Engineering Viral Immunity in *Lactuca sativa*

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Abstract

Lettuce mosaic virus is a common potyvirus that affects the growth and sale of *Lactuca sativa* within the Salinas valley. While it is non-harmful to humans, it can result in the loss of acres of lettuce once detected. We used CRISPR and Cas9 to perform a knockout on a sequence in the *eIF4e* gene that allows for the virus to stabilize and replicate within the plant. We designed our sgRNA as a DNA insert that was inserted into the Cas9 plasmid utilizing golden gate cloning. This Cas9 plasmid was inserted into *Agrobacterium tumefaciens* via electroporation. The lettuce seedlings were then dipped into the agrobacterium, transforming it with the modified plasmid, which we confirmed with sanger and PCR-based sequencing. After sequencing this part of the plants genome, we expect to see a high mutagenesis rate that results in the target sequence become non-functioning, thus immunizing the lettuce against Lettuce Mosiac Virus.

1 Introduction

1.1 Lettuce Mosiac Virus & the eIF4E gene

Lettuce Mosaic Virus (LMV) is a virus that infects many plant species in the Asteraceae family, one of which is *Lactuca sativa*, commonly known as lettuce [12]. LMV, a positive-strand RNA potyvirus, stunts the development of the lettuce, leaving it undesirable and unmarketable. [12]. The most notable characteristics of lettuce infected with LMV are stunted growth, distorted and yellowing of the leaves, and the failure to form the head of the lettuce [12]. However, ingesting an infected plant is harmless to humans.

Although the virus is a seed-borne disease that is passed hereditarily, the virus also spreads easily through insect vectors such as aphids. The presence of this virus in crop fields often results in the rapid infection of neighboring plants and growers subsequently need to destroy the affected acres and lose the investment of capital and natural resources for growing lettuce [38].

Currently, *Lactuca sativa* only has natural immunity from the virus with the recessive genes *mol*¹ and *mo1²*, which are mutant alleles of the *eIF4e* gene [12]. The eukaryotic initiation factor *eIF4e* gene has been shown to stabilize the virus and is needed for viral replication [27]. The modification of the *eIF4E* gene in other plant organisms has led to immunity against other potyviruses. Chandrasekaran et al. discovered that conferring widespread immunity to many potyviruses is possible by disrupting the *eIF4E* gene within cucumbers [7]. This was achieved using CRISPR technology and two gRNAs which targeted the *eIF4e* gene. Natural point mutations arose during DNA repair that ceased the ability of potyviruses to replicate in their host cell [7]. Four potyviruses were not able to infect homozygous mutants but heterozygotes still exhibited symptoms of the virus [7].

The *eIF4E* gene is a protein-encoding gene that helps recruit the ribosome during translation. It is part of a larger structure with multiple other elongation initiation factor (eIF) proteins such as *eIF4A*. The *eIF4E* protein binds to the 5’ cap of mRNA and begins to unwind the structure making it easier for the ribosome to bind to begin translation. Nicaise et. al. determined the high conservation of the gene via sequencing in many different let-


tuce genotypes [27]. They found that although highly conserved, there were a few sites that varied [27]. In all lettuce types susceptible to LMV, all contained the sequence AGGAGC from position 334-339 [27]. Modifications in this site, deletions or single nucleotide modifications correlated with mol1 and mol2 genes, those resistant to LMV. The eIF(iso)4E gene has near-identical sequences in different lettuce genotypes and is likely not related to LMV efficacy, however, the high variance in eIF4E suggests it might control infection. This site became our target site in Salinas lettuce to determine if a modification in dominant alleles could lead to immunity to LMV. The protein’s biological functional site is not the same as the variable site that correlates with the virus. The amino acid sequence correlating to the positions 334-339 in the DNA, -QGA-, is within a protein loop. This would allow the virus to correlate with the unit regardless of activity. Modification in this loop may lead to LMV resistance without interfering with the biological function of eIF4E.

