rapidly and precisely make targeted genetic changes resulting in improved agronomic traits. With the help of modern crop breeding technologies, like genetic transformation and gene editing, crops can be improved to provide higher nutritional value, have increased tolerance to abiotic stresses, and have greater resistance to pests and pathogens (Zhang et al., 2018). This project aims to use molecular tools to determine if all the components needed for gene editing in tropical maize are inherited correctly and functioning properly in multiple generations. Successful completion of this project will help to optimize the components used for

gene editing in tropical maize and potentially other crops. It will also contribute to the development of tools with the potential to improve sustainable crop production and food security locally and globally.

Background Information and Literature Review

Using Gene Editing to Drive Favorable Changes in Maize

There is a crucial need for increasing crop improvement rates to meet the rising global demand for food. Due to the rapidly growing population, agricultural pressures, and limited options for cultivable land, modifying crops to increase product yields is needed to ensure food security. Because it typically takes several months for crops to grow to a mature state, generating viable crops that have beneficial genotypic and phenotypic characteristics is vital.

The introduction or elimination of certain traits can be achieved with the CRISPR/Cas9 gene editing system. CRISPR/Cas stands for clustered regularly interspaced short palindromic repeats/CRISPR associated protein system. This biotechnological advancement is a type of immune system discovered in bacteria and has been adapted into a gene editing tool that makes site specific double-stranded DNA cuts. The RNA molecules crRNA and tracrRNA recognize a specific sequence or motif known as PAM and guide the Cas9 enzyme to a target DNA sequence adjacent to the motif. Then, Cas9 makes a cut in the double-stranded genomic DNA. Through non-homologous end joining, which is an error-prone DNA repair mechanism, imprecise repair of the cut DNA leads to the creation of genetic mutations (Figure 1) (Xu, 2013).

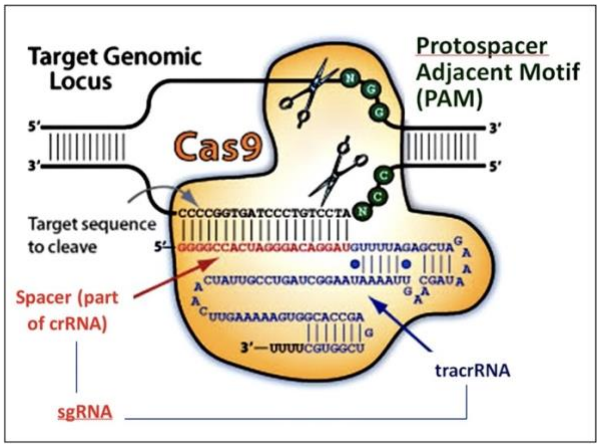

Figure 1. Schematic diagram of the CRISPR/Cas9 system used for gene editing. The target sequence, Cas9 nuclease (in orange), PAM motif, single guide (sg) RNA, and tracrRNA are noted.

In their research, Scheben and Edwards (2018) demonstrated how genome editing can be utilized to make plant breeding more predictable, particularly through the engineering of favorable qualitative and quantitative traits with the help of an allelic series. Allelic series refers to different mutant alleles of a gene that result in a phenotypic range. Each mutant allele contains a single point mutation in different locations within the same gene. Natural changes and chemically or physically induced changes can lead to unpredictable outcomes; however, this method of precise gene editing may be a more efficient way to improve the generation of more predictable qualitative traits. In their article, the authors show how using a Cas9-cytidine deaminase fusion technique can target qualitative traits and increase predictability within traditional breeding pipelines. For this method, called base editing, the researchers designed the Cas9 protein to target and modify the nucleotide bases of a target sequence, but not cut the genomic DNA. Because the Cas9 protein is fused with the cytidine deaminase enzyme, cytosine nucleotides within the target sequence are randomly converted to thymine. Targeting different regions of the gene using this method can lead to a variety of phenotypic changes, some of which

can improve crop production. Therefore, by using CRISPR/Cas-induced allelic series in *cis*-regulatory regions or non-coding regions that regulate gene transcription, researchers can rapidly create desirable traits in the crops, modifying them to be more resilient to agricultural stressors and allowing them to produce higher yields with the potential for fewer agronomic inputs (Scheben & Edwards, 2018).

