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Looking for Maize Genes Involved in Cold Response: Producing Knockouts for Arabidopsis Homologs of Maize Candidate Genes Using a CRISPR/Cas9 Approach

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Table of Contents

Abstract

Most of today's maize is cultivated outside its original climate zone, where yields are constrained by the changes in climate. Maize is especially vulnerable to high temperatures and drought stress, both of which negatively affect corn yields. An important strategy to combat this is early sowing, which avoids the effects of summer droughts and high temperatures in many places around the globe. However, maize is a cold sensitive species (Sanghera *et al.,* 2011), making improvement to cold stress crucial for its adaption. The relatively new system CRISPR (Clustered regularly-interspaced short palindromic repeats)/Cas9 offers the potential to study cold-stress related genes through targeted mutagenesis. Using an enzyme called Cas9 and guide RNA, scientists can target a specific region in the genome and make a double stranded break so that any DNA can then be added or removed through DNA repair mechanisms (Jiang, Yang, & Weeks, 2014). Our research project aimed at investigating cold response in maize through development of an application that can be used to analyze the function of plant genes. We designed and implemented CRISPR/Cas9 technology on a model organism *Arabidopsis thaliana* (rockcrest) to knock out eleven plant genes (from the website database arabidopsis.org) that would produce easily distinguishable phenotypic traits once mutated, or were homologs to potential stress candidates in maize. Using published CRISPR/Cas9 protocols (Čermák *et al.*, 2017) we selected appropriate gRNA regions to create approximately 200 bp out of frame deletions in coding parts of the genes and constructed transformation vectors, using golden gate cloning technology. The vectors at each of the cloning steps were analyzed by restriction digests, colony PCR, and sequencing, demonstrating the success of vector assembly. The T_0 plants were transformed with T-DNA transformation vectors for select genes and T_1 seeds were harvested and screened for transformants. Results showed the success of using CRISPR/Cas9 to create transgenic plants. Further investigation of mutant response to cold stress conditions is necessary to investigate the involvement of maize candidate genes in controlling cold tolerance in maize.

Background

The Importance of Maize

Maize is considered one of the most important crops in the world due to its versatility, its capacity for high yields, and its nutritional value. Although it originated in Mexico, maize is now a significant agricultural food source around the globe and has become the third main cereal crop after rice (*Oryza sativa L.)* and wheat (*Triticum aestivum)* (Wijewardana *et al.,* 2015). Aside from being well-suited for human consumption, maize is also a contributor to exports such as fuel and animal feed (USDA, 2018). Both directly and indirectly, maize is an essential crop in today's society. In developing countries such as South Africa, maize is the basis for food security, where it has become one of the most important staple foods (CIMMYT, 2016). This could be attributed to the fact that corn is able to, on average, provide the most calories per acre of any crop, narrowly beating out potatoes (USDA, 2018). What was once a wild grain of Mexico (Trtikova *et al.,* 2017*)* has evolved into a basis for both human and animal diets and is utilized as a key ingredient in a plethora of other products worldwide as well.

Over the past several decades, the production of maize has increased significantly. For instance, since the 1930s, corn production in the United States has expanded outside of the Corn Belt on a decadal-scale (Kucharik *et al.*, 2005). Corn remains on top of the agricultural production list. The United States alone dedicates nearly one-third of its cropland to maize, which equates to approximately 85 million acres (Barton *et al.*, 2014; USDA, 2018). This acreage represents close to twice as much corn production as there was in 1990. More surprisingly, outside of the U.S., China has more than doubled, while Brazil has more than

tripled, maize production since 1990 (USDA, 2018). This explosion of maize production across the globe shows its ever expanding importance to the world.

The global demand for agricultural crops for food, feed, and fuel has been expanding for many years (Edgerton, 2009). The increase in meat consumption in emerging economies, together with the demand for use of grain in biofuel production, is escalating pressure on farmers to keep up with the demand for grain (Edgerton, 2009*).* In fact, nearly 70% of maize is used for either livestock or fuel, with roughly 95% of ethanol-based biofuel in the U.S. being produced from corn (USDA, 2018; Davis *et al.,* 2011). If the demand continues to rise, more land area will have to be devoted to growing maize, or more efficient methods for growing it will have to be found. Devoting more land to producing maize, however, will come with environmental consequences. Increased use of nitrogen fertilizers, when utilized, can increase nitrous oxide emissions, reduce water quality, and increase the size of hypoxic zones (Edgerton, 2009). The Gulf of Mexico already has one such zone, widely known as the "Dead Zone," the largest hypoxic zone ever recorded at 8,776 square miles, roughly equivalent to the size of New Jersey (Rabalais *et al.,* 2002; NOAA, 2017*).* To combat consequences like this, making maize more resilient, rather than expanding the size of farmlands, is a necessity to our future environment.

Most of the world's main food crops are currently cultivated outside their original climate zones, where yields are constrained by the thermal thresholds for optimal growth (Rodriguez *et al.,* 2014). This means that crops need to be able to withstand all types of climates, with more time and resources having to be allocated to counteract constraints in each environment. This becomes true for maize in particular, with the cultivation of maize expanding into cooler regions (Fracheboud *et al.,* 1999). This, along with the implications due to climate change, puts a lot of stress on farmers. The variability of climate directly affects temperature, precipitation, length of

growing season, and timing of critical threshold events relative to crop development (Southworth *et al.,* 2000*).* The stress that varying weather puts on crops such as maize could mean lower yields and poorer crop quality for farmers. By modifying maize, we could adapt it to different growing seasons rather than battle the stress that comes with the constraints for optimal growth due to temperature variation.

Despite the widespread use and growth of maize, many environmental factors make it a challenging crop to grow. For instance, environmental factors that impose water-deficit stress, such as drought, salinity and temperature extremes, place major limits on plant productivity, as plants cannot withstand the stress (Cushman *et al.,* 2000). Maize is especially vulnerable during pollination and grain-filling stages, where high temperatures and drought stress negatively affect corn yields. Several strategies, such as early planting, have been implemented to overcome the problem (Wijewardana *et al.,* 2015). There is a critical window of time around tasseling where many places in the U.S. experience inadequate and inconsistent rainfall. By moving planting to early March, under normal growing conditions, the crop may initiate tasseling in May, a month with cooler temperatures, greater solar radiation, lower evaporative demand, and consistent, plentiful precipitation (Wijewardana *et al.,* 2015). Earlier planting would lead to higher yields by avoiding the stresses that come during the summer months and could be the key to the future of maize production.

The Impact of Cold Stress on Maize

Cold stress, which includes chilling (<20°C) and/or freezing (<0°C) temperatures, severely affects the growth and development of plants and significantly inhibits agricultural productivity (Chinnusamy *et al.,* 2007). Maize is a cold-sensitive species (Sanghera *et al.,* 2011),

making low temperatures a major factor in significant crop losses. Although chilling temperatures can affect maize development and physiology through its life cycle, cold is especially detrimental during germination and early seedling growth, when the optimal temperature threshold is above chilling temperatures (Rodriguez *et al.,* 2014; Prasad *et al.,* 1994). Due to this, early planting of maize is risky, and maturation of the plant is often constrained by cold snaps. Cold in early spring affects development of leaves, roots, shoots, and chloroplast function, thereby reducing photosynthetic capacity (Hund *et al.,* 2004; Rymen *et al.,* 2007*).* Cold temperatures are widely understood to negatively impact maize, making temperature tolerance an important characteristic that needs to be further investigated and improved.

Temperature tolerance is a multigenic trait, also called polygenic or quantitative, controlled by multiple genes involved in many different metabolic pathways and cell compartments (Sanghera *et al.,* 2011; Wijewardana *et al*., 2015). Many conventional breeding approaches are limited by the complexity of this multigenic trait, which often coincides with low genetic variance of yield components under stress conditions and lack of efficient selection criteria (Sanghera *et al.,* 2011). Conventional breeding is also labor intensive and it usually takes several years to progress from the early stages of screening phenotypes and genotypes to the first crosses into commercial varieties (Zhang *et al.,* 2018). Furthermore, genes linked to tolerance at one stage of development can differ from those linked to tolerance at other stages (Cushman *et al.,* 2000). All these factors lead to breeding programs that are often inefficient at assessing genetic variability and cold tolerance in crops (Wijewardana *et al*., 2015). It is therefore crucial that we find better strategies that are simple and consistent to develop cold tolerant crops.

In order to successfully develop cold tolerant maize, quantitative trait locus (QTL) analysis can be used to generate a list of candidate genes correlated with cold stress. During this

process, selection programs are used to screen the genome of an organism for regions containing genes associated with a specific phenotypic trait. This is done using a collection of DNA markers that arise from DNA mutations such as point mutations, insertions, or deletions (Collard *et al.*, 2005). By screening the genome, these markers are flagged, creating linkage maps derived from differences between two different parental lines. Linkage maps are important for identifying chromosomal locations containing genes and QTLs associated with a trait of interest (Collard *et al.*, 2005).

Previous research (Goering, 2017) used QTL analysis to identify a list of candidate genetic loci linked to cold stress between two common maize inbred populations, Mo17 and B73, where the parental maize lines are cold susceptible (B73) and cold resistant (Mo17). Through comparison of the two lines with differences in cold resistance, regions in the genome suspected to contain genes linked to cold stress were identified, with two main loci controlling as much as 40% of variation in response to cold stress. About 30 to 40 candidate genes were identified through RNA-Seq analysis within these loci and need to be analyzed individually to identify genes with the strongest connection to cold response in maize. One of the approaches to continue this line of investigation is producing mutants containing nonfunctional maize genes located in the identified cold response QTLs. Gene editing is a powerful tool that can be used to achieve this goal.

Genome Editing

Genome editing allows the addition, removal, or alteration of DNA at targeted locations in the genome, making it a powerful tool for specific modifications of targeted genes (Tsutsui *et. al.*, 2017). The use of gene editing is important for studying gene function and could serve as a tool for modifying genomes to correct defective genes as well as introduce new functionality to

the genome. Precise genome editing could also serve as the basis for studying biological processes (McManus *et al.,* 2015). Targeted genome editing has the potential to not only accelerate basic research but also plant breeding by providing the means to rapidly modify genomes in a precise and predictable manner (Zhang *et al.,* 2018). For farmers, this means that genome-editing techniques can be used to improvement crop quality and yield.

Until 2013, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the dominant genome editing tools (Bortesi *et al.,* 2015). Both were designed for targeted gene modification by making double stranded breaks (DSBs) in the genome so that DNA can be added or removed. Consisting of artificial DNA-binding enzymes that facilitate targeted editing, they are able to make DSBs with the help of a specific endonuclease, namely Fokl (Bortesi *et al.,* 2015). Guide-RNA (gRNA) is designed to recognize and bind to the target DNA sequence, allowing the endonuclease to cleave the desired DNA site. DNA repair mechanisms are activated, resulting in the insertion or deletion of genes at the break site, with the most frequent repair mechanism being done by non-homologous end joining (NHEJ) (Čermák *et al.,* 2017; Tsutsui *et al.,* 2017*).* A different approach is to repair by homology dependent repair (HDR), which copies information from a donor DNA template; however, this approach is less desirable because it requires introducing an organism's cell to both the donor DNA molecule and the endonuclease, making it more challenging to achieve stable mutants (Čermák *et al.,* 2017). ZFNs and TALENs have found targeted success in a number of species including rice, wheat, tomato, and maize (Bortesi *et al.,* 2015).

In the more recent years, a rapidly developing tool for gene editing called CRISPR (clustered regularly interspaced short palindromic)/Cas9 (see *Figure 1* for a schematic drawing) has proven to be an efficient and precise mechanism for editing targeted mutagenesis. First

discovered in the adaptive immune system of bacteria (Liang *et al.*, 2015), CRISPR/Cas9 consists of two key components: a Cas9 protein and gRNA. Cas9 is an RNA-guided DNA endonuclease, which serves as a molecular scissors by cleaving DNA. It acts alongside a piece of gRNA, which is a 20 base long sequence used to pinpoint and guide the Cas9 enzyme to the right target spot. As gRNA finds its matching target sequence, the paired components also needs to match to a protospacer adjacent motif (PAM) sequence (NGG) that follows the targeted DNA region in order for this Cas9-gRNA complex to induce a DSB at the target site (Tsutsui *et. al.*, 2017). This PAM sequence ensures the complex is cutting at the right place in the genome, and Cas9 will not cut unless it is present. Similar to other methods, the DSBs generated are repaired by NHEJ or HDR, resulting in an insertion or deletion or precise repair, respectively (Liang *et al.*, 2015). Using this paired mechanism, scientists can induce changes to one or more genes of a genome.

