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Title of dissertation: CRISPR/CAS 9 mediated mutagenesis of the eukaryotic translation initiation factor gene of *Nicotiana tabacum* for the induction of Potato virus Y tolerance.

By

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A research project submitted as a requirement for the Bachelor of Science Honors Degree in Biotechnology.

JUNE 2024

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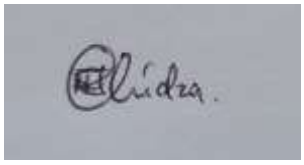
DECLARATION

I, Tanaka Nhidza hereby do declare that this project is a result of my original work, except where sources have been acknowledged. I declare that this is my work conducted at Tobacco Research Board (trading as Kutsaga) in the Crop Production and Molecular Technologies department. This work was funded by the Tobacco Research Board. The work described has never been produced by anyone seeking an award to the best of my knowledge.

The Tobacco Research Board rebranded on 30 July 2023 and is now trading as Kutsaga. Throughout this dissertation, the term TRB and Kutsaga will be used interchangeably.

Student's Signature:  Date: 7 JUNE 2024

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Chairperson of Department:……

DEDICATION

This research is dedicated to my parents, my brother and my future.

ACKNOWLEDGEMENT

I would like to acknowledge the Tobacco Research Board (TRB) as the originators of this research topic as well as the facilitators of this research. Their pioneering spirit in research is admirable and exemplary. I extend my gratitude to the Crop Production and Molecular Technologies department for the support and tutelage as well as their aid.

Heartfelt gratitude goes to my supervisors and family for their patience and unfailing support.

Abbreviations

BBL	Berberine bridge enzyme-like
CPMT	Crop Production and Molecular Technologies
CRISPR	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyl trimethyl ammonium bromide
DSB	Double stranded break
eIF4E	Elongation Initiation Factor 4E
GDP	Gross Domestic Product
gRNA	Guide RNA
HDR	Homology-directed/homologous recombination
LB	Luria Bertani
MES	2-Morpholinoethanesulphonic acid
MS	Murashige and Skoog
NBT	New breeding techniques
NCBI	National center for biotechnology information
nCBP	New Cap-Binding Protein
NHEJ	Non-homologous end joining
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
sgRNA	Single guide ribonucleic acid.
TALENs	Transcription activator-like effector nucleases
TMV	Tobacco Mosaic Virus
TRB	Tobacco Research Board
TYLCV	Tomato Yellow Leaf Curl Virus
URP	Universal Rice primers
VAM	Virgin A Mutant
VPg	Viral Protein Genome- Linked
WHO	World Health Organization
ZFN	Zinc Finger Nuclease

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ABSTRACT

One of the most significant viral infections affecting tobacco in Zimbabwe is the *Potato Virus Y* (PVY.). Currently, the most common method for controlling PVY is through the use of pesticides, which can have harmful effects on the environment and human health. By increasing resistance in plants, the need for pesticides could be reduced, leading to a more sustainable and eco-friendly approach to managing PVY. This study aimed at producing a PVY resistant tobacco genotype from a line ONC via Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR) mediated gene editing of the eukaryotic translation-initiation factors (eIF4E). The research utilized databases and web tools in the form of NCBI and CRISPOR to design the guide RNA (gRNA) DNA template. The gRNA DNA template was ligated into the pChimera plasmid and cloned in chemically competent *E.coli.* The gRNA insert was transferred as a construct into the binary vector pCas9-TPC and cloned in *Agrobacterium tumefaciens* for leaf disc transformation. The obtained construct was transformed into tobacco variety ONC leaf discs which developed into plantlets *in vitro*. The plantlets multiplied and after hardening were transferred to the greenhouse in a fumigated vermiculite/pine bark media. The plants displayed vigorous leaf development after 5 days with an increase in size. The *Agrobacterium* was also utilised in agroinfiltration of potted ONC plantlets and the genomic DNA of edited and wild type ONC plant was extracted and sequenced using the Applied Biosystems^{R} Genetic analyzer and alignment of the sequences showed a single nucleotide deletion at the 3'end. These sequences were used to generate a phylogenetic tree which illustrated the evolutionary relationships between different tobacco samples based on the sequencing of the eIF4e region. The close relationship between ONC Transformed and ONC Wild Type implies that the modification in the ONC Transformed sample altered the eIF4e region to some extent. It can be concluded that CRISPR/Cas9 technology can be utilised to mediate mutagenicity in the plant genome to induce PVY resistance.