Maize Transformation and Morphogenic Regulators

The process of maize transformation involves introducing foreign genetic material into the maize plant genome using genetic engineering techniques. Through biolistic particle bombardment or *Agrobacterium*-mediated transformation, maize can be genetically modified to carry new genetic information in its genome which can produce favorable traits such as pest/pathogen resistance or greater stress tolerance, allowing for increased crop yields and quality. Ishida et al. (1996) first described the *Agrobacterium*-mediated transformation method, which is now the preferred method for maize transformation. Generally, this process entails inoculating immature zygotic maize embryos with a standard binary vector system, which contains a transfer DNA (T-DNA) with the genes of interest and a helper plasmid. Additionally, a selectable marker like herbicide or antibiotic resistance is used to determine whether the genes of interest are properly incorporated in the genome of immature maize embryo cells, which are the typical explant tissue used for maize transformation. Following *Agrobacterium* infection using the standard maize transformation method, embryos that express the selectable marker are placed in a nutrient-rich environment, with a selective agent like an herbicide, in order to initiate somatic embryo generation that carry the inserted T-DNA. After a period of culturing, the herbicide or antibiotic resistant somatic embryos are transferred to regeneration media to induce the development of shoots and roots. After a complete plantlet is regenerated, it is placed in soil

and allowed to grow into a mature, fertile T0 plant. To test for inheritance and function in the next generation, the T0 plant is crossed with the wild type inbred of that transgenic plant to produce T1 progeny that are then assayed. This laborious process, shown in Figure 2 below, takes nine months or more to complete. To speed the generation of improved crops, faster and more efficient transformation methods are needed.

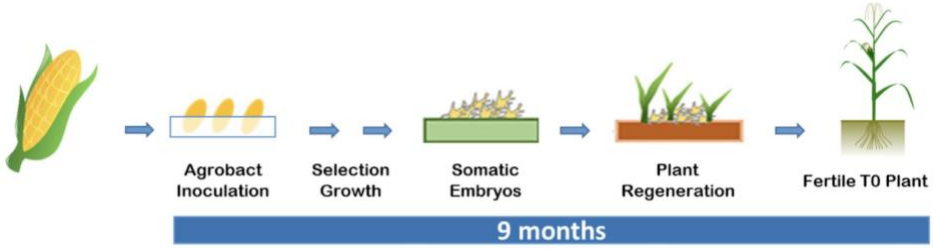

Figure 2. Standard method for *Agrobacterium*-mediated maize transformation. Image credit: Dr. Michael Muszynski.

Many cereal crops like maize, rice, wheat, and barley have been modified by genetic transformation technologies. Due to these genetic advancements in agriculture, there is an increased need for more efficient transgenic techniques that are genotype-independent. In other words, researchers are trying to find an improved transformation protocol that can be used for all genotypes of a specific crop. Currently, available transformation methods only allow for the successful regeneration of some plant species, and only certain varieties or cultivars within a species. The plant transformation process has many limiting factors including genotype, varietal dependence, explant sources, and specific bacterial culturing methods. In addition, it is also labor intensive, time consuming, and expensive. Morphogenic regulators, also called morphogenic genes, are powerful tools that can be used to overcome difficulties in plant transformation. They allow for the direct generation of multiple somatic embryos within a few days directly from the explant tissue source. This rapid induction of somatic embryos and fast plantlet regeneration

reduces the time frame of this process from nine months to four months (Mookkan et al., 2017). An overview of *Agrobacterium*-mediated maize transformation using morphogenic genes is shown below in Figure 3.