CRISPRs efficiency and simplicity are two of the main advantages over other editing technologies. Other tools, like ZFNs and TALENs, function through protein-DNA interactions, which means that targeting to a new site requires engineering and cloning a new protein (Wang e*t al.*, 2016). CRISPR/Cas9, on the other hand, is particularly useful because instead of using proteins that must be engineered for each new target, its target specificity is determined by short gRNAs (Čermák *et al., 2017).* Thus, CRISPR techniques are more efficient than previous modes of gene editing. As an alternative to other methods, such as TALENs, which encode DNA target specificity in the amino acid sequence of an organism's' DNA binding domain (DBD), Cas9 can be targeted to a large variety of DNA motifs, simply by co-expression of a target site-specific gRNA (Boettcher *et al., 2015;* Čermák *et al., 2017).* The easy programmability is what is

allowing CRISPR/Cas9 to increasingly become more popular than other genome editing techniques, including ZFNs and TALENs (Boettcher *et al., 2015*).

The CRISPR/Cas9 system has been demonstrated to function in a multitude of organisms, including species of both plants and animals (Svitashev *et al., 2015).* Previous studies have shown success in gene modification in a variety of plant species such as rice (*Oryza sativa;* Zhang *et al., 2018),* wheat (*Triticum aestivum;* Bhowmik *et al., 2018),* soybeans (*Glycine max;* Cai *et al., 2018),* and arabidopsis (*Arabidopsis thaliana;* Čermák *et al., 2017).* Further applications and methods for increasing efficiency and precision still need to be explored, specifically approaches that have been optimized for use in plants. Many gene knockout mutants and some gene replacement and insertion mutants have been produced through the use of genome-editing technologies in a wide variety of plants, and many of these mutants have been shown to be useful for crop improvement (Zhang *et al.*, 2018). Not only this, but CRISPR/Cas9 is also modified to target a single base letter instead of an entire gene. This new tool allows scientists to implement point mutations to evaluate the roles of specific amino acids in the function of a gene or protein (Inui *et al., 2014).* Understanding the role of specific amino acids in protein function can change the way we grow and sustain crops.

Arabidopsis thaliana is a model organism used in many plant genetic research studies. A small annual weed belonging to the mustard family, Arabidopsis has a small genome, is self-fertile, and is easy to mutagenize (Gepstein *et al.,* 1995), making it a desirable plant to work with in many laboratories. Compared to other plants, Arabidopsis has a relatively short generation time of about six weeks, causing it to produce thousands of seeds in approximately two months (Gepstein *et al.,* 1995). The main importance of this plant, in our study and many others, is that genes isolated from it can be used to find their homologs in crop plants. Additionally,

fundamental mechanisms that can be understood in this model plant can be applied in crop plants such as maize (Gepstein *et al.,* 1995). Since gene editing and making mutants in maize is resource-intensive, an alternative approach is to knock out homologs in Arabidopsis and see what happens.

In this study, the main goal was to understand cold response in maize. Quantitative genetic analysis provided the list of candidate genetic loci, identifying 30 to 40 genes located in these loci as potential candidates that could be helpful in moderating response to cold stress. Producing targeted maize mutants is a complicated and time-consuming process. Therefore, we decided to focus on genes from a plant model species, Arabidopsis, that are homologous to maize candidate genes identified in the previous QTL analysis. Using the CRISPR/Cas9 system, we studied Arabidopsis homologs of six candidate maize stress response genes from the list by inducing gene knockouts. The first step in this process, however, was to adapt established protocols developed by University of Minnesota researchers (Čermák *et al., 2017*). To achieve this goal, we selected five genes (making it eleven total genes in our study) that would produce easily distinguishable phenotypic traits or show significant responses to changes in environmental factors once deleted.

To produce gene knockouts in Arabidopsis using CRISPR/Cas9 approaches, published protocols were followed to construct transformation vectors for all eleven genes. Vectors were verified through colony PCR, restriction digest, and Sanger sequencing, and transformed into Arabidopsis seedlings via Agrobacterium-mediated transformation by the floral dip method. Transformed plants were allowed to develop and seeds were collected, germinated, and screened to find transformed plants and verify CRISPR-induced mutations. Our results demonstrate the ability to successfully use the engineered CRISPR/Cas9 system to achieve plant genome

modification. Further investigation of mutant response to cold stress conditions is necessary to elucidate the involvement of maize candidate genes in controlling cold tolerance in maize.

Materials and Methods

Planting materials and Growth Conditions

For this experiment, *Arabidopsis thaliana* seeds were sown into soil, covered with plastic wrap, and placed in the dark at 4°C. After three days of incubation, they were grown in controlled growth environments at 23°C, set to 16 hour days and 8 hour nights. Plastic wrap was removed when seedlings began sprouting. After 4-5 weeks, when flowers started budding, they were ready to be transformed using the floral dip method.

Strains, Cells, and Growth Conditions

Escherichia coli was used for plasmid cloning and genome editing of all transformation vectors. All *E. coli* strains were grown in Luria-Bertani (LB) medium and incubated at 37°C. For selection purposes, each vector contained specific antibiotic resistance genes and the bacteria containing each vector were grown in media containing the appropriate antibiotic (A, B, C → ampicillin; T → kanamycin). Vectors A and C were transformed into either regular DH5α cells with subcloning efficiency or high-efficiency DH5α cells. For selection following golden gate assembly, vectors B and T contained a ccdB gene, which codes for a normally toxic protein, and were transformed into ccdB Survival T1R cells. During cloning, this ccdB gene should be replaced with our insert making any cells containing non-recombinant vectors, thus still expressing this gene, die after transformation.

Following final vector construction, vectors were introduced into Agrobacterium transformation strain Gv310 using a published protocol (Neece, *2013*). Competent cells of Agrobacterium were prepared according to manufacturer's instructions. Following transformation, cells were plated on plates containing gentamycin and kanamycin and incubated at 28°C.

Selecting Genes to Knockout

Eleven genes (see *Figure 2; Table 1)* were selected for producing knockout mutations. To establish the CRISPR techniques, four marker genes were selected, each of which produced easily distinguishable characteristics when mutated: root alteration (*ARK2),* resulting in twisted roots; dwarfism (*DWF5)*; misshapen leaves (*PLL5),* or discoloring (*SDP).* The remaining seven genes were related to cold stress, where six of which were identified through QTL analysis as candidate stress response genes in maize. It is important to note that because producing knockouts in maize is complex and time-consuming, for our study we used the Arabidopsis homologs for each to investigate their role in stress response. Two of the selected homolog genes had identified functions involved in stress response. *PAL1* encodes an important protein involved in response to oxidative stress, cold stress, and drought recovery, while *ATIPS2* encodes a protein involved in response to heat and high-intensity light. The remaining four homolog genes had unknown functions. The last gene was a marker gene related to cold stress (*ERD14).*

Primer and gRNA Design

Online resources, *http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex* (*Čermák et al., 2017),* were used to design primers for vector construction. For Vector B, initial gRNAs (refer to

Table 2 for sequences) were created to direct the Cas9 enzyme to make two cuts surrounding the gene selected for deletion. They were designed using *http://www.rgenome.net/cas-designer/,* making sure they were 200-500 bps away from each other, 50-65% in GC content, and avoided GG sequence combinations whenever possible. Screening primers were designed using Primer3 (*http://bioinfo.ut.ee/primer3-0.4.0/).* See *Table 3* for a complete list of primers used.

Plasmid Construction

Four basic starting plasmids were selected using a published website, *http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex,* and used in this experiment: pModA vector pQS pA0102 (Vector A), pModC vector pC000 (Vector C), pMod_B2103 vector pCO056 (Vector B), and transformation vector pTrans220d (Vector T). Each construct contained different components necessary for gene modification. Vector A construct contained the gene necessary to produce the Cas9 (under Ubiquitin 10 promoter) enzyme needed to make the DSB. Vector C construct was used as an empty vector allowing for customization of mutagenesis protocol, such as additional gRNAs, if desired. Transformation Vector T served as the vector backbone, in which all other vectors were assembled into. Finally, vector B construct was made to contain the specific gRNAs used for gene deletion.

These vectors were combined into a final transformation vector using a Golden Gate protocol (Čermák *et al., 2017).* All starting plasmids were transformed and plasmid DNA was isolated using QIAGEN Plasmid kit. Each were digested with *MspA1I* restriction enzyme to verify correctness. Correctly transformed plasmids were used directly for golden gate cloning, with the exception of vector B as it required an additional step before assembly of the final vector.

For the vector B construct, a set of PCR fragments containing AtUBI10 promoter, designed gRNAs, and Csy4 were assembled using golden gate cloning (*Figure 3*). To obtain the fragments, a set of three PCR reactions were ran as follows:

Reaction #1: Primer 46 + CSY_gRNA1;
Reaction #2: REP_gRNA1 + CSY_gRNA2;
Reaction #3: REP_gRNA2 + Primer 45

Amplicons were verified on a gel and directly used in the golden gate reaction. During the golden gate reaction, restriction enzyme *SapI* was used to open up the backbone for assembly of prepared PCR products, creating 3 bp overhangs, and release the ccdb gene, promoter, and gRNA scaffold. Restriction enzyme *Esp3I* was used to create 4 bp overhangs on both ends of all remaining PCR products. In combination, these enzymes, along with T4 DNA ligase, allow the vector to be constructed so that each PCR product replaces the ccdb gene. After assembly, cells were transformed, and correct clones were identified by Sanger sequencing using primers 47 and 48, and by restriction digest using *MspA1I*.

To assemble the final transformation vector, each initial construct was cut using the *AaRI* enzyme, producing a specific 4 bp overhang. In order to combine the required parts in the specified order (A→B→C), this overhang is necessary. The DNA fragments released from each construct were assembled into the *AaRI* sites of the transformation backbone using T4 DNA ligase, replacing the ccdB gene, and thus creating the final transformation vector, shown in *Figure 4*. After assembly and transformation of the final vector, correct clones were identified by Sanger sequencing using primers 49, 50, and 51 (*Table 3)* and by restriction digest using *EcoRI*.

Plant Transformation

Plant transformation was done using the floral dip method (*Figure 5*). Overnight cultures of agrobacterium cells containing vector D were suspended in an Infiltration Medium containing water, sucrose, and Silwet L-77. Arabidopsis flowers were dipped in the solution and held there for thirty seconds. Immediately following the floral dip, plants where placed in the dark overnight and returned to growth chambers the next day. Seeds were collected and germinated until seedlings were mature enough for screening.

Germination of T_0 Seeds

Seeds were sterilized in a 50% bleach solution and sown into plates containing a MS medium and kanamycin. Plates were incubated at 4°C in the dark for two days, followed by 4-6 hours of high intensity light at 22°C. Plates were then wrapped in aluminum foil and allowed to incubate for 2 days at 22°C, high intensity light. In the remaining two days of incubation, aluminum foil was removed and plates were incubated at 22°C, set to 16 hour days and 8 hour nights, although germination was successful in constant high intensity light.

DNA Extraction and Verification of Seedlings

DNA extraction of T_1 plants was done using a Cetyltrimethyl Ammonium Bromide (CTAB) Extraction Solution containing beta-mercapthoenthanol and CTAB buffer. T_1 leaves were crushed into a powder carefully using liquid nitrogen and prepped according to manufacturer's instruction. Following DNA extraction, DNA was screened for transformants using primers 52-59, according to which plants grew. Three regions where screened in each plants, two of which were located within the T-DNA borders of the plasmids, around the

promoter and kanamycin resistant gene. The last set of primers was designed to surround the gRNAs to check for deletions.

