

CRISPR/CAS9-MEDIATED GUS GENE KNOCK-OUT IN THE TOBACCO PLANT

Rose Weinstein, Linda Do, Annalise Wellman, Nicholas Nifakos,
Justin Kirke & Xing-Hai Zhang
Charles E. Schmidt College of Science

Abstract

CRISPR/Cas9 technology facilitates gene editing by removing or adding nucleotides at specific DNA sequences. This results in a gene removal (knock-out) or insertion (knock-in), thus causing a cell to manipulate gene expression. Using *Agrobacterium*-mediated DNA delivery, CRISPR/Cas9 was utilized to genetically modify a transgenic RM-1 tobacco plant that expresses a reporter gene, β -Glucuronidase (GUS). The CRISPR/Cas9 integrated plants, named HaG, were regenerated, and the presence of Cas9 gene in these plants was confirmed by qPCR. The T1 and T2 progenies of the HaG plants were screened for effective gene editing by GUS staining assays. The GUS negative plants were selected and grown; the DNA was then extracted, sequenced, and compared to the original GUS gene sequence. Our results demonstrate that GUS expression had diminished in the CRISPR plants. DNA sequencing showed a deletion occurred in the expected coding region of the GUS gene, this resulted in a gene knock-down instead of a complete knock out.

Introduction

CRISPR/Cas systems were initially discovered in bacteria as the immune response to foreign DNA, such as bacteriophages or plasmids. It is facilitated by RNA-guided nucleases to target, edit, and degrade invading phage genomes. This natural mechanism can be used as an accurate gene editing system which targets and cleaves specific sites of nucleic acids [1].

The CRISPR/Cas9 system consists of guide RNA (gRNA), a 20-nucleotide targeting sequence; a 3-nucleotide protospacer adjacent motif (PAM), and a Cas9 enzyme. The Cas9 protein is an endonuclease commonly utilized in biotechnology that results in genome modification. The gRNA and Cas9 enzyme work in unison to find the specific gene of interest, the gRNA directs the Cas9 nuclease to the DNA sequence that is identical to the targeting and PAM sequences. Once the DNA is cleaved by Cas9, the double stranded DNA undergoes two forms of repair. One way of repairing is known as nonhomologous end joining (NHEJ), which is error-prone and results in an insertion or deletion of genes. The other form is homologous recombination (HR)-directed repair; in contrast to NHEJ, a DNA template is required. Results of HR-directed repair consist of a new gene knock-in or full gene restoration, depending on the template applied [1][2].

Agrobacterium-mediated infection is commonly implemented to deliver DNA construct into plant cells to produce genetic modification. It transfers bacterial plasmid DNA into host cells, allowing recombination to occur. The *Agrobacterium* exploits the factors of the host to transfer the gene of interest from the bacterium plasmid T-DNA into the host genome. In the natural world, gene transfer from *Agrobacterium* in plants can result in a crown gall formation, this tumor growth is due to the tumor-inducing characteristic of the Ti-plasmid. However, the *Agrobacterium* used in research has a modified Ti-plasmid possessing an absence in tumor formation expression. The *Agrobacterium*-mediated transformed cells spontaneously regenerate and produce transformants that carry cellular T-DNA sequences from *Agrobacterium* [3][4].

Within the T-DNA gene construct utilized for transformation, a hygromycin phosphotransferase gene (HPT) served as a selection marker. Derived from *Streptomyces hygrosopicus*, it expresses an enzyme that functions as a hygromycin B detoxifier by phosphorylation. Hygromycin B is known as an inhibitor of protein synthesis in ribosomal translocation and aminoacyl-tRNA recognition, in both prokaryotes and eukaryotes [5]. Successful transformants would display HPT gene expression, thus showing resistance to this inhibitor.