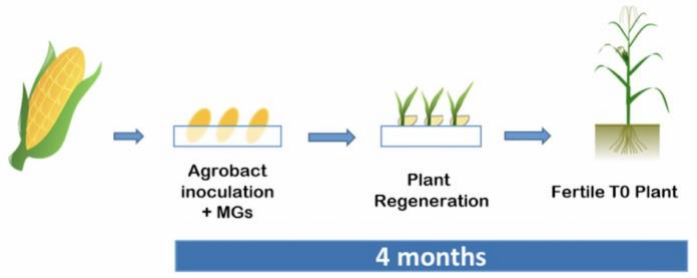

Figure 3. *Agrobacterium*-mediated maize transformation with morphogenic genes. Image credit: Dr. Michael Muszynski.

Thus far, morphogenic genes have significantly simplified the steps needed in transformation by decreasing plant regeneration time and allowing for genotype-independent transgenic T0 plant recovery with high frequencies (Anand et al., 2018). For example, Lowe et al. (2016) confirmed that the overexpression of the morphogenic genes *Wushel2* (*Wus2*) and *Baby boom* (*Bbm*) in some monocot crops resulted in stimulated growth of embryogenic tissue. Additionally, they found that this enhanced transgenic plant recovery, specifically for marginally transformable plant varieties of maize, rice, sorghum, and sugarcane. The researchers found that the overexpression allowed for the direct *Agrobacterium*-mediated transformation of embryo axes derived from mature seeds or leaf segments without the need for tissue culturing. When studying the early growth phenotypes of the transient expression of *Wus2* and *Bbm*, they observed that growth stimulation was highly pronounced with constructs that were cobombarded with the morphogenic genes, while using maize-optimized GFP (moGFP) as a selectable marker. When the morphogenic genes were expressed for longer periods of time, the authors noted that this contributed to the transgenic plant recovery due to their effects on growth rates. Improved

transformation frequencies were also observed across some of the inbred lines, meaning that the longer-term use of these genes extended their ability to transform some of these lines, causing them to go from marginally useful levels to practical levels for transformation (Lowe et al., 2016).

Although morphogenic genes are used to trigger rapid somatic embryo induction, they must be excised to allow for normal growth and development of regenerating plantlets and mature plants. Retention of the morphogenic genes in the inserted T-DNA during plant regeneration can lead to undesired pleiotropic effects, like distorted leaf growth, shoot overproliferation, and sterility. Precise excision, however, requires a lot of skill, can be difficult to time, and is not always achieved completely. In their study, Hoerster et al. (2020) describe a technique called altruistic transformation, in which the excision of the morphogenic genes is no longer required, making the transformation process more efficient and producing higher regeneration frequencies. For altruistic transformation, the researchers used two *Agrobacterium* strains, one with a T-DNA binary plasmid with a *Wus2* expression cassette, and the other with a selectable T-DNA binary plasmid. By ensuring that the morphogenic genes are expressed on a different T-DNA than the selectable T-DNA, they eliminate the need to excise the morphogenic genes before plant regeneration. This further increases the frequency of regenerated stable T0 plants that only contain the selectable T-DNA and not the *Wus2* gene, which can significantly affect regenerated plant growth (Hoerster et al., 2020).

In 2018, Lowe et al. sought to improve the maize transformation process by finding suitable transcription promotors to regulate the expression of morphogenic genes. Although the morphogenic transcription factors *Bbm* and *Wus2* can be utilized to increase the efficiency of transformation, their constitutive expression, which refers to the genes that are transcribed at a

constant level, can introduce sterility and phenotypic abnormalities, like wrinkled leaves and thickened roots with decreased function, to the regenerated plants. To find a way around this, the researchers conducted a genome wide search to find promoters that could drive specific patterns of expression. Based on their search, the researchers found that only one gene, the phospholipid transferase protein gene (*Zm-PLTP*) met their criteria. The authors tested the morphogenic transcription factors together and independently and also observed the effects of altering whether expression in certain parts of the plant was restricted. They discovered that using a combination of the *Zm-Axig1* promoter driving *Wus2* and the *Zm-PLTP* promoter driving *Bbm* resulted in normal plants that did not require any excision. Their findings allowed for the regeneration of fertile plants without the need to excise any of the morphogenic genes used in the cassette (Lowe et al., 2018).