Molecular Biology Techniques

Polymerase chain reaction (PCR) was used to amplify the segments of DNA. Master mix, select primers, DNA, and water were mixed and placed in a PCR cycle of 95°C 2min + 30x (95°C/30sec + 60°C/45sec + 72°C/45sec) + 72°C/2min + 10°C hold. For ligation of our vectors, we used golden gate cloning technology. Both golden gate reactions were ran at a cycle of 10x (37℃/5min + 16℃/10min) + 37°C/15min + 80°C/5min + 4°C hold. Plasmid purification was done according to Qiagen manufacturer's instructions. Sanger sequencing was done by the University of Minnesota Genomics Center. During restriction Digest, DNA, desired enzymes, buffer, and water were prepped according to manufacturer's instruction. All restriction enzymes were ordered from NEB. One method we used for screening plasmids for correct transformation was Colony PCR. DNA, master mix, select primers, and water for colony PCR were prepped according to manufacturer's instructions.

Results

Eleven genes were initially selected for this study (*Table 1*). Of the eleven, six were Arabidopsis homologs of maize candidate genes involved in response to cold stress. Many had unknown functions in relation to cold response or were examined to not directly relate to cold stress but instead other biotic and abiotic stresses, however, that does not mean they are not indirectly related to cold response or contribute to multiple pathways in the organism. The remaining five were maker genes used to establish plant transformation technologies. The

marker genes selected produced distinguishable traits allowing us to easily identify and select the transformed progeny.

After gene selection, the first step was to construct correct transformation vectors. Four vectors were preselected for this experiment: A, B, C, and T. Vectors were selected using online resources and each initial plasmid was transformed into *E. coli*. Following transformation, they were purified from bacterial clones, and digested with restriction enzymes to verify the identity of each of the vectors. For vector A, we expected to get three fragments, with sizes being 245, 977, and 6574 bps. For vector B, we expected to get three fragments with sizes being 245, 977, and 3102 bps. Vector C expected sizes were 245, 897, and 977 bps. Vector T was expected to cut into 18 different fragments, with sizes ranging anywhere from about 50 to about 1100 bps. Results from *Figure 6* show the expected number of fragments for each vector, providing verification of initial vector construction. Correct clones were isolated and used for the rest of the experiment. Vectors A, expressing the Cas9 gene, C, serving as a necessary place holder, and T, the transformation backbone, were ready to use in golden gate assembly following transformation. Vector B, however, required an additional step before golden gate cloning, as it still needed to be constructed to contain our select gRNAs for gene knockout.

Following initial vector construction and transformation, the next step was to construct our vector B so that it contained our designed gRNAs. In order to target select genes, we designed sets of gRNA for sites located on various exons of the genes. These gRNAs attach to the complementary target sequence of DNA in the genome, as well as the Cas9 enzyme. Upon binding of the gRNA, the Cas9 enzyme is able to recognize the DNA sequence of the gRNA, bind to it, forming a complex, and cut at the targeted location. gRNAs were designed so that Cas9 would make two cuts, 200-500bps away, on these exons enabling DNA repair mechanisms

to induce a deletion, causing loss of gene function. *Figure 7* shows a visual schematic of our gRNA locations on each of the eleven genes.

To clone the gRNAs into vector B, a golden gate assembly of PCR products carrying unique elements apart of the vector, as well as our designed gRNA was established. A set of three PCR reactions were done. For the first reaction, primers were designed to amplify Csy4 as well a promoter contained in the template vector B. The second reaction used a different set of designed primers to amplify one of our gene-specific gRNAs and Csy4 located in the vector B. Finally, the third reaction used a third set of primers designed to amplify the second of our gene-specific gRNAs and Csy4. The set of three amplicons were assembled into construct B replacing the ccdB gene. To avoid the amplification of an alternative, wrong product, plasmid B was digested using *BanI* before being used in reaction one. Digestion is verified in *Figure 8.* Each PCR product, along with vector B, was then ligated using golden gate cloning.

After ligation, vector B plasmids were transformed into *E. coli.* The next step was to select for transformants. Colony PCR suggested select colonies from eight of the eleven genes contained our recombinant plasmid. To further confirm these results, suggested bacteria colonies were cultured, after which plasmid DNA was extracted and purified. Following purification, a restriction digest using the enzyme *MspA1I* was done. Digestion with *MspA1I* predicted different fragments for each gene. *DWF5* and *PAL1* was expected to cut into four fragments, with sizes being 245, 750, 977, and 1854 bps. *SDP*, *ERD14*, *PLL5*, *ARK2*, *GA2OX8* and *ATIPS2* were expected to produce three fragments with sizes being 245, 977, and 2604 bps long. Results from *Figure 9* showed digestion with *MspA1I* resulted in different sized fragments for many of the genes. Four plasmids from *DWF5*, five from *SDP*, three from *ARK2*, six from *ERD14*, two from *PLL5*, three from *GA2OX8*, and two from *ATIPS2* were all shown to properly cut. Plasmids were

then sent for sequencing to further confirm our results. Correct plasmids were isolated and select ones were used further in the study. It is also important to note that the remaining three genes were disregarded at this point due to time constraints.

After confirmation that the initial vectors A, B, C, and T were correct, golden gate cloning technologies were used to assemble the final transformation vector. Plasmids from each vector were cut using restriction enzyme *AaRI* and ligated together using T4 ligase, where they were again transformed into *E. coli.* Following transformation, they were purified from bacterial clones, and digested with restriction enzymes to select for transformants. Digestion using *EcoRI* was expected to cut each vector into three fragments with sizes being 546, 5776, and 10072 bps long. Results from *Figure 10* indicate select plasmids from each gene were correct. Plasmids were sent for sequencing and select ones were isolated. *PAL1* transformation was unsuccessful and was disregarded at this point.

Once final transformation vectors were verified, plasmids were transformed into *Agrobacterium*. Competent cells from agrobacterium were made, and cells were transformed so that they contained our plasmid DNA. Bacterial clones were sequenced. Arabidopsis plants were transformed into *Agrobacterium* using a floral dip method. Once the plants had flowers, a filtration medium was made using sucrose, water and silwet. Agrobacterium cells containing our plasmids were spun down and collected and then submerged into the medium. Plant flowers were dipped and left to grow overnight in the dark. After which, they were replaced in the growth chamber and treated T_0 plants were allowed to develop and resulting seeds were collected and stored in the dark until used for screening. *Figure 11* shows a timeline of plants during and after the floral dip method.

Seeds produced from T_0 plants were germinated, 200 per plate, on plates containing MS medium and the appropriate antibiotics. T_1 generation was analyzed to confirm inheritance of mutagenized genes resulting from Cas9 knockout. Only approximately 1% of T_0 seeds collected germinated in the presence of kanamycin. *Figure 12* represents the progression of each before screening. Two genes, *SDP* and *PLL5*, germinated and stayed alive along enough to screen. DNA was extracted and designed primers were used to screen the mutants. Three regions within the genome were targeted for screening. The first region was around our gRNAs. If there was indeed a deletion, we would expect the fragment in the mutant plant to be shorter than the one in our control plant. The remaining two regions were selected for verification of the CRISPR system in our T_1 plants. Two regions with the T-DNA borders of the plasmids, one around the promoter and the other around the kanamycin resistant gene, were selected. We would expect there to be a band present in the mutant plants compared to the control plants if we successfully made transgenic plants. Results from *Figure 13* indicate we made transgenic plants, but we did not induce a detectable deletion. Amplification products for the *SDP* gene was sent for sequencing and results confirmed no deletion, not even a small base point mutation, was present. Amplification products for the *PLL5* gene still needs to be sent for sequencing.

Discussion

Out of the initial eleven genes, the most progress was made with *SDP* and *PLL5*. Seeds containing potential mutations in these genes successfully germinated and grew to a point where they could be screened. Screening for those genes showed that although we made transgenic plants, a large deletion was not detectable. The *SDP* gene was sequenced in transgenic plants and it was confirmed that no mutation was made. This does not mean, however, that there was not

small base pair mutations for the *PLL5* gene, and sequencing should be done to further confirm these results.

Our results exemplify the challenges presented with the CRISPR/Cas9 system, as it can be a difficult system to work with. Successful gene deletion in our offspring showed limited success, which could be due to low heritability or the complexity of the entire process of plant transformation. In plants, high mutation efficiencies have been reported in primary transformants following gene editing, however, many of the mutations analyzed were somatic and therefore not heritable (Jaganathan *et al.,* 2018). A study done in tomato plants showed low mutations rates ranging from 18% to 40%, depending on the promoter used (Hashimoto et al., 2018). Whereas a study done in Arabidopsis showed higher mutation rates, ranging from 58% to 79% (Feng *et al.,* 2014). Results from these studies show that transformation comes down to a numbers game, and that more seeds need to be screened in order to increase the chance of finding mutants expressing gene deletion. Additionally, plants could be transformed again. With more time, we should be able to increase the number of transformants expressing gene deletion.

Confirmed mutants for cold related genes were not yet produced, so this work needs to continue. After confirmation, the next step would be to investigate the changes in expression of these genes in cold stress conditions. Changes in the physical response in mutants would also have to be tracked. Confirmed mutants should be tested for response to cold and other environmental stresses, as many stress response genes are known to react to various abiotic stress conditions. Subjecting the mutated plants to a multitude of different conditions in a controlled environment could provide insight into the genes' roles.

If the selected genes are indeed involved in cold response, further research would need to be done to reproduce these results in maize. Investigating allele variation for these genes in

different cold-tolerant and cold-susceptible maize varieties could provide insight into which alleles are desirable for cold tolerance. From there, breeding strategies incorporating the desired alleles into many maize varieties would need to be done. One such option is to develop molecular markers that facilitate this type of breeding. If plants showed a positive correlation to cold resistance, another option could be to take that gene and overexpress it in maize, possibly through CRISPR or another method. If the gene proves to negatively affect resistance, it would need to be silenced. Data from our study could be beneficial for future breeding strategies.

A limited number of cold stress related genes have so far been reported. A study done by Xia *et al.* (2018) showed evidence of the *ZmSiR* gene being important to cold stress tolerance, as knock-downs of *ZmSiR* in maize plants decreased cold stress tolerance. However, many studies have shown QTLs associated with cold response, but have yet to identify specific genes linked to cold stress. One reason may be that many maize breeders face the complexity of the response to low temperatures (Rodriguez *et al.,* 2014). Even if a gene is confirmed as cold stress related in maize, *it is* likely that many genes contribute to stress response. It is also likely that a gene produces an epistatic gene effect, where its presence suppresses the effect of another gene. In addition, genetic variance between maize lines could mean that confirmed cold stress related genes in one line might not be the same in another. Taking these factors into consideration, finding one gene may not be enough to make a difference in the grand scheme of things. It is therefore likely that a list of favorable alleles from many genes would have to be compiled in order to actually make a difference. The limited success of current breeding programs means work needs to continue to develop strategies to overcome the complexity of cold tolerant genes.

Our project faced a long list of limitations, some of which have already been noted. There were design limitations from the beginning, as our study used Arabidopsis homologs to maize

genes, and cold stress might have different effects across species. It should also be noted that our plants were not grown in growth chambers, which could have limited the success of our results. Using a more controlled growth environment in the future could be beneficial. Another limiting factor may have been the design of our gRNAs, therefore, it might be worthwhile to redesign gRNAs for any genes that didn't show clear progression. Increasing the number of gRNAs for a given gene might also maximize our chances of success.

In addition to the design limitations, there were also experimental limitations. Due to time and resources, no mutants for cold response genes have been confirmed. This means further investigation needs to be done in order to determine to their role in stress response. Along with this, multiple steps along the way had to be redone, as it proved to be difficult to find and confirm transformed cells. Further techniques needs to be established in order to utilize the CRISPR/Cas9 technique in our lab. Initial growth of T_1 seeds also proved to be a challenge that is taking a lot of time and resources to perfect. This resulted in many transformants not growing thus far. Jiang *et al.* (2014) found only a 1% germination rate using the floral dip method, meaning that perhaps our recovery rate is typical, and more seeds need to be grown in order to increase the chance of germination. However, changing the way we grow the plants could also increase their growth percentage. For instance, using slightly different mediums or growth conditions that provide nutrients more keen to our plants could be advantageous.