1.2 The Impact of Lettuce in Salinas Valley & its Economy

Lettuce has been an economical crop of California and the Salinas Valley since the 1920s. Since 1949, lettuce has been shipped from the Salinas Valley to the other contiguous US states. The Salinas Valley is unique in that its operation and distribution systems can last 8 months due to its unique climate and environment that protect it from midsummer diseases and pestilence that arise in other growing locations [13]. In the 1950s, the lettuce industry in Salinas almost disappeared due to the unknown introduction and proliferation of LMV. Grogan, Welch, and Bardin studied the effects and transmissibility of LMV-carrying aphids. It was noted there was a high percentage of the LMV-free lettuce seeds and subsequent offspring had become infected with LMV due to the aphid carriers traveling by winds across fields and infecting other local farmland [14]. To combat the spread of LMV, Monterey county implemented new guidelines such as increased weed host removals, lettuce-free growing periods, and the screening and planting of LMV-free lettuce seed to ensure that this disease would not result in an economic loss [14]. Pryor published a report on the effectiveness of these efforts because of the reappearance of localized cases of LMV [29]. Two hypotheses were proposed: New undocumented LMV strains had appeared, or the disease had evolved new carriers to transmit the virus [29]. Both of these hypotheses were subsequently tested. Zerbini, Koike, Gilbertson characterized LMV isolates acquired from the outbreaks in Salinas including 5 necrosis-inducing strains [41]. After the strains were characterized, the collective results from each strain did not indicate the presence of a new highly virulent LMV strain [41]. Zerbini, Koike, Gilbertson identified Gazania spp. as a carrier of LMV that can inoculate the lettuce fields and result in sporadic outbreaks of LMV in the Salinas Valley [42]. LMV in the Salinas Valley is still managed by Monterey county via the guidelines set back in 1952 with the inclusion of pesticides.

Pesticides are used in agriculture to control pests that spread viruses. Aphids are the most common vectors that spread LMV. The most common pesticide in the United States is Chlorpyrifos, an organophosphate insecticide [1, 8]. Organophosphates are poisonous to insects and can also be fatal to mammals. Pyrethrin and azadirachtin are natural pesticides that are considered toxic to mammals by the FDA, but are less likely to be fatal to humans [21]. Over 90% of people in America have pesticides in their system from eating fruits and vegetables [15].
The city of Salinas resides in Monterey county and contributes to a $3.9 billion per year Monterey industry, as well as 76,000 jobs to the community. 61% of the total agricultural output of Monterey was lettuce and Salinas, the “Salad Bowl of the World,” produced the majority of the crop for the county [6]. The UC Davis Department of Agricultural and Resource Economics (2017) released a cost-analysis report on the production of iceberg lettuce in the central coast region. Lettuce was grown on 250 to 1200 acres on a 1500 acre farm with lettuce rotated with other crops to ensure soil replantation. The total cost of growing lettuce was $12,400 per acre, and the cost per carton of lettuce ranged from $12 to $17, with the price decreasing as devoted acreage increases [36]. With the pandemic hitting the United States mainland in late 2019, the extra strain had been put on the agriculture sector and caused an increase in the price to produce and harvest lettuce in the Salinas Valley. The Grower-Shipper Association of Central California (GSA)(2021) commissioned a report to be made on the cost of lettuce in regards to the pressure being applied on agriculture by COVID-19 and other external forces. A baseline of $14.99 was set as the average cost per carton, and factoring in the additional costs of an increase in costs of packaging, labor, and COVID, the price per carton is raised to $17.11 on average [9, 10]. This applied more pressure to farmers and packagers with the inflation of their price of goods, on top of maintaining LMV-removal practices.

1.3 Previous CRISPR work with Other Plants

Several experiments on other plants have been carried out using CRISPR to target other genes. Xu et al. used Agrobacterium tumefacien to deliver CRISPR/Cas9 systems into rice [40]. Xu et al. designed guide RNAs meant to target two sites within the rice’s BEL gene, which encoded for resistances against bentazon and sulfonylurea herbicides [40]. Knockout or partial mutation of the BEL gene resulted in the phenotype of leaves withering when sprayed with bentazon herbicides. Xu et al. noted mutagenesis efficiency was small with target site 1 showing 14 mutants from 90 lines (15.6% success rate), and target site 2 only showing two mutants out of 96 sampled plants (2.08% success rate) [40]. The low rates of mutagenesis were speculated to be related to the higher levels of Cas9 protein detected in mutant plants with target site 1. The nucleotide composition of the target sites also matters with the GC content of target 1 (65%) being lower than target 2 (85%) [40]. Targeting lower GC target regions within a gene will help improve the chance of mutagenesis as lower GC-rich regions are associated with less stable portions of DNA in a gene.