DNA Extraction and Purification

The presence of gene editing components in plants can be analyzed through PCR and agarose gel electrophoresis. However, the genetic material must first be obtained and purified using genomic DNA extraction techniques. Regardless of the sample type from which the DNA is being extracted from, all DNA extraction protocols follow several basic steps: tissue disruption, physical and chemical cell lysis, addition of reagents and buffers to provide optimal conditions for the DNA to bind to the spin column membrane, washing of contaminants and enzyme inhibitors, and addition of an aqueous buffer to elute DNA from the membrane into a new vial to store for downstream applications. This process is shown in Figure 4.

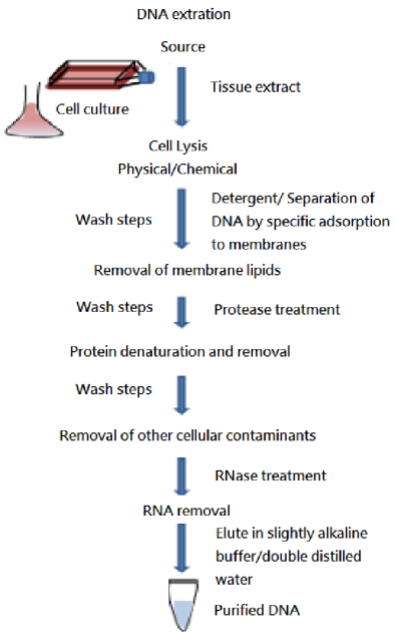

Figure 4. Basic steps involved in DNA extraction methods (Dhaliwal, 2013).

The accuracy of PCR results is greatly influenced by the quality and purity of the isolated genomic DNA. Although various commercial kits are available for DNA extraction, they do not all generate products of equal PCR detection sensitivities. Kit selection is determined based on several factors: the origin of the sample, how the sample is prepared, what the isolated DNA will be used for, what the sample contains, the sample size, the expected DNA yield, and the experience of the user. All successful DNA extractions should result in sufficient amounts of high quality and pure DNA without the presence of any contaminants. The purity of the isolated DNA can be evaluated using ultraviolet absorbance. If the absorbance ratio at 260 and 280 nm (A260/A280) is over 1.8, then this indicates that the sample is contaminated. There are several different commercial kits available that aid in the isolation of DNA from microbes, animal cells and tissues, plant cells and tissues, blood, and combinations of these. Many differences exist between the extraction kits mentioned due to the variations in sample types. Therefore, it is vital that the extraction kit and the reagents being used to isolate the DNA from the samples are

chosen carefully to ensure that the quantity, quality, and purity of the DNA extractions are viable for other applications and processes (Dhaliwal, 2013).

Polymerase Chain Reaction

Polymerase chain reaction is a quick and efficient molecular biology tool that is used to exponentially amplify specific DNA segments millions to billions of times so that it can be further studied. It relies on thermocycling, which involves the repetition of three stages: denaturation, annealing, and extension/elongation. At a high heat, usually around 95°C, the denaturation of DNA can occur, causing the double stranded molecule to split into 2 single strands of DNA. When the temperature is decreased to around 5°C below the melting point of the molecule, primers can anneal to targeted complementary sequences on the single stranded DNA templates. The temperature can then be increased to 72°C, which is the optimal temperature for DNA polymerase activity, to allow for the extension or polymerization of new DNA strands from the template strands, as shown in Figure 5.