Despite all the limitations, several successful outcomes were achieved. Being able to establish the Cas9 system was a goal of its own, so the fact that we did see transgenic plants was a success in itself. We also successfully made transformation vectors for eight of the eleven genes, showing our establishment of using CRISPR/Cas9 techniques. All five marker genes were integrated into agrobacterium and transformed into plant cells, where we were able to produce

and collect seeds. You may refer to *Table 4* for a complete progression timeline for each gene. Future research will only continue to confirm and add to all the successes we saw.

In the last decade, CRISPR has shown success in a variety of different crops. It has been adopted in nearly 20 crop species so far for various traits including yield improvement, shelf life, biotic and abiotic stress management (Jaganathan *et. al, 2018).* CRISPR/Cas9-based genome editing has been utilized to increase crop disease resistance and also to improve tolerance to major abiotic stresses like drought and salinity (Jaganathan *et. al, 2018).* Heritability of mutant rice lines were evaluated to show the efficiency of the CRISPR/Cas9 system in inducing targeted mutagenesis (Zhang *et. al,* 2014). More recently, *Theobroma cacao,* a major source of cocoa, through of use of CRISPR engineering, developed crop disease resistance to a pathogen causing significant crop loss (Fister *et. al,* 2018). Soyk *et al*. (2017) used CRISPR to improve fruit yield in tomatoes. These studies show the importance of applying efficient and precise CRISPR methodologies in the background of basic biological trait information to obtain desired crop traits.

Currently, five crops edited with the CRISPR/Cas9 approach have been declared to be released to the public, free from regulatory monitoring by the United States Department of Agriculture (USDA) (Jaganathan *et. al,* 2018). This list includes a white button mushroom (*Agaricus bisporus)* that has shown resistance to browning once the CRISPR/Cas9 system knocked out a gene polyphenol oxidase (Waltz, 2016), along with a drought-tolerant soybean (*Glycine max)* variety, waxy corn (*Z. mays)* with enriched amylopectin, green bristlegrass (*Setaria viridis)* with delayed flowering time, and camelina developed with enhanced oil content (Waltz, 2018). This means that CRISPR does not fall under the definition of a GMO under regulatory regimes, holding true in many countries. Being able to cultivate and sell crops without

having to go through regulatory monitoring means several million dollars can be saved. In addition, it also reduces time, as it usually takes several years to release a GMO crop (Jaganathan *et. al,* 2018). The CRISPR/Cas9 system provides opportunities to create genetically modified crops with high precision of genetic modification but without needing to go through the tedious and time consuming process commonly associated with GMOs.

Using CRISPR to genetically modify crops could redefine the definition of GMO, making it more accepted to many people. One of the advantages of using CRISPR in gene editing is that instead of using selective breeding to produce plants with a desirable trait, you can now use genetic engineering to turn off or on genes that are already there, ultimately changing the GMO debate. Research shows that CRISPR is an alternative, more natural method to creating better, genetically modified crops than the traditional method (Zhang *et al,* 2018). This new technique could save time spent crossing generations of plants to get the desired trait, as well as reduce the controversy over crossing species that do not breed together naturally. It also means using an efficient method of genome editing without the use of antibiotic and herbicide resistant markers common for GMO crops today.

However, with this newly advanced method, comes its own caveats. For instance, one problem with CRISPR is that it produces off-target mutations, which creates unintentional side-effects when targeting specific regions of the genome, thus impacting precise gene modification. Several modifications of the Cas9 enzyme have already been developed to increase target specificity and reduce off-target cleavage, while another strategy in minimizing off-target cleavage has been to increase the PAM length (Jaganathan *et. al,* 2018). Further research to understand Cas9 properties could improve its efficiency and precision. Another problem with this system is low heritability. Cold-tolerance-related traits in particular usually show low

heritability, mostly because of an important genotype by environment interaction, for which a main factor is seed origin (Rodrigues *et. al*, 2008)). Increased research also needs to be done to show consistency in mutations generations down from the T_0 transformed plants.

Incorporating gene editing tools into research offers the potential to change the way we breed and grow crops. Future crops for sustainable productive agriculture are those which have better pest resistance, with enhanced nutritional value and quality, and that are able to survive in changing climate (Jaganathan *et. al*, 2018). Understanding the functions of genes involved in stress response could help produce more resilient crops that require less regulation and that increase crop yield. Moreover, instead of expanding the environmental and disease tolerance of already domesticated crops, plant species that are already well adapted to different environments could be domesticated with high-value traits (Gao, 2018). This technology could offer numerous possibilities to improve crops for better nutrition and food security. Adapting cold tolerant maize lines could mean longer growing seasons, as well as provide improvement for cultivation of maize outside of their normal climate zones.

Conclusion

CRISPR/Cas9 is a powerful tool used to modify target genes. In combination with other techniques, it has the ability to assemble single and multi-gene knockouts, as well as conduct gene modification or gene replacement (Čermák *et al., 2017)*. While several labs were successful in using the CRISPR/Cas9 system in a wide variety of plants, transferring this complicated technology between laboratories is not easy. We were interested in knocking out Arabidopsis homologs for maize candidate genes related to cold stress response. However, before doing so, we needed to show the effectiveness of the approach in our hands.

In this study, we designed and implemented the CRISPR/Cas9 system to create transgenic plants by targeted mutagenesis. Eleven genes were selected for gene editing, through which gRNAs were designed to make short deletions on select exons of each gene. We successfully constructed and verified transformation vectors for eight genes. From there, two marker genes successfully grew and were screened for mutations. Screening results indicated that although we produced transgenic plants, we did not successfully induce the intended deletions. Sequencing needs to be done to confirm whether or not a small point deletion occurred. Future research needs to be done to investigate cold stress related genes in maize. If data suggests they are in fact cold tolerance genes, they could be advantageous to improving future farming.

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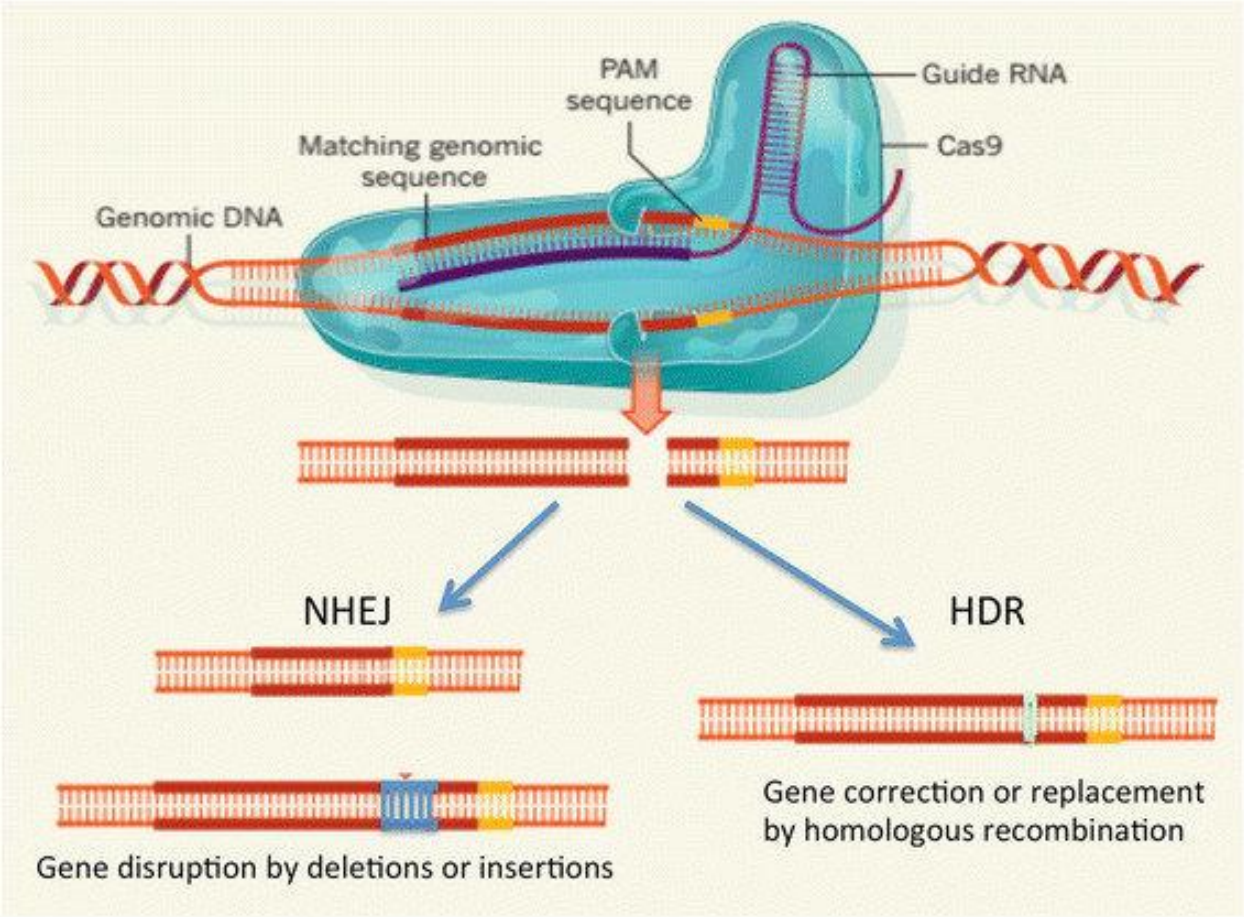
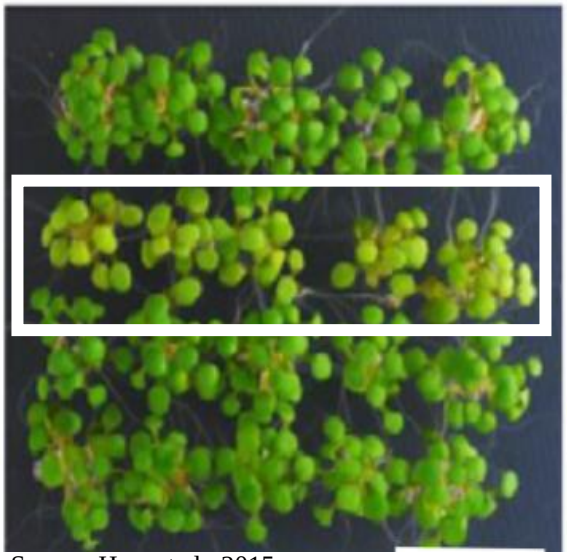


Figure 1. Photo credit goes to Tu *et al.*, 2015. Cas9 forms a complex with gRNA in a cell, where the complex than attaches to a matching DNA sequence adjacent to a PAM sequence (yellow). Binding of this complex results in a DSB in the genome where DNA repair mechanisms can cause gene disruption by deletions or insertions or gene correction or replacement.

A

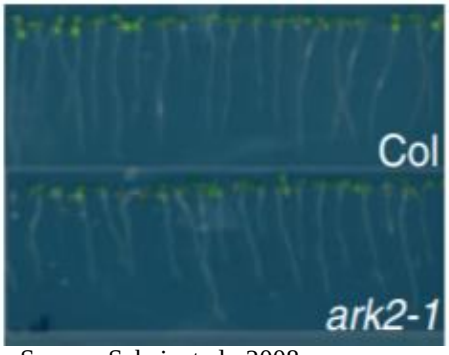


Source: Han, et al., 2015

B

Source: Song, et al., 2005

C



Source: Sakai, et al., 2008

Figure 2. Predictive phenotypes of select genes. Previous research showed expected phenotypes for three of the eleven genes. **A)** Shows mutant phenotypes compared to control plants for the gene *SDP*. Mutants were suggested to include reduced chlorophyll content, poor growth, yellow leaves, and abnormal chloroplasts. **B)** Shows mutant phenotypes compared to control plants for the gene *PLL5*. Mutants were suggested to show abnormal leaf shape. **C)** Mutant phenotypes for the gene *ARK2*. Mutants were suggested to show twisted roots.