Zhang et al. evaluated CRISPR technology on its ability to make double-strand breaks in the rice (*Oryza sativa*) genome [43]. Zhang et al. targeted several genes in this study: PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, and YSA [43]. Some of these genes do not have immediate effects on phenotypes when modified. When they looked at the first generation of plants, many displayed homozygous or bi-allelic forms of the mutated genes with an average mutation rate of 44% overall [43]. However, not every target was mutated with the presence of chimeras and heterozygotes detected in said plants [43]. In T1 plants, wild-type target sequences persisted which was concerning given the high mutagenesis rates found in T0 plants. Zhang et al. hypothesized that this could be due to the location of the host’s genome during the CRISPR/Cas9 transgene insertion [43].
et al. believed that low concentrations of the Cas9 protein resulted in low mutation events [43].

A third study by Brooks et al. targeted the SlAGO7 gene in tomatoes (Solanum lycopersicum) to show the efficacy of the CRISPR/Cas systems in plant cells [5]. The SlAGO7 gene was selected because it would show an immediate, distinct mutant phenotype with the first leaflets displaying no petioles and later leaves lacking developed laminae. Two sgRNAs were designed to target two sites that would result in a 62 base pair deletion. PCR was performed with several deletions of varying sizes were found in the alleles of 14 of the 29 T0 plants, indicating a 48% mutagenesis efficacy [5]. Brooks et al. noted that although there was a high mutagenesis rate, there was only one that displayed a proper cut from the two sgRNAs designed [5]. Brooks et al. hypothesized this could be due to the low efficiency of simultaneous DNA cutting required, and the probability of a proper cut occurring lowers as asynchronous cuts and repairs at the target site occur[5]. This high efficacy rate can also be due to such a small sample size selected in the T0 generation of plants. Jacobs et. al. showed that CRISPR/Cas systems could perform gene knockouts on green fluorescent protein (GFP) transgenes inserted into soybeans [17]. Several other homologous genes within the soybeans genome were targeted with mutagenesis rates shown to be greater than 70% [17]. MicroRNA (MIR) genes were then targeted because of their relatively small nucleotide sizes; Within the soybean, two MIR genes, miR1514 and miR1509 were targeted with indel frequencies greater than 95%, but these indels did not affect the mature mRNA produced during translation [17]. Pyott et al. utilized CRISPR/Cas9 technology to engineer viral resistance into Arabidopsis thaliana against Turnip mosaic virus (TuMV) via knockout of the eIF(iso)4E gene because the eIF gene family had been previously identified as recessive resistance alleles against potyviruses [30]. Successful induced mutations of the eIF(iso)4E gene resulted in complete resistance against TuMV in the plants [30].

1.4 Vectors to Genetically Modify Plants

The delivery system for CRISPR/Cas9 experiments is extremely important. For plants, common delivery methods of CRISPR/Cas9 systems have included particle bombardment, agrobacterium-mediated, floral-dip, de-novo maristem induction, and virus-mediated sgRNA delivery [24, 25]. Agrobacterium tumefaciens and Agrobacterium rhizogenes have been the two most common strains used in agrobacterium-mediated CRISPR/Cas9 delivery systems. Numerous studies have used A. Tumefaciens mediated delivery as a reliable method for genetic manipulation in Lactuca sativa [23]. A. Tumefaciens have typically been used for transgenic experiments but have also been successfully used in base-editing experiments [31]. A. Tumefaciens that contained a tumor-inducing plasmid coding for kanamycin resistance have been used to select for transformed bacteria before introduction into the plant [11].