Keywords: CRISPR, PVY, eIF4, *Nicotiana tabacum* (Tobacco), *Agrobacterium*.

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1. INTRODUCTION

1.1. Background

Tobacco Research board (TRB) t/a Kutsaga is the primary agricultural research institution founded in 1938 and reconstituted in 1950 under the Tobacco Research Act (Chapter 18:21), it has the mandate to;

a) Direct, control and carry out tobacco research in Zimbabwe;
b) establish, enhance and harness technologies for industrial applications, inclusive of biopharming;
c) research climate-proofing crops;
d) mainstream sustainable and environmentally benign crop production methods; and
e) safeguard national self-sufficiency in potato and horticulture micro-propagation.

As the primary research organization for the crop in Zimbabwe, the TRB conducts a wide range of studies on tobacco. The creation of tobacco varieties targeted at specific markets, the application of best practices for tobacco planting, handling, and curing, and the provision of knowledge and support to tobacco farmers are all included in this endeavor. The Research and Extension Services department at TRB is divided into several specialized divisions namely Plant Breeding, Crop Production and Molecular Technologies, Plant Health Services, Seed Production, Statistical Services and Analytical Services. Although the research will often be division-specific, cross-divisional cooperation is carried out to ensure strong research and output. The Plant Breeding division's main responsibility is to produce new elite varieties. To date, the division has produced over 70 premium burley and flue-cured tobacco types that are resistant to multiple diseases. By providing tobacco growers with a higher output quantity and quality at a reduced cost of production, these initiatives aim to improve the tobacco industry's overall viability and sustainability. Furthermore, efforts have been made to develop varieties that are appropriate for current weather patterns and regulatory authority criteria. While traditional breeding methods are still used to create new varieties, the Plant Breeding division collaborates with the Crop Production and Molecular Technologies (CPMT) department to adopt new breeding techniques (NBTs) that modify crops at the molecular level.

When PVY was firstly observed in 1930, it was thought to be a virus spread by aphids (Scholthof *et al*., 2011). It belongs to the *Potyvirus* species, which is one of the six genera in the *Potyviridae* family. It is among the most important infectious viruses affecting Zimbabwean tobacco in terms of commerce (Scholthof *et al*., 2011). To date, locally produced tobacco varieties in Zimbabwe lack PVY resistance and multiple PVY outbreaks have been experienced with the most recent being in 2017-2018 season when several farmers lost their tobacco crops (Takakura *et al.*, 2018). During that season tobacco output fell by at least 30 percent and this was a tremendous loss given that tobacco is one of Zimbabwe's most important cash crops (Takakura *et al.*, 2018).

There are two main strains of PVY; one that causes symptoms similar to mosaicism, and the other that causes necrosis of the leaves and veins. It is well known that strains of the mosaic type result in lower leaf quality, in dried tobacco, the mosaic patterns cause the loss of the golden leaf (Tajima *et al*., 2002). On the other hand, the necrotic strain can cause large yield losses and is quite harmful. In Zimbabwe, late-planted tobacco is still plagued by this strain. PVY causes considerable economic losses by changing the cured leaves' chemical composition, especially the amount of nicotine (Tajima *et al*., 2002).

Because PVY is transmitted in a non-persistent way by more than 50 aphid species, controlling the virus's spread with pesticides is ineffective. *Myzus persicae* is its most effective PVY vector (Tajima *et al.*,2002). This insect feeds on tobacco sap while also inoculating virions on the surface of the epidermis of the leaf or stalk of tobacco (Takakura *et al.*, 2018).