Table 1. Selective Reports on Selected Genes

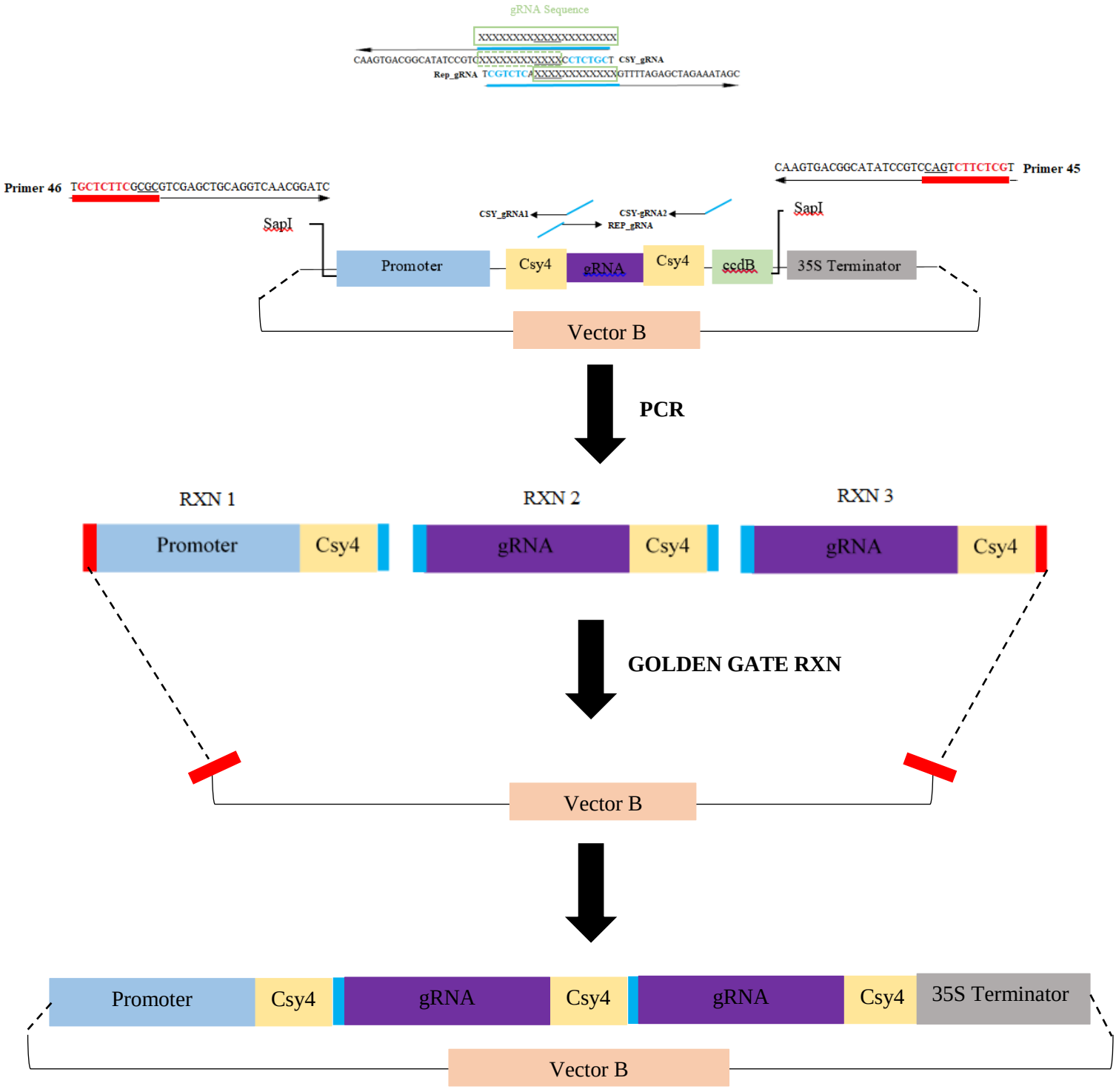
Gene	Alternative Names	Length (bps)	Exons	Gene Function	Mutant Description
AT1G12800	*SDP*	3781	13	RNA binding protein	Mutant defects include reduced chlorophyll content, poor growth, yellow leaves, and abnormal chloroplasts
AT1G50430	*DWF5*	3721	13	Reductase	Dwarfism
AT1G76180	*ERD14*	1256	2	Involved in calcium ion binding, encodes a protein involved in early response to dehydration, involved in response to cold	Response to cold
AT1G01950	*ARK2*	5304	20	Involved in root development and microtubule-based movement [kinesin]	Mutants have twisted Roots
AT1G07630	*PLL5*	2885	4	Encodes a protein phosphatase 2C like gene involved in leaf development	Mutants have abnormally shaped Leaves
AT2G22240	*ATIPS2*, ATMIPS2, MIPS2	2696	7	Encodes protein involved in response to heat, phosphate starvation, high light intensity, and defense mechanisms	Response to stress
AT1G76340	*GGLT1*	2088	2	Encodes a nucleotide-sugar transporter [Golgi GDP-L galactose transporter]	Unknown
AT1G74240	N/A	2944	9	Mitochondrial substrate carrier family protein	Unknown
AT2G37040	*PAL1*	3389	2	Encodes a phenylalanine ammonia-lyase involved in response to oxidative stress and drought recovery	Unknown
AT4G21200	*GA2OX8*	4089	3	Encodes a protein with gibberellin 2-oxidase activity	Unknown
AT1G07090	*LSH6*	1087	1	Involved in responses to light	Unknown

Gene	gRNA	Sequence
SDP	gRNA 1	GCAGCAACAGCCTCCACCAATGG
	gRNA 2	CCAGCCTGTCAATGTAGAAGAGG
DWF5	gRNA 1	ATCGATGCGTAAGTAACGATCGG
	gRNA 2	ATTCTTCAGCTGCTTCTGCCTGG
ERD14	gRNA 1	TGCTCAAACTCTGAAGCGATCGG
	gRNA 2	GGACACAAGAAACCTGAAGACGG
ARK2	gRNA 1	GGATCCATGAGACCAGTCTCTGG
	gRNA 2	TTGTGTCGAGTTGCAACCTGAGG
PLL5	gRNA 1	GTCTGGTCGGACGTAGCAGAAGG
	gRNA 2	CTCTTTCGATCGGACCTGAAAGG
ATIPS2	gRNA1	GAAGAAGCGGTTGTTGTCGTGGG
	gRNA 2	GTCGTCCACGAGAATCGTAACGG
GGLT1	gRNA 1	CAGAACCAAGCCCAGAGACTTGG
	gRNA 2	TAGCTGGGCTCTTGCGTATCTGG
AT1G74240	gRNA 1	ACGCTCTTACGAAAACTCGGAGG
	gRNA 2	CGGAATCGAATAGTTGCAAGTGG
PAL1	gRNA 1	GCTGCAGCGGAGCAAATGAAAGG
	gRNA 2	AAACGGTGTCGCACTTCAGAAGG
GA2OX8	gRNA 1	AAGCTCCACCTCCTCGACGACGG
	gRNA 2	CGCCGTCAGCCACTTCTATCCGG
LSH6	gRNA 1	CACCACCAGCTACACCTAGCAGG
	gRNA 2	GTAAATCCGAACCGCACGTGCGG

Table 2. Designed gRNA sequences for selected genes. Pam sequences are underlined.

Table 3. Designed Primer Sequences

Primer ID	NAME	PRIMER SEQUENCE
Primer 1	CSY_AT1G12800_GRNA1	TCGTCTCCGGCTGTTGCTGCCTGCCTATACGGCAGTGAAC
Primer 2	REP_AT1G12800_GRNA1	TCGTCTCAAGCCTCCACCAAGTTTTAGAGCTAGAAATAGC
Primer 3	CSY_AT1G12800_GRNA2	TCGTCTCCTTGACAGGCTGGCTGCCTATACGGCAGTGAAC
Primer 4	REP_AT1G12800_GRNA2	TCGTCTCATCAATGTAGAAGGTTTTAGAGCTAGAAATAGC
Primer 5	CSY_AT1G07630_GRNA_1	TCGTCTCCGTCCGACCAGACCTGCCTATACGGCAGTGAAC
Primer 6	CSY_AT1G07630_GRNA_2	TCGTCTCCCGATCGAAAGAGCTGCCTATACGGCAGTGAAC
Primer 7	REP_AT1G07630_GRNA_2	TCGTCTCAATCGGACCTGAAGTTTTAGAGCTAGAAATAGC
Primer 8	REP_AT1G07630_GRNA_1	TCGTCTCAGGACGTAGCAGAGTTTTAGAGCTAGAAATAGC
Primer 9	CSY_*DWF5*_GRNA1	TCGTCTCCCAGCTGAAGAATCTGCCTATACGGCAGTGAAC
Primer 10	REP_*DWF5*_GRNA1	TCGTCTCAGCTGCTTCTGCCGTTTTAGAGCTAGAAATAGC
Primer 11	CSY_*DWF5*_GRNA2	TCGTCTCCTTACGCATCGATCTGCCTATACGGCAGTGAAC
Primer 12	REP_*DWF5*_GRNA2	TCGTCTCAGTAAGTAACGATGTTTTAGAGCTAGAAATAGC
Primer 13	CSY_*ARK2*_GRNA1	TCGTCTCCTCTCATGGATCCCTGCCTATACGGCAGTGAAC
Primer 14	REP_*ARK2*_GRNA1	TCGTCTCAGAGACCAGTCTCGTTTTAGAGCTAGAAATAGC
Primer 15	CSY_*ARK2*_GRNA2	TCGTCTCCAACTCGACACAACTGCCTATACGGCAGTGAAC
Primer 16	REP_*ARK2*_GRNA2	TCGTCTCAAGTTGCAACCTGGTTTTAGAGCTAGAAATAGC
Primer 17	CSY_*ERD14*_GRNA1	TCGTCTCCAGAGTTTGAGCACTGCCTATACGGCAGTGAAC
Primer 18	REP_*ERD14*_GRNA1	TCGTCTCACTCTGAAGCGATGTTTTAGAGCTAGAAATAGC
Primer 19	CSY_*ERD14*_GRNA2	TCGTCTCCTTTCTTGTGTCCCTGCCTATACGGCAGTGAAC
Primer 20	REP_*ERD14*_GRNA2	TCGTCTCAGAAACCTGAAGAGTTTTAGAGCTAGAAATAGC
Primer 21	CSY_AT2G22240_GRNA1	TCGTCTCCTGTTGTCGTGGGCTGCCTATACGGCAGTGAAC
Primer 22	REP_AT2G22240_GRNA1	TCGTCTCAAACAACCGCTTCGTTTTAGAGCTAGAAATAGC
Primer 23	CSY_AT2G22240_GRNA2	TCGTCTCCCTCGTGGACGACCTGCCTATACGGCAGTGAAC
Primer 24	REP_AT2G22240_GRNA2	TCGTCTCACGAGAATCGTAAGTTTTAGAGCTAGAAATAGC
Primer 25	CSY_AT1G76340_GRNA1	TCGTCTCCGGCTTGGTTCTGCTGCCTATACGGCAGTGAAC
Primer 26	REP_AT1G76340_GRNA1	TCGTCTCAAGCCCAGAGACTGTTTTAGAGCTAGAAATAGC
Primer 27	CSY_AT1G76340_GRNA2	TCGTCTCCAGAGCCCAGCTACTGCCTATACGGCAGTGAAC
Primer 28	REP_AT1G76340_GRNA2	TCGTCTCACTCTTGCGTATCGTTTTAGAGCTAGAAATAGC
Primer 29	CSY_AT1G74240_GRNA1	TCGTCTCCTCGTAAGAGCGTCTGCCTATACGGCAGTGAAC
Primer 30	REP_AT1G74240_GRNA1	TCGTCTCAACGAAAACTCGGGTTTTAGAGCTAGAAATAGC
Primer 31	CSY_AT1G74240_GRNA2	TCGTCTCCTATTCGATTCCGCTGCCTATACGGCAGTGAAC
Primer 32	REP_AT1G74240_GRNA2	TCGTCTCAAATAGTTGCAAGGTTTTAGAGCTAGAAATAGC
Primer 33	CSY_AT2G37040_GRNA1	TCGTCTCCCTCCGCTGCAGCCTGCCTATACGGCAGTGAAC
Primer 34	REP_AT2G37040_GRNA1	TCGTCTCAGGAGCAAATGAAGTTTTAGAGCTAGAAATAGC
Primer 35	CSY_AT2G37040_GRNA2	TCGTCTCCGCGACACCGTTTCTGCCTATACGGCAGTGAAC
Primer 36	REP_AT2G37040_GRNA2	TCGTCTCATCGCACTTCAGAGTTTTAGAGCTAGAAATAGC
Primer 37	CSY_AT4G21200_GRNA1	TCGTCTCCTCAGTCACAGCCCTGCCTATACGGCAGTGAAC
Primer 38	REP_AT4G21200_GRNA1	CGTCTCACTGAGAAGCTAAGTTTTAGAGCTAGAAATAGC
Primer 39	CSY_AT4G21200_GRNA2	TCGTCTCCTGGCTGACGGCGCTGCCTATACGGCAGTGAAC
Primer 40	REP_AT4G21200_GRNA2	TCGTCTCAGCCACTTCTATCGTTTTAGAGCTAGAAATAGC
Primer 41	CSY_AT1G07090_GRNA1	TCGTCTCCTAGCTGGTGGTGCTGCCTATACGGCAGTGAAC
Primer 42	REP_AT1G07090_GRNA1	TCGTCTCAGCTACACCTAGCGTTTTAGAGCTAGAAATAGC
Primer 43	CSY_AT1G07090_GRNA2	TCGTCTCCGTTCGGATTTACCTGCCTATACGGCAGTGAAC
Primer 44	REP_AT1G07090_GRNA2	TCGTCTCAGAACCGCACGTGGTTTTAGAGCTAGAAATAGC
Primer 45	CSY_TERM TC222	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC
Primer 46	ATUBI10_*SAPI*	TGCTCTTCGCGCGTCGAGCTGCAGGTCAACGGATC
Primer 47	SCREENING ATUBI10_SCREENING	TTCTCGTGACCTAGTCGTCCTC
Primer 48	SCREENING REVERSE CSY4_REVERSE	GGAACCCTAATTCCCTTATCTGG
Primer 49	220D_PRIMER_1	CTTTACACTTTATGCTTCCGGCTC
Primer 50	220D_PRIMER_2	GTTGGATCTCTTCTGCAGCA
Primer 51	220D_PRIMER_3	GGATGTGCTGCAAGGCGATTAAG
Primer 52	SCREENINGREVERSE_PROMOTER	TCGACGAGCGATCTACCTG
Primer 53	SCREENINGFORWARD_PROMOTER	TGTGGAATTGTGAGCGGATA
Primer 54	SCREENINGFORWARD_KANR	AGCGAAACCCTATAGGAACC
Primer 55	SCREENINGREVERSE_KANR	TCTCTCGAGCTTTCGCAGAT
Primer 56	SCREENINGFORWARD_GRNA_AT1G12800	AAGCCTGTTCTGAAAGATGGAG
Primer 57	SCREENINGREVERSE_GRNA_AT1G12800	CTCAAGCTCTGGCCTTGCT
Primer 58	SCREENINGFORWARD_GRNA_*PLL5*	GCCAAAAGACATCTCCGTTCT
Primer 59	SCREENINGREVERSE_GRNA_*PLL5*	GCTTATCAAGCGGACCTGAG