1.5 Why Lettuce?

Although multiple experiments have shown that modification of the eIF4E gene has conferred widespread immunity to potyvirus in multiple crops, lettuce has not yet been tested. Due to the economic importance of lettuce in the world, our group attempted to prove modification of the eIF4E gene in lettuce would lead to LMV immunity. Other CRISPR and
RNAi experiments have proved to be beneficial in lettuce such as making seeds more resistant to higher temperatures during germination. Bertier et al. found that the LsNCED4 gene in *Lactuca sativa* can be modified to control the thermoinhibition of seed germination [3]. Bertier et al. knocked out the gene and enable modified seeds to germinate at much higher temperatures for lettuce [3]. CRISPR was used to create a homozygous mutant that contained the target gene by silencing it using RNAi molecules in tandem with the Cas9 mechanisms. Although the germline was edited using CRISPR, there was a low frequency of successful edits [3]. Nguyen et al. targeted the Lettuce gene LsVAR2, which controls variegation and displays albino discoloration when knocked out [26]. Although success rates with CRISPR may be low, it is possible to alter the genes beneficially with more understanding of the biological systems in place which only comes with further research and experiments. Eventually, this technology could provide better, cleaner seeds that grow easier and with fewer chemical additives.

Seeing as LMV is a common reason for the loss of Salinas lettuce, our team wanted to immunize the crop from it, leading to a reduction of waste from destroying the affected lettuce and an increase in the amount of water saved. The most efficient way of achieving this was to use CRISPR and knock out the *eIF4E* gene, making the subsequent generations of the altered lettuce immune as well. It was the most cost-efficient method by only requiring a single altered lettuce plant, because lettuce is self-pollinating, while producing a lifelong supply of the crop to the consumer By making genetic alterations, there is no more need to use pesticides to save Salinas Lettuce from LMV.

## 2 Methods & Materials

### 2.1 Oligonucleotide Design

Three different oligonucleotide sequences were designed for the purpose of this project. The guide RNA that would be produced by the pHSE401 plasmid had to be designed as a DNA oligonucleotide to be inserted into the backbone using golden gate cloning. We decided to target the sequence \[5'\text{-CATCGACCAAGCAAGTTGGCTCAAGG-3'}\] because it lies in the open reading frame of the *eIF4E* gene. Nicaise et al. found a sequence in lettuce types susceptible to LMV that was absent in those immune to the virus [27]. We hypothesized this sequence might provide stability to the viral RNA during its replication, and subsequent modification could provide immunity. The bolded region is the NGG PAM site required for Cas9 to cut the DNA. To introduce this sequence to the backbone of our plasmid, we flanked the sequence with BsaI cut sites for golden gate cloning. We designed the sgFoward and sgReverse primer pair to amplify our oligonucleotide via PCR. The final sequence used can be found in Appendix A.1.

Two sets of primers were designed, one set for PCR amplification of the DNA insert and one set for the amplification of the lettuce genome. The first set of primers, the sgFoward primer \[5'\text{-CGATCGATTTAGCCTAGCCGT-3'}\] and the sgReverse primer \[5'\text{-GCTAACGCATATGGCCCTCA-3'}\], were designed using NCIB’s primer design tool with the default parameters except for amplicon size. The primer melting temperature (Tm) of these primers were both within half a degree of 60°C. Our second set of primers were designed to
amplify a region of the lettuce genome. Although the forward primer would amplify both transgenic and wild-type lettuce, the reverse primer overlapped the CRISPR cut site. This way, if modifications were made in the genome, the reverse primer wouldn't be able to anneal and not exponentially amplify the DNA in a PCR reaction. This second set of primers were also designed using NCIB’s primer design tool with default parameters except for amplicon size.

2.2 Plasmid miniprep, PCR of DNA insert, Golden Gate cloning

The Cas9 plasmid used was the pHSE401, which was a gift from Qi-Jun Chen (Addgene plasmid 62201; http://n2t.net/ncbi:Addgene:62201; RRID: Addgene_62201) [39]. We used this plasmid as it contained all the genes need to assemble the Cas9 protein and the guideRNA in the plant cell, and kanamycin and hygromycin selective markers. The pHSE401 came in an E. coli stab and was cultivated at 37°C and selected using LB-Kanamycin media. Plasmid purification was performed using the plasmid miniprep kit provided by Thermo Fisher Scientific [33]. Verification of the plasmid was done with a nanodrop and with gel electrophoresis. The DNA insert was PCR amplified and verified with gel electrophoresis. The golden gate cloning protocol was based on the protocol from the 2017 iGEM Every Paris-Scalay team [2] and placed in the thermocycler for 20 cycles and held at 4°C indefinitely. We used the BsaI restriction site to insert our DNA fragment with the respective restriction enzyme purchased from New England Bio labs [22]. Verification was preformed via gel electrophoresis and the reaction was cleaned using magnetic bead-based purification [32].