The T-DNA construct also contains the Cas9 gene and gRNA sequence to target the gene of interest, β -Glucuronidase (GUS enzyme), in order to produce GUS gene knock-out in this experiment. The GUS gene does not naturally occur in plants but is found in *E. coli* and other organisms. An RM-1 tobacco plant that was modified prior to this experiment served as the model organism due to its expression of GUS activity. In the presence of the X-Gluc substrate (5-bromo-4-chloro-3-indolyl-D glucuronide), cleavage occurs by the GUS enzyme. Following cleavage of glucuronide, oxidative dimerization arises that is stimulated by O₂, resulting in a blue colored precipitate. In the application of a GUS staining assay, blue coloration indicates the presence of the GUS gene, the absence of blue indicates that GUS activity is not present [2]. If proper execution of CRISPR/Cas9-mediated GUS knock-out occurs, the transgenic progenies will express hygromycin resistance and an absence of blue precipitate in the GUS staining assays.

Methods and Materials

Gene construct

The cloning vector utilized in this experiment contained the genes of interest within the left and right border of the T-DNA, which was then transferred into the plant cell, facilitated by *Agrobacterium*. The recombinant T-DNA construct in Figure 1, contained three critical genes: a hygromycin phosphotransferase gene (HPT) for hygromycin resistance, the Cas9 enzyme coded gene, and the gRNA GUS-targeted sequence. HPT served as a selection marker gene; it was used to detect and select the transformed cells. The GUS gene in the host plant coincided in serving as the knock-out target and a reporter gene to confirm the success of gene removal. The right border begins with the U6 promoter driving the gRNA, followed by the appropriate CaMV35S promoter that drives the Cas9 enzyme sequence, which is then tailed the NOS terminator. To the left of this terminator is the 35S promoter driving the HPT gene that is then concluded by the 35S terminator.

Agrobacterium infection

With the noteworthy capability to infect hosts through DNA transfer, an *Agrobacterium tumefaciens* bacterial infection was vital to the investigational transformation. To exploit this mechanism, a co-cultivation process was applied, infected explants were placed onto treated media; this allowed for proper T-DNA integration into the tobacco genome. A previously prepared overnight culture of *A. tumefaciens* that contained the T-DNA plasmid was grown, constantly aerated and incubated at a controlled environment of 28 °C for 1-2 days. The culture was then transferred into a sterile 50 ml tube and centrifuged at 3,000 rpm for 10 minutes at 25 °C. Excess liquid was properly discarded under a fume hood while keeping the pellet undisturbed. The pellet was resuspended with 5 ml of a previously prepared co-cultivation solution. Preparing the plant host to be infected, RM tobacco leaves were cut into ten 5x5 mm sections (per experimental sample) using a sterilized scalpel. The leaf discs were incubated in the bacterial solution for 15 minutes, and then were removed from the solution, blotted dry by sterile paper towel, and placed on the filter disk over the co-cultivation medium without hygromycin. The leaf sections were positioned with the abaxial surface facing upwards. The filter disk served to avert undesirable bacterial growth. The filter disks containing the infected leaf tissues were incubated for four days in a dark environment.

Transformation & regeneration

Following the four-day incubation, the filter paper-placed leaf sections were removed and replaced on selection/regeneration media containing both hygromycin B (30 mg/L) and timentin (500 mg/L). The application of timentin prevented any unwanted overgrowth of *Agrobacterium* on this media, the bacterial overgrowth would have potentially caused necrosis which would

threaten proper transformation. About six shoots from each regeneration dish (lacking contamination) were removed from the callused tissues that showed HPT resistance-linked transformation and were inserted in prepared rooting media boxes containing hygromycin B (30 mg/L). Sterilized scalpel and forceps were applied in isolating the shoot from the surrounding callus. It is important to note that the shoots were not entirely submerged in the media to allow proper root formation and continuous growth. The boxes were placed and incubated in a controlled growth chamber with light and a temperature of 26°C for approximately five weeks.

Promising plantlets from these preliminary rooting boxes were aseptically transferred into new individual rooting boxes and placed in the equivalent environment for about two weeks. At significant root formation, small sections of leaf tissue were cut from each plantlet for DNA extraction and GUS knock-out analysis. Nineteen suitable transformant juvenile plants (T1 HaG) were transferred into potted soil to reach maturity.

Cultivation of T2 seeds and Mendelian segregation ratio

The seeds of the mature 19 T1 HaG lines (T2 HaG) were collected. The seeds were folded into fabric squares containing about 30 seeds each, labeled appropriately with the genotype of the parent T1 HaG plant line, and paperclipped closed. The compartmentalized seed packets were sterilized in a flask containing a solution of 10% bleach. The sterilized T2 HaG seed packets were rinsed with DI water and were inserted into seed germination media containing hygromycin (30 mg/L) (one Petri dish for each seed line) by sterile forceps. The dishes were properly labeled with the correlated T2 genotypes and placed inside of the growth chamber for a duration of approximately two weeks.