Picture and description adapted from Cermak *et al.*, 2017

Figure 3. Construction of vector B. *SapI* was used to open up the backbone for assembly of prepared PCR products, creating 3 bp overhangs, and release the ccdb gene, promoter, and gRNA scaffold. The 3 bp (underlined and labeled in red) overhangs generated by *SapI* are complementary to the plasmid backbone on the 5' end of the first and 3' end of the last PCR product. Restriction enzyme Esp31 creates specific 4 bp (underlined and labeled in blue) overhangs on 3'end of the first, 5' end of the last and each end of all remaining PCR products. The 20 bp of each specific gRNA sequence is split between two primers used to create consecutive parts – e.g. the first half of the first gRNA spacer will be in the 3' end of the first PCR product (also containing the promoter) and the second half of the first gRNA will be in the 5' end of the second PCR product. The 4 bp overhangs created by the Esp31 enzyme are positioned in the middle (positions 9-12) of each gRNA spacer, such that after their ligation a full length (20 bp) gRNA spacer is created.

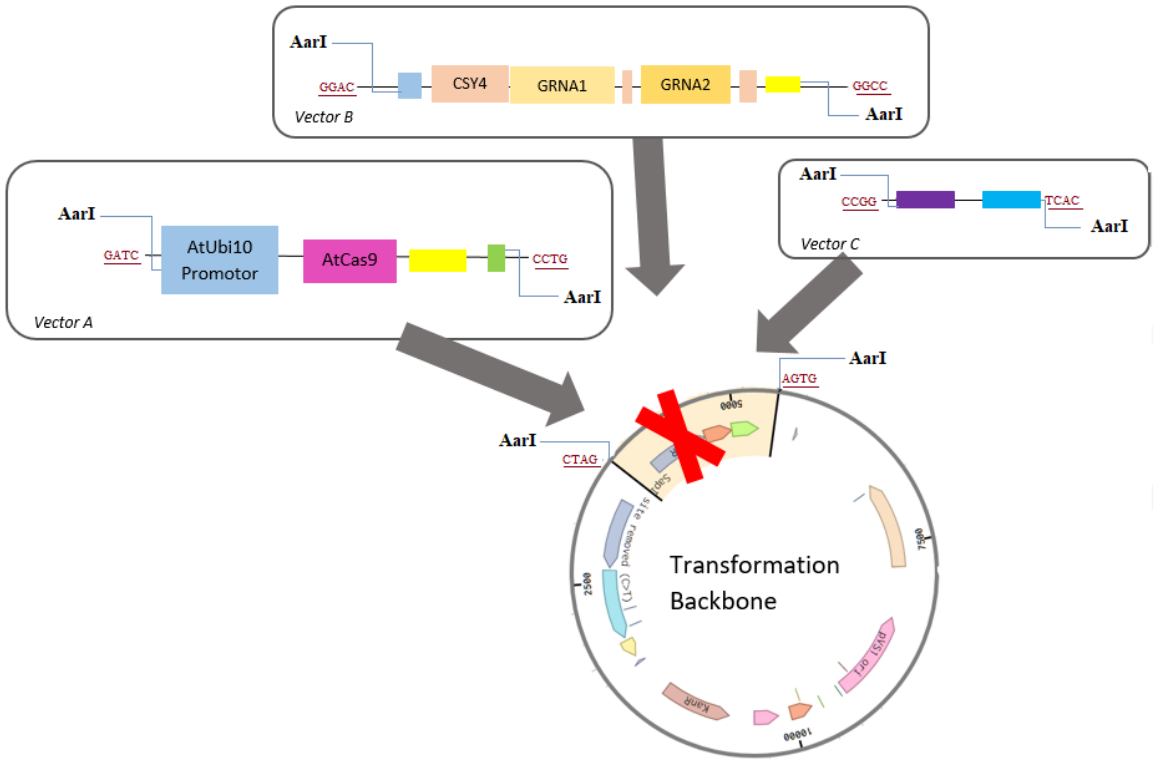
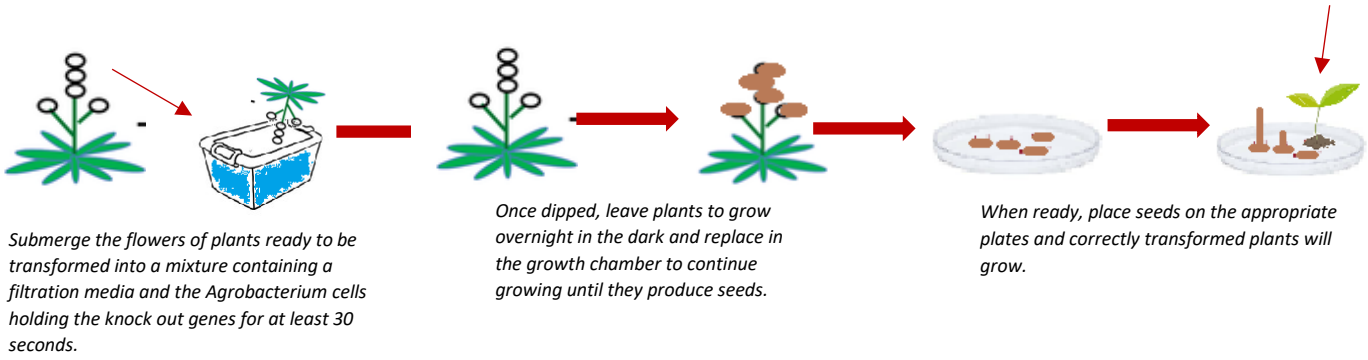


*Figure 4.*The golden gate process of removal and addition of select parts of each vector into our final vector D using the restriction enzyme *AaRI*. 4 bp overhangs used to correctly construct the final vector are underlined.



Submerge the flowers of plants ready to be transformed into a mixture containing a filtration media and the Agrobacterium cells holding the knock out genes for at least 30 seconds.

Once dipped, leave plants to grow overnight in the dark and replace in the growth chamber to continue growing until they produce seeds.

When ready, place seeds on the appropriate plates and correctly transformed plants will grow.

Figure 5. Floral dip method.

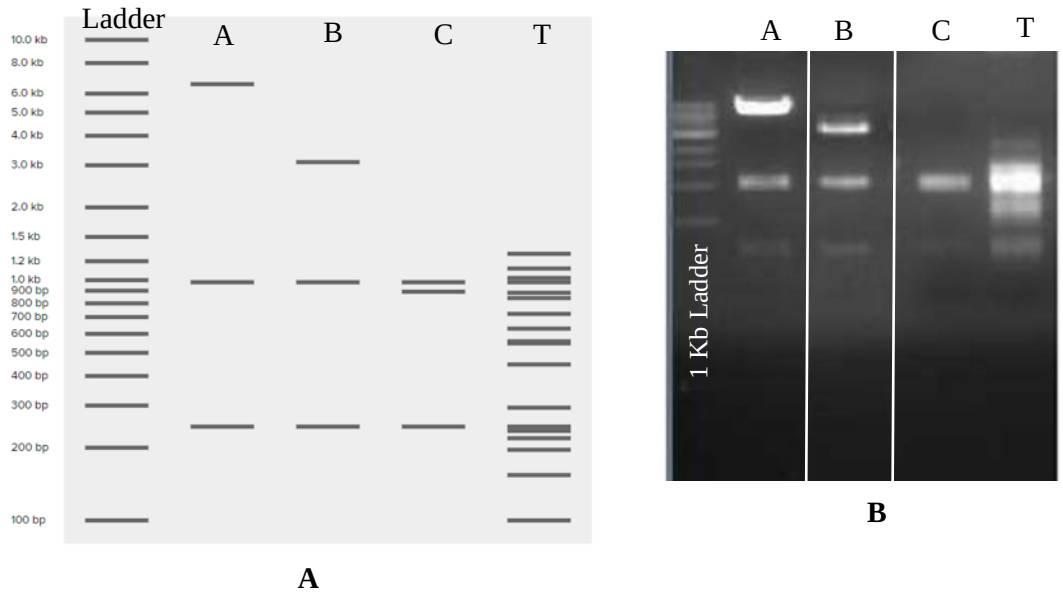


Figure 6. Restriction digest of vectors A, B, C, and T. Verification of transformation of Vectors A, B, C, and T was done using restriction enzyme MspA1I. **A)** MspA1I was expected to cut each vector into different sized fragments. For vector A, we expected to get three fragments, with sizes being 245, 977, and 6574 bps. For vector B, we expected to get three fragments with sizes being 245, 977, and 3102 bps. Vector C expected sizes were 245, 897, and 977 bps. Vector T was expected to cut into 18 different fragments, with sizes ranging anywhere from about 50 to about 1100 bps. **B)** The gel shows correct transformation of each initial vector.