2.3 Electroporation of A. Tumefaciens

Electrocompetent LBA4404 A. Tumefaciens cells were purchased from Takara Bio Inc [16]. The agrobacterium was plated on Yeast Extract Broth purchased from PhytoTech Labs [20]. These cells were kept on -80°C storage until we were ready to move forward. Electroporation was carried out based on Bio-Rad’s provided protocols for agrobacterium [4]. Agrobacterium cells were mixed with the completed plasmids from Golden Gate cloning in a 0.1cm cuvette. It was then electroporated using the Bio-Rad MircoPulser electroporator and its preprogrammed 2.2kV setting ”Agrobacterium”. The cells were then transferred to YEB liquid media to recover for 3 hours at 30°C, then streaked on YEB-Kanamycin plates and placed back in for 48 hours at 30°C. Positive colonies were then selected and transferred to YEB-Kanamycin liquid cultures in 15mL falcon tubes and left to incubate for 48 hours at 30°C. Any growth detected in the media was transferred to a larger Erlenmeyer flask containing the same selective media and incubated at 30 °C for another 48 hours and moved into temporary storage at -4 °C.

2.4 L. Sativa Cultivation Transformation

Murishage and Skoog (MS) media were purchased from Caisson Labs [19] and mixed with Plant Preservative Mixture (PPM) from Plant Technology[35] and agarose was poured onto plates. Lettuce seeds were pushed into the agar gently and left in direct sunlight for 7 to 10 days, or when the first two leaves had sprouted.
For the transformation of the plant, we decided to follow protocols provided by Wayne Curtis and Rahul Patharkar [28, 37]. The liquid media cultures previously prepared were centrifuged down into a pellet with the YEB media supernatant being poured off. We used three different methods of agrobacterium-mediated transformation on the plants: floral dipping, cotyledon splicing, and transfer to both solid MS media and liquid MS media. Plants that were floral dipped were moved to the greenhouse into the soil, while those that were cotyledon spliced were moved to back into the sunlight on their new plates. Plants were left for 10 days to recover and grow in their respective environments.

2.5 Sampling & Sequencing of Plants

The extraction of pure DNA from plant samples was preformed from a modified protocol from Integrated DNA Technologies and Keb-LLanes et al. [18, 34]. Elution buffers were created according to said protocol and the lettuce leaves were grounded in a mortar and pestle to allow for better access of the plant cells. After following the steps highlighted in the protocol the plant DNA was stored at -20°C for PCR-based sequencing and Sanger sequencing.

3 Results & Discussion

3.1 Plasmid Confirmation & DNA Insert Confirmation

The presence of our Cas9 plasmid post-miniprep was confirmed via a nanodrop spectrophotometery and gel electrophoresis. The nanodrop spectrophotometer was used to confirm the presence of DNA and its purity in its environment, while the gel was used to confirm its approximate length. The results of the gel can be seen in Figure 1. The gel and tracking and loading dye are noted in the caption of these filters. Positive bands indicated the presence of DNA in our sample and that it was the approximate size of our plasmid, 16653bp. Likewise the PCR amplification of the DNA insert was also confirmed via nanodrop spectrophotometery and gel electrophoresis. The results of the gel can be seen in Figure 2 and we see a positive match in well 4 with the band approximately 100bp, which was our expected DNA insert size.