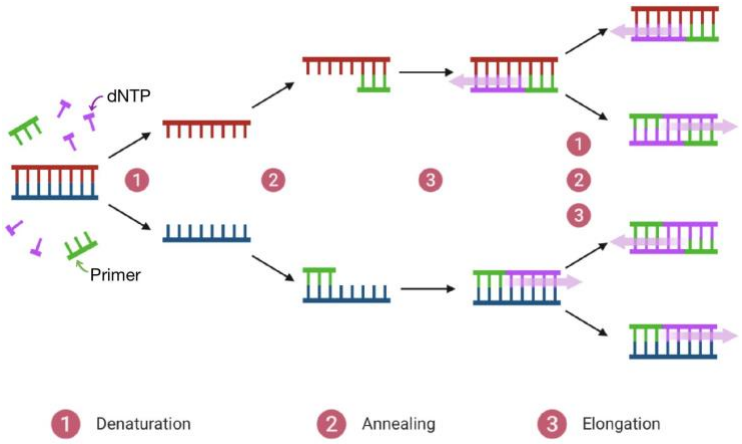

Figure 5. Schematic diagram of PCR cycles. Created with BioRender.com.

In a study conducted by Safaei et al. (2019), the researchers sought to test the use of conventional PCR as an accurate and reliable screening method for detecting genetically modified (GM) rice. Compared to other screening methods, DNA-based PCR has higher

specificity and can be used as a cost-effective assay for detecting transgenic events in crops. GM crops can be analyzed at a molecular level to determine the presence or absence of certain gene editing components. To do this, the researchers extracted the DNA from their samples using the DNA Extraction kit from Plant Materials, evaluated the concentration and purity of their isolated DNA using a NanoDrop spectrophotometer, performed PCR on their samples, ran their PCR products on an agarose gel using gel electrophoresis, and then visualized and analyzed their gel results using a UV transilluminator. For their positive controls, the authors used samples with known genetic elements and also sequenced the primer target sites to verify the specificity of their primers. The positive controls were used to ensure that their protocols and reagents were valid and working properly. For their negative control, they used sterile water instead of genetic material for PCR amplification. This was to determine whether the reagents used were contaminated. If the negative control PCR product did not amplify, then there would be a reduced probability of having false positive results. Based on the overall results of their study, the authors concluded that the methods they used for the DNA extraction, conventional PCR, and gel electrophoresis can be used to accurately screen crops for GM ingredients (Safaei et al., 2019).

Agarose Gel Electrophoresis

Gel electrophoresis is a laboratory method that is used to separate biological macromolecules such as DNA, RNA, and proteins based on their size. With this method, researchers can utilize agarose gels to sort longer molecular fragments and polyacrylamide gels to sort shorter molecular fragments (Lee & Bahaman, 2012). Particularly focusing on agarose gels, choosing an appropriate concentration of agarose in the gel in relation to the size of the fragment is of very great importance and is shown in Table 1.

Table 1. Appropriate agarose concentrations for separating DNA fragments of different lengths (Sambrook & Russell, 2001).

Agarose (%)	Effective range of resolution of linear DNA fragments (kb)
0.5	30 to 1
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

In response to a direct current power supply, the molecules and particles migrate towards the electrode that has the opposite charge of the sample. For the separation of the aforementioned macromolecules, they run from the negative electrode (black) to the positive electrode (red) due to their overall negative charges. As the samples travel through the gel, the agarose gel acts like a sieve through which the molecules can go through. Solidified agarose has a 3D mesh of channels, and the pore size varies with changes to the concentration of agarose powder in the buffer. Different molecules separate based on their size and mass, with the shorter fragments moving faster and therefore further on the gel (closer to red). Longer fragments, on the other hand, have greater frictional drag since they have an easier time getting entangled in the agarose fibers. Sample mobility across the gel is affected by the following factors: gel concentrations, buffer conditions, and sizes/conformations of the samples. Because the composition and ionic strength of the electrophoresis buffers affect the electrophoretic mobility of the samples, it is important to choose a buffer that has an ionic strength suitable for the samples. Using buffers of really low ionic strength may lead to minimal electrical conductivity, whereas using buffers of really high ionic strength may cause the electrical conductance to be too efficient, resulting in a melted gel or denatured sample. The voltage that the gel runs at should be determined based on the distance between the electrodes. Also, as a general rule, the higher the applied voltage, the faster the samples migrate towards the oppositely charged electrode. During the analysis of the

gel, the intercalating dye ethidium bromide can be used as a tool to visualize DNA in agarose gels via a UV illuminator. When UV light is not present, the dye shows up as light, colored markings on the gel, which can be used while the gel is running to monitor electrophoresis progress (Lee & Bahaman, 2012).