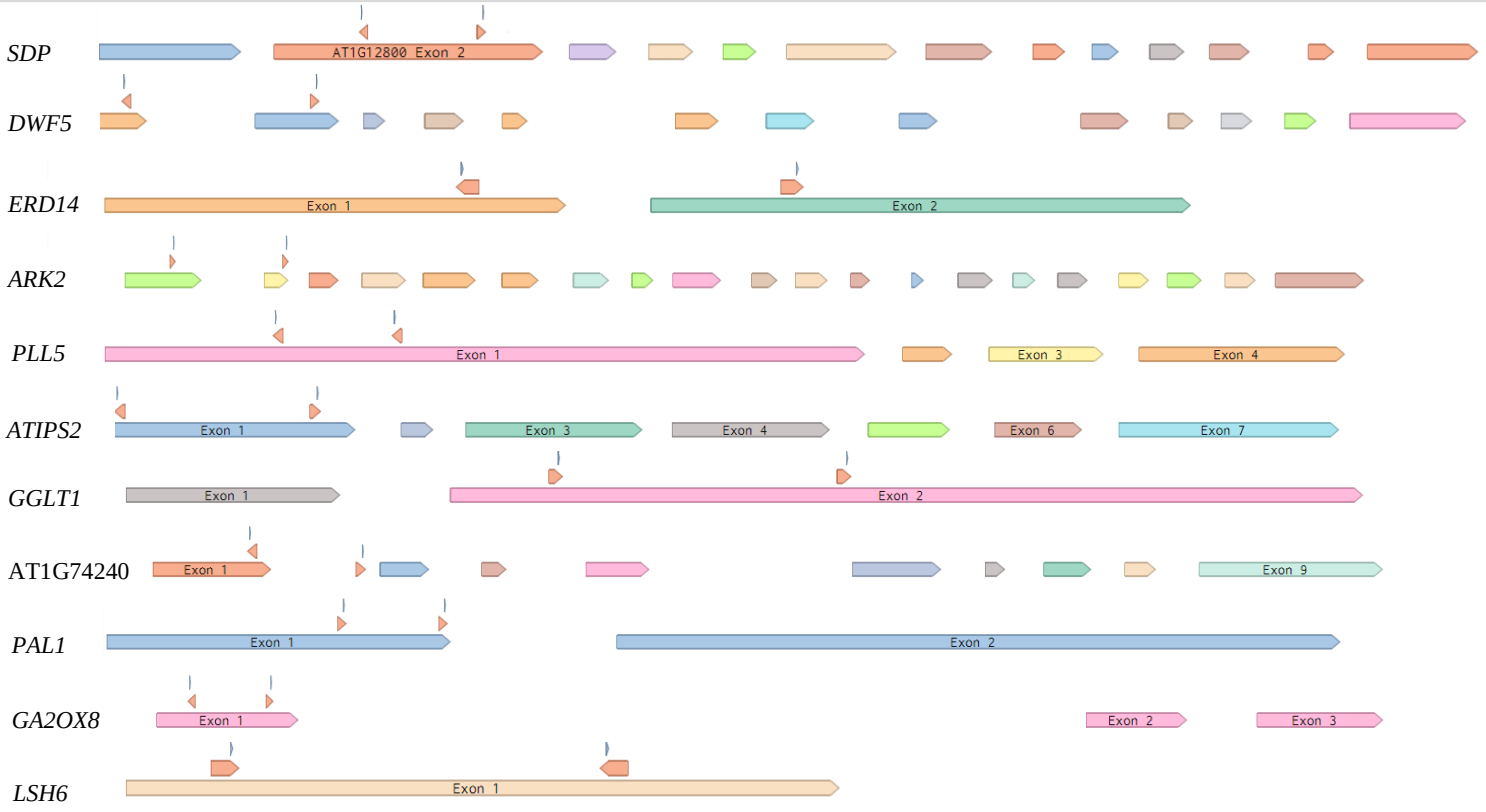


Figure 7: Visual representation of selected genes designed gRNAs. The position of each gRNA is designated with the orange arrows, with cut-sites labeled in blue. Arrow direction represents forward and reverse gRNAs.

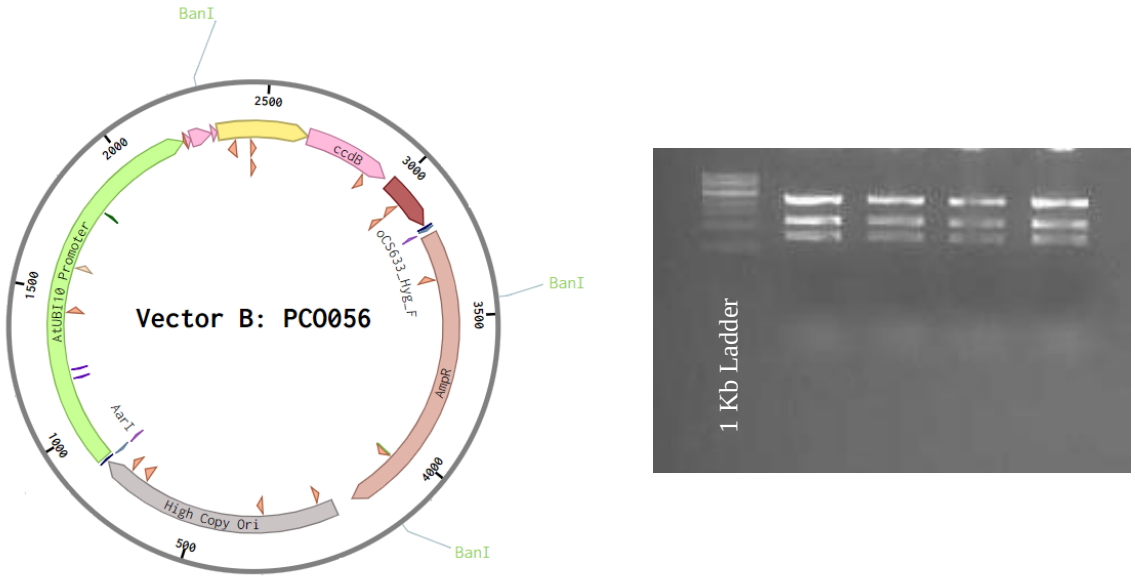


Figure 8. Restriction digest of Vector B. Before vector B could be used for ligation by golden gate assembly, it needed to be cut with BanI due to possible the presence of two possible attachment sites. We expected BanI to produce three fragments and results from gel match the expectation.

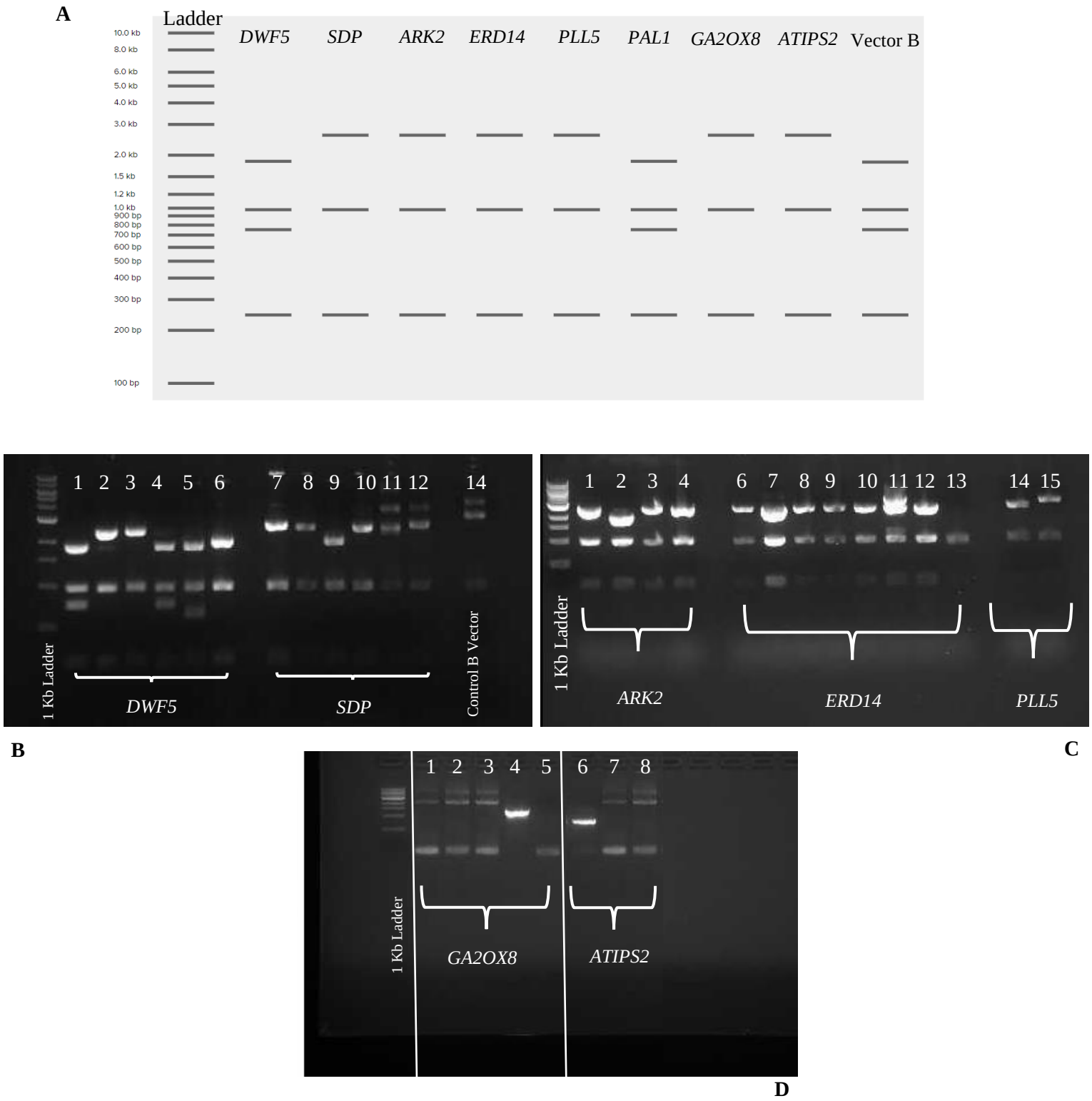


*Figure 9.*Verifiation of Vector B Transformation. After colony PCR, plasmid DNA was isolated and purified. After which, a restriction digest using MspAI1 was done to verify successful ligation and transformation. A non-transformed vector B was used as a control. **A)** Expected fragment sizes for each vector resulting from being cut. *DWF5* and *PAL1* was expected to cut into four fragments, with sizes being 245, 750, 977, and 1854 bps. *SDP*, *ERD14*, *PLL5*, *ARK2*, *GA2OX8* and *ATIPS2* were expected to produce three fragments, with size of fragments being 245, 977, and 2604 bps long. **B)** *DWF5*- 1, 4, 5, and 6 and *SDP*-1, 2, 4, 5, 6 were properly cut. **C)** *ARK2*- 1, 3, and 4, *ERD14*- 6, 7, 8, 9, 10, and 12 and *PLL5*- 14 and 15 were properly cut. **D)** *GA2OX8*- 1, 2, and 3 and *ATIPS2*- 7, and 8 were properly cut. Results from *PAL1* are not shown.