3.2 Golden Gate Cloning & Agrobacterium Electroporation

After confirming the presence and amounts of these two DNA components, we preformed golden gate cloning. We checked with gel electrophoresis, as seen in Figure 3, that the reaction had taken place and after cleaning it with the magnetic bead-based cleanup, we used nanodrop photospectrometry to confirm that there was purified DNA. Electroporation of *A. Tumefaciens* with the modified plasmid was successful. After plating the liquid media recovery vials, a few colonies were spotted on the YEB-kanamycin agar plate. These were transferred to selective liquid media and incubated for usage during the plant transformation. This is shown in Figure 4.
Figure 1: SYBR green tracking dye and DNA loading dye were used on a 2% agarose and 1X TBE mixed gel. Well 1 was loaded with the 1kb ladder and well 2 was loaded with a negative control. Well 3 and 4 were loaded with the plasmid and are a positive indication of the presence of large DNA as there were bands fluorescing when exposed to UV light. The bands present in wells 3 and 4 correspond to a rough approximate size of the plasmid, approximately 16kb.

3.3 Plant DNA Sequencing

Following the agrobacterium-mediated transformations, the floral-dipped plants all survived. However, the cotyledon spliced plants in the liquid MS media and the solid MS media did not have a high survival rate in the first round with only one cotyledon survived. A second round of cotyledon spliced plants were dipped and placed on media. The last step of the verification was to send our sample to get sequenced, which we expect to show a successful knockout of the eIF4E gene.
Gel results of DNA Insert

Lane 1: 2μL 1kb ladder
Lane 2: 4μL 1kb ladder
Lane 3: Neg. Control
Lane 4: DNA insert (~100bp)

Gel of DNA insert PCR

Figure 2: The gel was a 0.7% agarose mixed with 1x TAE and EtBr. Samples were mixed with TriTrack loading and stain dye and set to run at 100V for 25 minutes. A single faint band was noted in well 4 that correlated with the ladder’s fragment size to be near 100bp.
Gel of golden gate cloning

Figure 3: The gel used was a 2% agarose and 1x TBE mixture. Samples were mixed with TriTrack loading and stain dye and set to run at 90V for 30 minutes. As highlighted in red, there are two bands detected with a possible third band near the end of the ladder. These bands indicate the presence of DNA and that there are fragments related to the size of our plasmid and the DNA insert.
Figure 4:  A) A positive agrobacterium colony is identified on and selected post-electroporation and recovery.  B) The positive colonies are transferred to the falcon tubes with 10 mL of YEB-Kanamycin media on a shaker incubator at 30°C.  C) The agrobacterium growth after 48 hours compared to a negative control.  D) A large Erlenmyer flask filled with the selective media, and one of the vials emptied into it beginning its 48 hour incubation period.
4 Conclusion

The successful modification of the eIF4E gene in Salinas Lettuce to confer immunity to the Lettuce Mosaic Virus will result in a decrease in agricultural waste, economic benefits, and will further the understanding of CRISPR/Cas9 use in crop experiments. The verification of the experimental procedures carried out so far projects a successful outcome, dependent upon the uptake of the transformed A. Tumefaciens in the propagated lettuce leaves and growth after transformation. The final procedure to confirm this consists of extracting the plant DNA and sending it to get sequenced at UC Berkeley. From this, it can be determined if the gene was successfully knocked out by comparing the sequence returned by UC Berkeley against the Salinas Lettuce genome. It is also possible to test for a modification by attempting to PCR amplify the DNA extracted from the transformed leaves with the primers used for the unmodified sequences. If the DNA is amplified with the old primers, the modification did not take place. However, this test will only be performed in addition to the outsourced sequencing. If the sequencing results return with an unsuccessful knockout, an in depth review of possible explanations will be included in the final report as feedback for future experiments.

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References


[36] Laura Tourte et al. “Sample Costs To Produce And Harvest Iceberg Lettuce”. In: (2017), p. 17.


A Appendix

A.1 DNA Insert Sequence

>sgRNA_eIF4E
5’- GGATCGATCGATTTAGCCTAGCCGTAGACGATGCTT
CAGGCGTGGTCTCGATTGCACCACCGAAGCAAGCAAGT
CGCTCAAGGGTTGGAGACCACCATCGTACCGTACGTTTGAG
GGCCATATGCGTATCCTATCTCACTCTAAGG - 3’