Methodology/Research Design

PCR-based assays will be developed to analyze T1 and T2 plants transformed with different molecular elements, including the Cas9 enzyme that cuts the DNA, the guide RNA that targets the Cas9 enzyme to the correct genomic location, an herbicide resistance gene, an antibiotic resistance gene, an additional selectable marker, like a colored fluorescent protein, and the promoter elements driving expression of any of the previous components. To do this, PCR primers will be designed and tested so that they can be used to amplify the specific DNA sequences for each of these elements. T1 plants will then be grown in the greenhouse and leaf tissue samples will be collected after two to three weeks to be used for DNA isolation (Dhaliwal, 2013). PCR assays (Safaei et al., 2019), and gel electrophoresis analysis (Lee & Bahaman, 2012) will be used to assess the presence of each genetic element. DNA sequencing will be used to confirm the integrity of the elements. Herbicide and antibiotic resistance and colored fluorescent protein assays will be conducted to assess the functionality of these genes. T1 plants will be crossed to produce T2 progeny, which will be grown and analyzed using the same methodology applied to the T1 plants. New PCR primers will be designed and tested for the amplification of novel genetic elements used for new transformation studies. A new generation of T1 plants will be grown and analyzed, again using the same methodology. These T1 plants will be crossed to produce new T2 progeny, which will be grown and analyzed as previously described. The results

from these assays will be summarized for my Honors thesis and Undergraduate Showcase for Research and Creative Work presentation.

Laboratory Trainings Completed

As a student at the University of Hawai‘i at Mānoa, I comply with all accepted research protocols. I have completed the required online UH Biosafety courses for my project: ORC 101 (Initial General Biosafety) and ORC 103 (Transportation of Biological Substances Initial and Refresher). My project does not include any animal or human experimentation. It does, however, involve hazardous chemicals and working with plant material. Special precautions will be taken when dealing with the hazardous and biological materials. I have completed the UH Safety Solutions Laboratory Hazard Assessment and the Hazardous Waste Generator Training. Additionally, I have completed the UHM EHSO Lab Safety Checklist and Training and the online training modules for Responsible Conduct of Research (Biomedical and Biological) and Institutional Biosafety Committee (IBC). My laboratory supervisor Dr. Muszynski has an approved IBC registration (B22-100361), and the laboratory passed its annual biosafety inspection in September 2022.

Role of the Researcher

For this project, I will be the sole administrator, responsible for writing the proposal, planning out the experiments, conducting the laboratory work, compiling data, and analyzing the results under the guidance of my laboratory supervisor and faculty mentor Dr. Michael Muszynski who has assigned this project to me. Under his supervision, I have written this proposal that describes my research goals and how I plan to accomplish them. During my time in Dr. Muszynski’s laboratory, I have learned several molecular biology techniques including the following: DNA extraction, PCR, and gel electrophoresis. I have also gained experience in

growing, measuring, and dissecting maize plants for phenotypic and molecular analysis. By completing this project, I expect to gain more knowledge on maize genetics and gene editing, as well as different laboratory techniques and molecular analysis. Being an integral part of the Muszynski laboratory will also allow me to develop valuable skills such as professionalism, better time management, self-confidence, improved presentation skills, and critical thinking.

Resources and Materials

All resources and materials will be available and provided by the Muszynski Laboratory in the St. John Plant Sciences Laboratory building at the University of Hawai‘i at Mānoa. Additional resources needed for this project will be funded by the Undergraduate Research Opportunities Program.

Timetable

Conduct Research	Present - March 2024
Outline Thesis	December 15, 2023
Write ½ of Thesis	January 15, 2024
First Draft Thesis	February 15, 2024
Final Draft Thesis and Submit to Mentor and Committee Member	March 15, 2024
Upload Personal Statement and Resume	April 2024
Final Honors Project Submission	April 2024
Undergraduate Showcase	May 2024
Graduation	May 11, 2024

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