PVY virions have a diameter of 12 nm and a length of 730 nm. They are flexible and filamentous (Quenouille *et al*., 2013). The genomic RNA is 9.7 kb long and single-stranded in the messenger sense. It is covalently attached to a 3' polyadenylated tail and a virally encoded protein (VPg) at the 5' end. The genome is produced as a polyprotein with about 3062 amino acid residues, which is then broken down into 11 mature proteins by three virus-specific proteases (Quenouille *et al*., 2013).

Notably for *Potyviridae*, the eIF4E family of eukaryotic translation initiation proteins is a significant RNA virus resistance component (Zlobin and Taranov, 2023). The eIF4E family in flowering plants consists of three members; eIF4E, its isoform eIF(iso)4E, and nCBP (new cap-binding protein), all of which are 7- methylguanosine triphosphate (m7GTP) cap-binding

proteins, however nCBP does not appear to act in canonical translation (Mazier *et al*., 2011). Significantly, the roles of eIF4E and eIF(iso)4E substantially overlap. Although the specific role(s) of eIF4E in potyvirus infection is still being debated (Julio *et al.*, 2015), the interaction between an eIF4E and the VPg of various potyviruses is strongly tied to infection success.

Gene editing with the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Cas9 system can modify tobacco resistance to PVY. This gene editing method functions in concert with the endonuclease enzyme protein Cas9 (Zhou *et al*., 2014). The gRNA directs the Cas9 enzyme to a precise site for cleavage. By cutting DNA in a specific manner and allowing natural DNA repair to take over, CRISPR/Cas9 modifies genes (Jiang *et al*,, 2013).

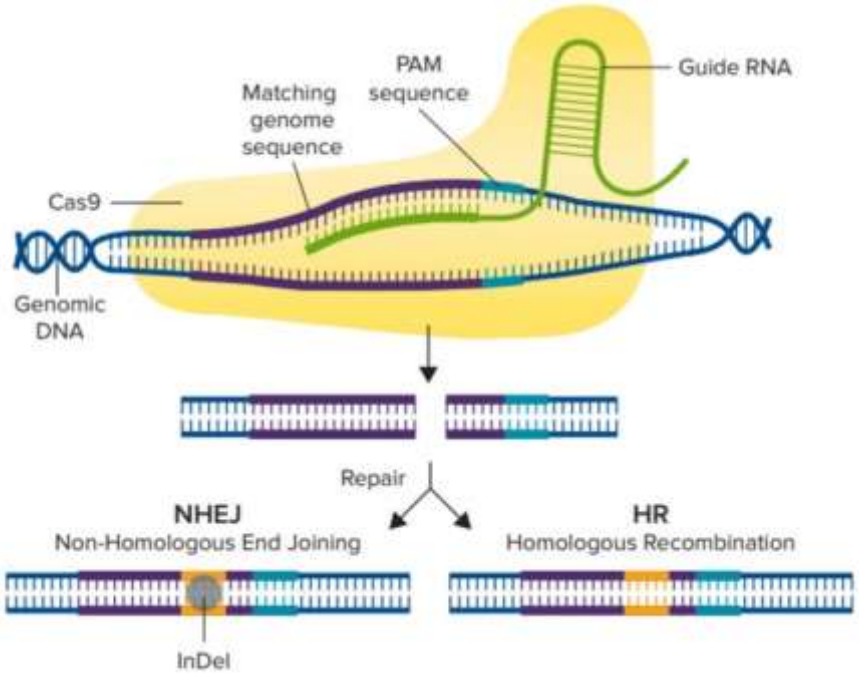

Figure 1: Mechanism of CRISPR-Cas9 and the pathways of double-stranded DNA cleavage (Molecular Devices, 2023).

DNA segments known as CRISPR are present in the genomes of prokaryotic organisms and are thought to have originated from the bacteriophage that had previously infected the prokaryote. When an endonuclease enzyme like Cas9 is combined with the complementary CRISPR sequences, it makes up the guide RNA, /a system that can recognize when bacteriophage nucleic acid is re-entering the cells and cleaves it, effectively changing and stopping the bacteriophage's replication. This mechanism of action has been compared to the

bacterial immune system (Molecular Devices, 2023; Nidhi *et al.,* 2021). This mode of action has been described as the immune system of bacteria.