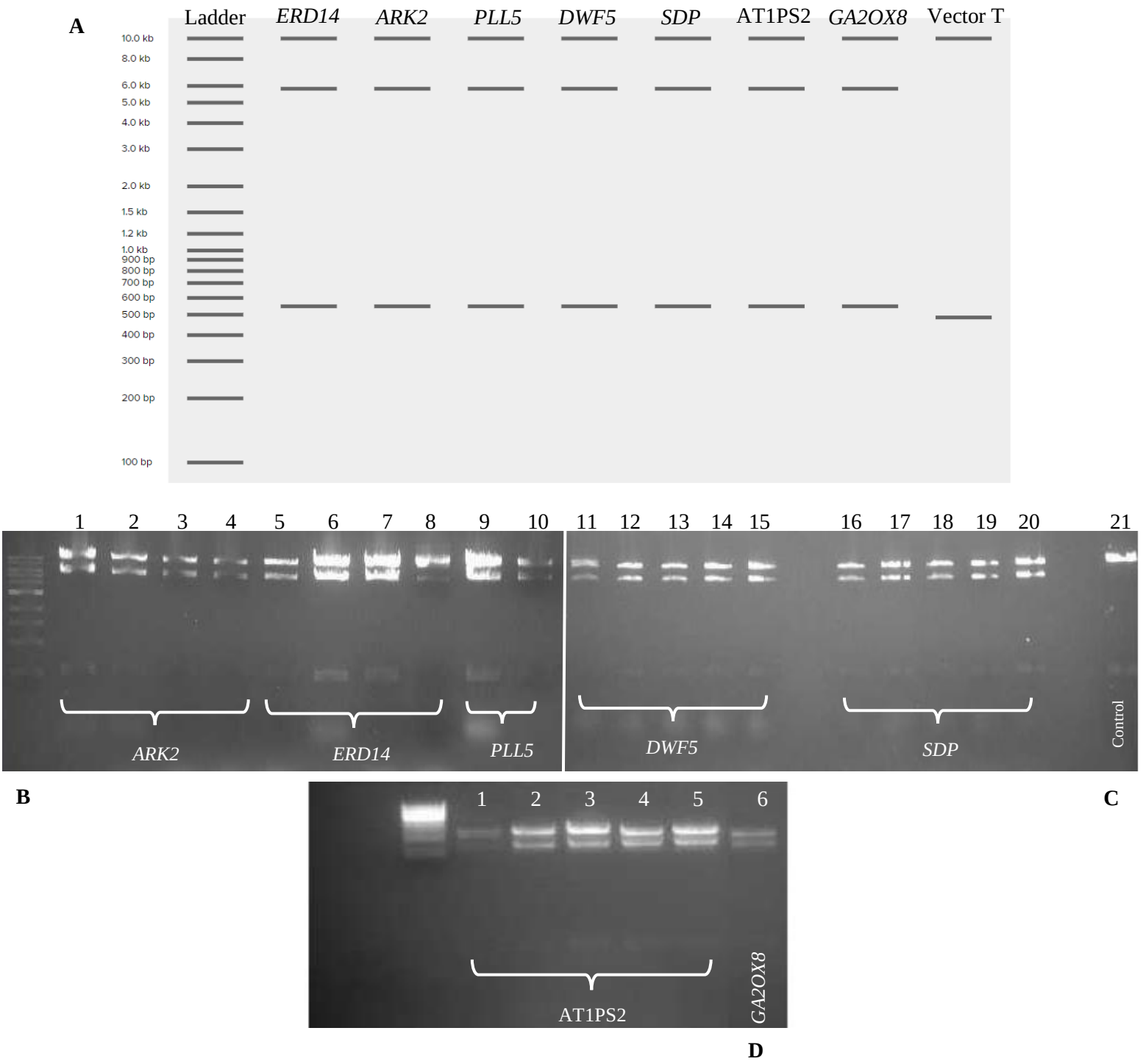


Figure 10. Verification of Correct Clones after Final Transformation. After colony PCR, plasmid DNA was isolated and purified. After which, a restriction digest using *EcoRI* was done to verify successful ligation and transformation. A non-transformed vector T was used a control. **A)** Expected fragment sizes for each vector resulting from being cut. *EcoRI* was expected to cut vectors into three fragment, sizes of fragments being 546, 5776, and 10072 bps long. **B, C,** and **D** show all genes were properly cut.

Day of Floral Dip

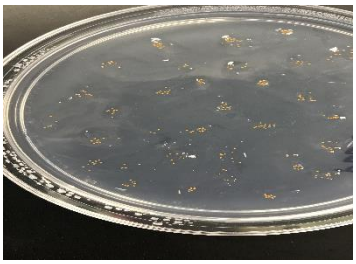
During Floral Dip

One Day after Floral Dip

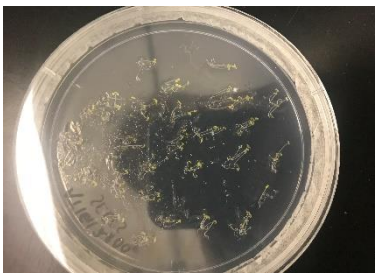
Three Days after Floral Dip

Figure 11. Step-by-step visual of floral dip process; before → during → after

Day of Planting

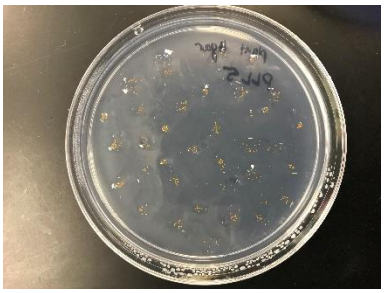


Two Weeks Later

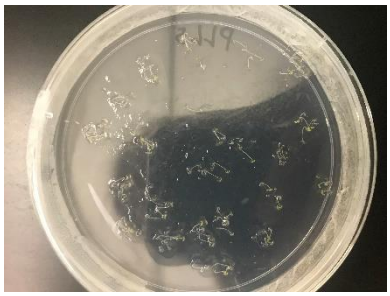


One Month Later

Two Days after Transferring to Soil



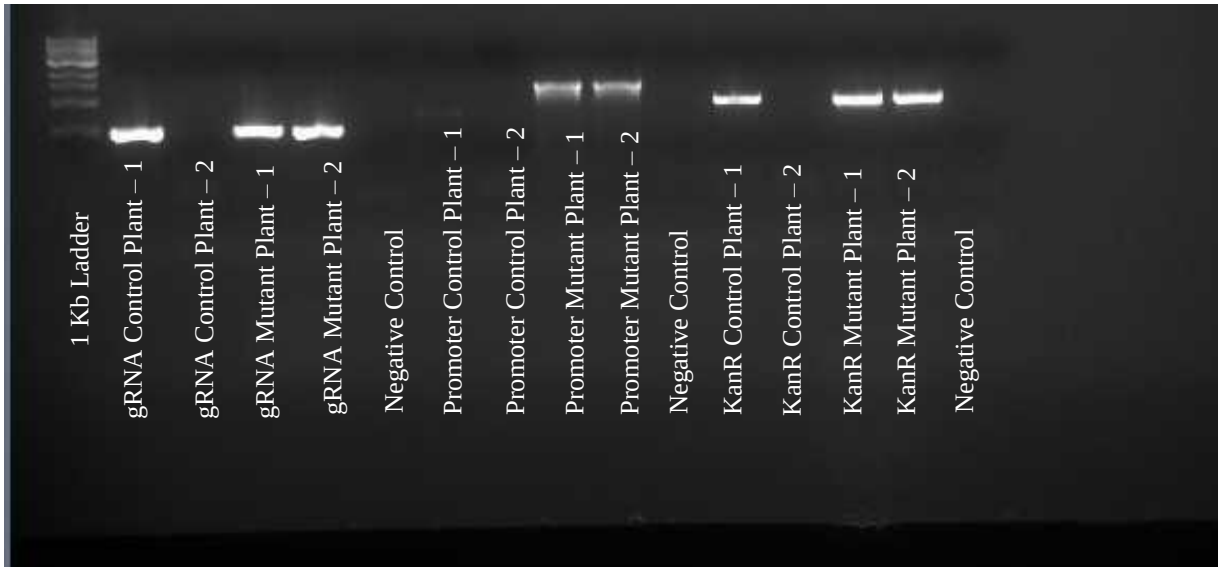
Day of Planting



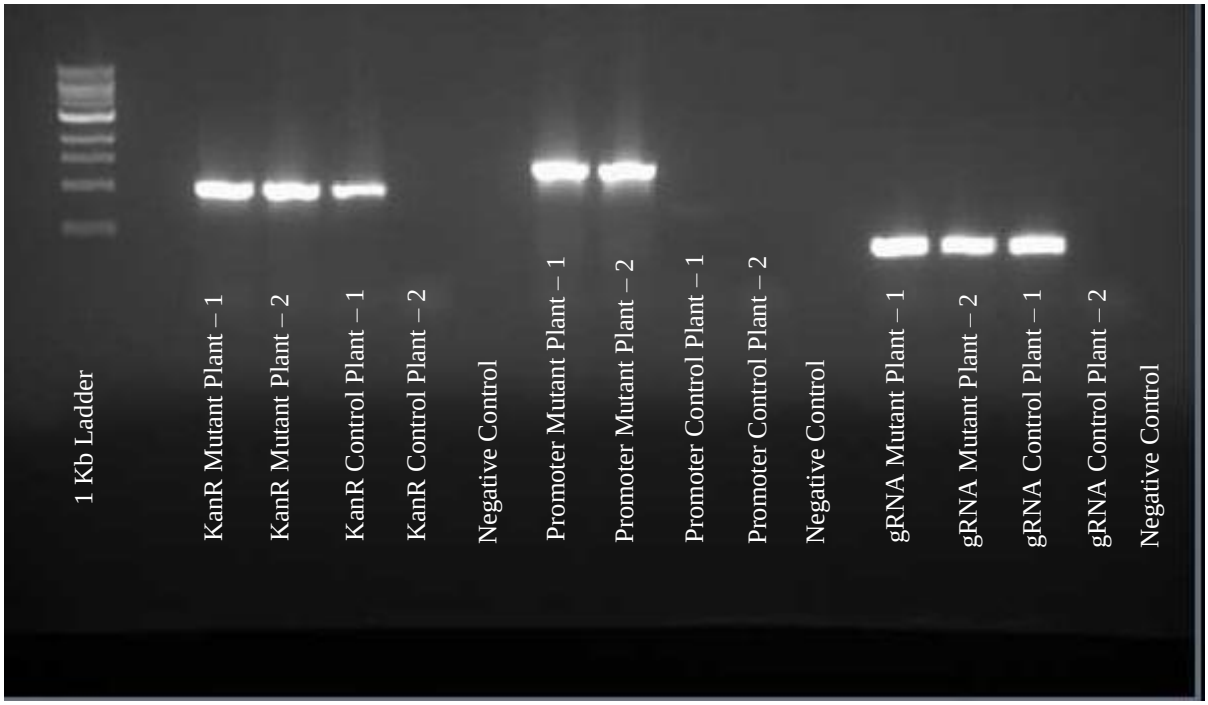
Two Weeks Later

One Month Later

Figure 12. Timeline of the mutant transformation process; day of planting → two weeks later → one month



A) *SDP* Mutant Screening



B) *PLL5* Mutant Screening

Figure 13. Screening for mutants in transformed plants. Primers were designed around three regions of the plant's genome: the designed gRNAs from the Arabidopsis genome, and the AtUbi10 promoter and kanamycin resistant gene within the T-DNA borders of the T-DNA vector. If a deletion was present, the mutant band would be smaller than the control band. **A)** Gel showing screening of mutant *SDP* T_1 seedlings. The presence of a band for both the kanamycin resistant gene and the promoter indicate successful transformation. Results indicate that no large deletion was detected. **B**) Gel showing screening of mutant *PLL5* T_1 seedlings. The presence of a band for both the kanamycin resistant gene and the promoter indicate successful transformation. Results indicate that no deletion was detected.

Table 4. Progression of each gene.

Gene	Vector B Construction	Final Vector Construction	Agrobacterium Integration	Floral Dip	Production of T_0 Seeds	T_1 Germination	T_1 Screening
SDP	✓	✓	✓	✓	✓	✓	✓
DWF5	✓	✓	✓	✓	✓		
ERD14	✓	✓	✓	✓	✓		
ARK2	✓	✓	✓	✓	✓		
PLL5	✓	✓	✓	✓	✓	✓	✓
ATIPS2	✓	✓	✓				
GGLT1							
AT1G74240							
PAL1	✓						
GA2OX8	✓	✓	✓				
LSH6							