Following the growth period, the Petri dishes were surveyed for hygromycin resistance by determining which seedlings displayed resistance with dark green cotyledon and root initiation; as well as nonresistance with pale yellow cotyledon and no roots. The Mendelian ratio, involving resistance versus nonresistance, was determined. 12 out of the 19 T2 seed lines proliferated and were selected to be applied towards further analyses.

GUS staining assay of RM, T1 and T2 plants

To screen for GUS knockout in the experimental tobacco, GUS staining of the original, untransformed RM-1 plant served as a key visual reference and control. Small segments of leaf tissue were cut from the RM and T1 HaG plants, and immediately placed into individual microcentrifuge tubes. 100 µl of X-Gluc solution was added to each tube, ensuring complete submergence of the leaf tissues. The tube was incubated in a 37°C-water bath overnight. Following, the X-Gluc staining solution was removed. To increase the appearance of blue staining, a substantial amount of 70% ethanol was added to each tube, immersing the leaf sections for two days.

The leaf tissue samples were then observed and photographed under a low-power dissecting microscope. This GUS assay protocol was duplicated on the T2 HAG plants. It was noted that the T2 leaf tissues were inserted into 96-well plates in place of microcentrifuge tubes. Four leaf samples of the T2 plants (two HaG-LD2 and two HaG CT), that showed predominant GUS knock down, were chosen to continue to the DNA extraction and sequencing phase of this research.

DNA Extraction and purification

A GenCatch protocol was executed towards an Epoch extraction kit for effective purification of genomic DNA, approximately 100 mg of fresh leaf sample from each T1, T2 and RM plant were ground to a fine powder by a small pestle with the assistance of liquid nitrogen in a microcentrifuge tube. Four hundred μ l of PX1 buffer and 4 μ l RNase A stock solution were added to each tube and vortexed to homogenize. Subsequent to the vortex and a 10-minute 65 incubation period, 130 μ l of PX2 buffer was added to the lysate of each sample. After another vortex, the samples incubated on ice for 5 minutes, the lysates were then added to shearing tubes (inserted in the collection tubes) to be centrifuged for 2 minutes at maximum speed. The flow through from each tube was carefully transferred to a new tube without disrupting the pellet nor obtaining unwanted cellular debris. Dependent on the amount of flow through for each tube, half of the flow through volumes worth of PX3 buffer and one volume of 100% ethanol were added to the clear lysate and mixed by pipetting. Six hundred and fifty μ l of each sample were added to individual Plant Genomic DNA Mini Columns sitting in a collection tube. Tubes were closed and centrifuged at 10,000 rpm for 1 minute, the filtrate was properly discarded. Two washing periods consisting of 0.7 ml of WS buffer with 30 second centrifuge periods and immediate filtrate disposal were applied. Centrifuging the columns for an additional 2 minutes to allow all residual WS buffer to be removed from the columns; the columns were then transferred to new 1.5-ml tubes. Two hundred μ l of warmed ddH₂O was added to each tube, tubes were centrifuged for 1 minute and stored in -20°C. It is noted that the concentrations of the kit solutions were not disclosed.

DNA Analysis & qPCR of T1 and RM

DNA concentration was measured for each T1 extraction by Nanodrop spectrophotometer. Each sample was diluted to 2 ng/ μ l to achieve standardization. Applying the qPCR protocol, each sample was aliquoted into six wells; three wells paralleled to target Cas9, with the remainder three corresponding to EF-alpha (internal standard). Each well contained 11 μ l of

mastermix, this consisted of 2X fluorescent SYBR Green Dye, forward and reverse primers, and 9 μ l of loaded sample. The qPCR ran for about two hours. The Cas9 relative gene dosage was quantified for each T1 sample with RM as reference.