When it comes to gene editing, CRISPR is far more precise than traditional methods like Zinc finger nucleases (ZFN) and TALENs (transcription activator-like effector nucleases). By inducing a double strand break (DSB) at a particular genomic site, Cas9 enables genome editing. Following the cleavage of the target region by the Cas9 enzyme, the DNA can be repaired using either the high-fidelity Homology Directed Repair (HDR) or the error-prone Non-Homology End Joining (NHEJ) (Betermier *et al*., 2014). The NHEJ technique is effective when there is no repair template (Yan *et al.,* 2020). This method can be applied to mediate frameshift and premature stop codon modifications resulting from indels that occur inside a coding exon and create gene knockouts (Waters *et al.,* 2014).

Although ZFN and TALENs were used as alternative genome editing techniques before CRISPR/Cas9 gene editing, the Cas9 gene editing method has many advantages. For instance, the Cas9 system has the ability to provide targeted DNA editing at specific locations of interest. HDR or NHEJ facilitate the efficiency of the editing (Mali *et al.,* 2013). Additional advantages of this strategy include high targeting efficiency, flexibility, and the ability to facilitate multiplex genome editing. The Cas9 approach, however, has a number of shortcomings. Cas9 is frequently directed by a 20 nucleotide sgRNA to particular genomic locations (Cao *et al.,* 2021). This only occurs when the 20-bp target sequence is 3' of a PAM sequence. Off-target mutagenesis incidence represents an additional potential limitation.

In this project the CRISPR/Cas9 gene editing method was used to alter the eIF4e gene which leads to PVY tolerance in tobacco.

1.2. Problem statement

PVY has been shown to be a major concern to tobacco farming in Zimbabwe. The current PVY control methods have proved to be insufficient in controlling tobacco losses that are currently being experienced. Conventional breeding methods for PVY resistance are time-consuming and often result in incomplete resistance. There is need for an effective and rapid approach to generate a PVY tolerant tobacco cultivar and CRISPR/CAS 9 serves and a gene editing tool to enhance efficiency and precision.

1.3. Justification

According to the World Health Organization (2021), Zimbabwe is the top tobacco producer in Africa, producing up to 25.9% of Africa's total output in 2018, with this output translating to 10% of the national gross domestic product (GDP). This sets tobacco as one of the major contributors to the Zimbabwe economy. The production of tobacco is carried out by both contract and independent farmers, which means the tobacco industry injects wealth both into the industrial sector as well as directly into the farming population (Chingosho *et al.,* 2021).

PVY has been a problem in late planted tobacco for almost a century now. The major problem being the availability of more than 50 species capable for transmitting the virions amongst tobacco plants. This makes it difficult to get rid of this viral disease by means of pesticides. This means that there is need to come up with a permanent solution against PVY (Takakura *et al.,* 2018).

Current control measures for PVY, such as chemical treatments, often have adverse effects on the environment, including the pollution of soil and water systems. By developing PVY-resistant tobacco varieties, we can reduce the reliance on chemical interventions, promoting environmentally friendly agricultural practices. Mutagenesis of the eIF4e gene offers a targeted and precise approach, minimizing the use of broad-spectrum pesticides and their associated environmental risks (Tajima *et al.*,2002).

The use of CRISPR/Cas9 to induce mutagenesis in tobacco genome in order to induce resistance against PVY has many advantages because CRISPR/Cas9 has efficiency and simplicity. The fact that CRISPR/Cas9 targets the specific genes which causes susceptibility and interaction with the virus and alter them reduces the possibility of off targets. The CRISPR/Cas9 system has been widely used in altering tobacco traits, including quality, disease resistance and yield and have had successful results thus CRIPSR/Cas9 system can be used in this current study. Therefore, this study aims to produce PVY resistant/tolerant tobacco and the produced varieties could be used in future breeding problems (Rajput *et al.,* 2021).