PCR for GUS gene from T2 plants

The extracted and purified DNA from each T2 HaG-LD2 and HaG-CT leaf samples were thawed out along with the appropriate reagents. Each PCR tube, four in total, contained 25 μ l of 2x PCR mastermix, 1 μ l of plant DNA, 1 μ l each of GUS forward and reverse primers and 22 μ l of nuclease-free water. The tubes were centrifuged to ensure proper mixing of the template and reagents which were placed inside of the thermocycler. The PCR was initiated at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 25 seconds. After completion, the tubes were stored in -20°C.

Gel electrophoresis of T2 PCR products

Gel electrophoresis was performed on the T2 PCR products to purify the DNA prior to sequencing. The PCR tubes were taken out of storage and thawed. A 1.2% agarose gel was made using TBE (Tris borate-EDTA) buffer and the PCR samples were loaded. The electrophoresis was run at a constant 80V for an hour. The DNA bands were excised from the gel while on top of a UV light box and placed into the microcentrifuge tubes.

GUS DNA clean up and gel extraction

The PCR product (GUS DNA fragments) were extracted from the gel with the addition of 0.6 ml of GEX buffer. The tubes were incubated in a 50°C water bath for approximately 10 minutes while being inverted every 2 minutes until the gel completely dissolved. The dissolved gel solution containing the DNA and buffer were transferred into new individual columns and centrifuged for 30 seconds at 5,000 rpm, the filtrate was discarded. This step was repeated. The columns were washed by adding 0.5 ml of WN buffer and centrifuged for 30 seconds at 5,000 rpm, the flow-through was discarded. The columns were washed with 0.5 ml of ethanol containing WS buffer and centrifuged for 1 minute at 5,000 rpm, flow-through discarded. The columns were centrifuge at 12,000 rpm for an additional 3 minutes to remove residual ethanol to ensure DNA quality. The columns were placed into new labeled 1.5-ml centrifuge tubes and the DNA was eluted by applying 15 μ l of elution buffer directly to the center of the column membranes. The columns remained undisturbed for 2 minutes, and then centrifuged for 1 minute at 12,000 rpm. Six μ l of DNA from each tube were transferred into individual and labeled tubes that were sent out to be sequenced. The remainder of the tubes containing the gel extracted DNA were stored in -20°C for future use. Upon sequence completion, the edited GUS sequences were analyzed and compared to the GUS sequence of the original transgenic RM plant.

Discussion and Results

Nineteen transformant plants were created by implementing *Agrobacterium* mediated infection of T-DNA containing hygromycin resistance and a CRISPR/Cas9 system engineered to target the GUS sequence. The first generation (T1), labeled as the HaG genotype, were primary transformants derived from RM-1 tobacco prevent overgrowth of *Agrobacterium*; if this were to occur, it would have been detrimental to the potentially regenerated tissue by inducing necrosis. In the process of regeneration, the leaf sections grew ample in size, along with the formation of callused tissue; maintenance was mandatory to provide an optimal environment of this process (Figure 2.1C). Once green buds formed and shoots began to proliferate (Figure 2.11D), suitable shoots were removed from the callused tissues and inserted in rooting media boxes containing

Figure 9. Quantification of relative Cas9 gene dosage of RM-1 (-C) versus T1 HaG plants. hygromycin (Figure 2.2). Juvenile plants leaf tissues. Upon infection, the tissues were placed on media containing hygromycin to ensure that only the plant cells that express Figure 7. Sequence data analysis of CRISPR T2 HaG plants HPT resistance gene to flourish (Figure 2.1A). The plasticity of the plant tissue enabled successful regeneration of the selected tissues when being placed in regeneration medium consisting of hygromycin and timentin (Figure 2.1B). The addition of timentin was served to displaying strong root formation were removed from the rooting boxes and placed in potted soil to reach maturity (Figure 2.2). Seeds were harvested from the 19 mature, transgenic tobacco T1 HaG plants, which were then sterilized, and planted in petri dishes of seed germination media containing hygromycin to examine the segregation of HPT gene according to Mendelian law

(Figure 4). These seeds germinated, rooted and grew into T2 seedlings. A Mendelian ratio was determined according to their sensitivity to hygromycin. The hygromycin sensitive seedlings were properly discarded, and the resistant seedlings were selected and utilized in further experimental analyses.

GUS stain assays were performed on the leaf tissues of the RM-1 and T1 plants. RM-1 served as the control which demonstrated a strong histochemical localization of GUS activity (Figure 3). The highly saturated blue precipitate signified the presence of the GUS gene due to enzyme-substrate cleavage activity. The T1 plant tissue (Figure 3, top right) displayed a great decrease in blue precipitate, this qualitatively represented a reduction in GUS activity. To ensure CRISPR/Cas9 editing process continued in the progenies of the T1 plant, GUS staining was also performed on the leaf tissue of T2 seedlings. Figure 3 (bottom) shows a visual reduction of GUS activity as well. It was noted that even though there was a drastic qualitative contrast between the RM and transformed progenies, there was still a residue of activity. This indicated there was not a complete silencing of the gene, but the Cas9 system did indeed successfully

target and disrupt the gene of interest; leading to a significantly decrease in gene activity. Due to time constraints, only 14 out of the 19 T1 plants were utilized for qPCR analysis for Cas9. The forward and reverse primers used in the application of qPCR towards the T1 plants were designed to target the newly inserted Cas9 sequence in the host genome. RM-1 was also applied in this procedure and served as the negative control. In Figure 5, quantitative measurement of the relative Cas9 gene dosage was determined, which showed increased gene presence among the T1 plant lines versus the RM plant (-C). A presence of the inserted Cas9 gene was noted and inferred successful transformation, but this did not indicate whether Cas9 enzyme had edited the host genome.

Two HaG-CT and two HaG-LD2 plant lines were chosen for further analysis because they showed the greatest reduction in GUS staining. DNA was extracted from T2 leaf samples of these four plants. Instead of applying qPCR towards an end goal of quantitatively measuring gene dosage, it was directed towards the GUS sequence. The forward and reverse primers used in this application were engineered to target and amplify the GUS gene sequence flanking the CRISPR editing site. GUS sequence in RM 1 served as reference in order to determine differences between sequences. Following the completion of PCR, the amplified DNA of each sample was extracted from the gel and sequenced. Comparison of the GUS sequence from the HaG plants with the sequence from RM-1 revealed a "T" (thymine) nucleotide deletion in the boxed target site, illustrated in Figure 6. This edit resulted in a frameshift mutation of *L, leucine, the 318th amino acid of a 603 amino acid sequence of the GUS protein to a stop codon. As a result, the GUS protein was truncated at the middle of the protein, resulting in the GUS enzyme inactivation. The reason why traces of GUS stains remained in the plant tissues is that since gene editing is the result of both Cas9 enzyme activity at the specific site and DNA repair process in the host cells. Not all the cells in the plant undergo the editing process at the same rate and to the same extent. The cells that have not yet completed CRISPR mediated editing will retain the GUS activity, thus showing blue when stained. Since our HaG plants possess the CRISPR/Cas9 gene system permanently, we expect that editing for GUS gene will continue during plants growth and development until the targeting site is exhaustively edited.

Overall, the transgenic tobacco plants served as an achievable exemplification of biotechnology and genetic engineering through the exploitation of the CRISPR/Cas9 system. Regardless of the resulting data and correlating figures not necessarily displaying a complete removal and absence of the GUS gene, it was confirmed that CRISPR/Cas9 effectively targeted and disrupted the sequence of interest. The histochemical stain assay visually exhibited a robust blue coloration of the unedited RM-1 control plant, allied with active gene expression; whereas the T1 and T2 HaG plant tissues displayed a marked inhibition of blue precipitation which

correlated to GUS gene inactivity. qPCR analysis of the T1 HaG plants quantitatively solidified transformation by showing a predominant increase of Cas9 gene dosage but not indicating functionality. The sequence analysis of T2 HaG plants showed a thymine deletion resulting in the abolishment of leucine at the 318th amino acid of the GUS sequence, which resulted in a creation of a stop codon from the frameshift mutation. The mutation inactivated the gene and has led to GUS gene knock-down in place of a total knock-out due to the possibility of the RM-1 plant possessing multiple gene copies of GUS. This is a plausible explanation for why there was not a complete execution in targeting every GUS gene.

The applications for this technology are enormous as it can be used as a possible way to genetically modify plants in order to be resistant to various diseases and stresses that cause crop shortages around the globe. By creating more plants that are genetically resistant to top abiotic and biotic pressures, it is possible to prevent food shortages in poorer regions including a reduced loss of capital in the field of agriculture.

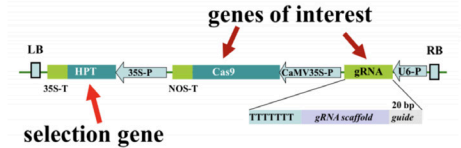


Figure 1. Gene construct diagram [2].

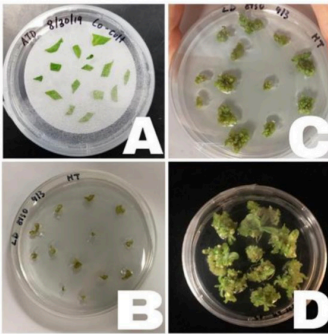


Figure 2.1. Transformation process. (A) Infected tobacco tissue placed on filter paper. (B) Tissue transferred 10 days later to regeneration media. (C) Callus formation on tissue and mass growth on day 17, budding began to form. (D) Shoots began to proliferate from budding callused tissue on day 28.



Figure 2.2. Regeneration process. Transferred shoots acclimated in rooting media box, day 37. Juvenile plant translocated to potted soil, day 50; transformed tobacco plant reaching maturity, about to produce flowers.

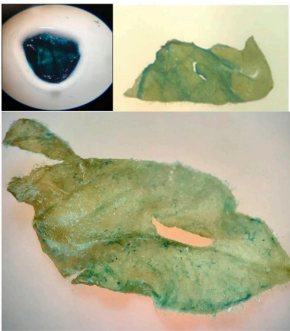


Figure 3. GUS stains of RM-1 tissue (top left), T1 HaG-RW (top right) and T2 HaG-CT (bottom).



Figure 4. Mendelian ratio determination of hygromycin resistant T2 transformants.

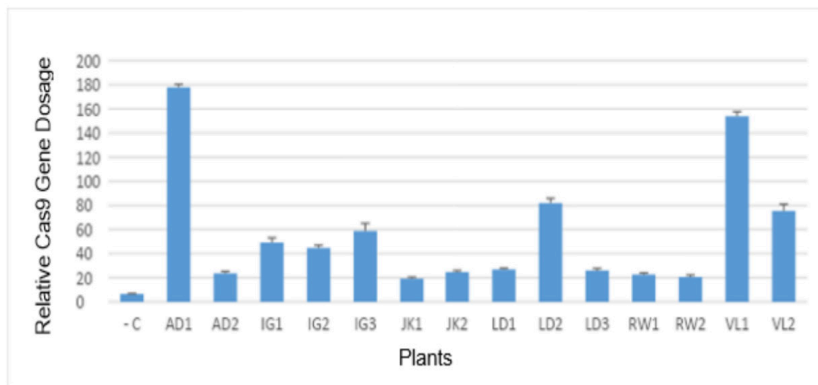


Figure 5. Quantification of relative Cas9 gene dosage of RM-1 (-C) versus T1 HaG plants.

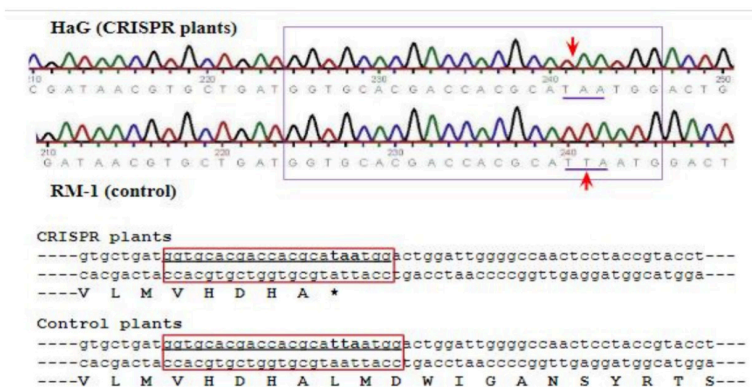


Figure 6. GUS sequence analysis of T2 HaG plants versus RM-1 displaying a thymine deletion indicated by arrows, resulting in a frameshift mutation. *Deletion of leucine.

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