1.5. Aim of the study

The aim was to carry out site specific mutagenesis of the eIF4 gene on tobacco parental line (ONC) using CRISPR/Cas9 technology.

1.4. Specific Objectives

- Designing of the single guide RNA (sgRNA) to target the eIF4 genome.

- Cloning of sgRNA DNA template in a chimeric plasmid and subsequently transforming the gRNA + scaffold in binary vector (Cas 9).

- Transformation of *Agrobacterium* using pCas9-TPC plasmid.

- Tobacco leaf disc transformation and stable transformation by agroinfiltration of tobacco.

- Sequencing to identify mutation in eIF4e gene.

1.6. Research questions

- Can guide RNA sequences be systematically designed and evaluated to effectively target and disrupt the eIF4E gene using CRISPR-Cas9 technology in plants?
- What is the optimal method for efficiently and accurately cloning a desired guide RNA (gRNA) sequence into a Cas9 expression vector to enable CRISPR-Cas9 genome editing?
- What are the optimal strategies for stably transforming *Agrobacterium* with a pCas9-TPC binary vector to facilitate efficient and targeted genome editing in plant cells?
- What is a reliable protocol for generating stable transgenic tobacco plants through agroinfiltration and leaf disc mediated transformation?
- What specific mutations in the eIF4E gene of *Nicotiana tabacum* are introduced through CRISPR/Cas9-mediated mutagenesis?

1.7. Limitation

The scope of the study will be limited to the one gene of interest which is responsible for interaction with PVY. Due to the limitation of time, statistical tests for successful PVY resistance in successive generations of plant offspring as well as determination of complete segregation of the Cas9 plasmid will not possible, this may render the study to function as a proof of concept.

1.8. Significance of study

CRISPR/Cas9 mediated mutagenesis of the eukaryotic translation initiation factor (eIF4) gene in *Nicotiana tabacum* is a significant advancement in inducing tolerance to *Potato Virus Y* (PVY). This gene plays a crucial role in the initiation of translation, and its targeted mutation

through CRISPR/Cas9 allows researchers to understand better how viruses like PVY exploit the plant's translational machinery. By identifying specific resistance pathways, this approach aids in developing virus-resistant plants, which is vital for enhancing crop productivity and food security, especially for economically important crops like potatoes. The precision and efficiency of CRISPR/Cas9 technology offer rapid and reliable development of resistant varieties, promoting sustainable agricultural practices by reducing reliance on chemical treatments. Insights from this research can be applied to other crops, broadening the scope of virus resistance strategies in plant biotechnology. This innovation not only provides cost-effective solutions for farmers by decreasing crop protection expenses and losses due to viral infections but also contributes significantly to food security by enhancing the resilience of staple crops. Thus, CRISPR/Cas9 mediated mutagenesis of the eIF4 gene in tobacco represents a transformative approach in agricultural biotechnology, with profound implications for global food security and sustainable farming practices.

2. LITERATURE REVIEW

2.1. Eukaryotic translation-initiation factor

The eukaryotic translation-initiation factor 4 (eIF4) family regulates mRNA translation, which influences a variety of cellular functions such as growth, development, and stress response. Recent research has linked certain eIF4 genes to plant defense against viral infections, including PVY. These genes regulate translation efficiency, ribosome loading, and antiviral protein expression, all of which influence viral infection outcomes (Le *et al.*, 2022).

To investigate the evolutionary relationships of eIF family genes, a phylogenetic tree was constructed by Huang *et al*., (2022) using 23 and 63 eIF protein sequences from *A. thaliana* and *B. rapa*, respectively (Figure 2). The image does not include the protein sequences of nine *B. rapa* genes because they are too short and non-conservative. Together with 23 eIF family genes in *Arabidopsis,* 54 eIF genes were primarily categorized into six categories. Class IV (eIF4 family genes) was the largest group, which included 24 genes, and it was followed closely by class III, which included 21 eIF3 genes. Class I was the eIFiso family genes, which included seven genes, and class II/V/VI had the same and the fewest genes, containing three genes each (Huang *et al*., 2022).

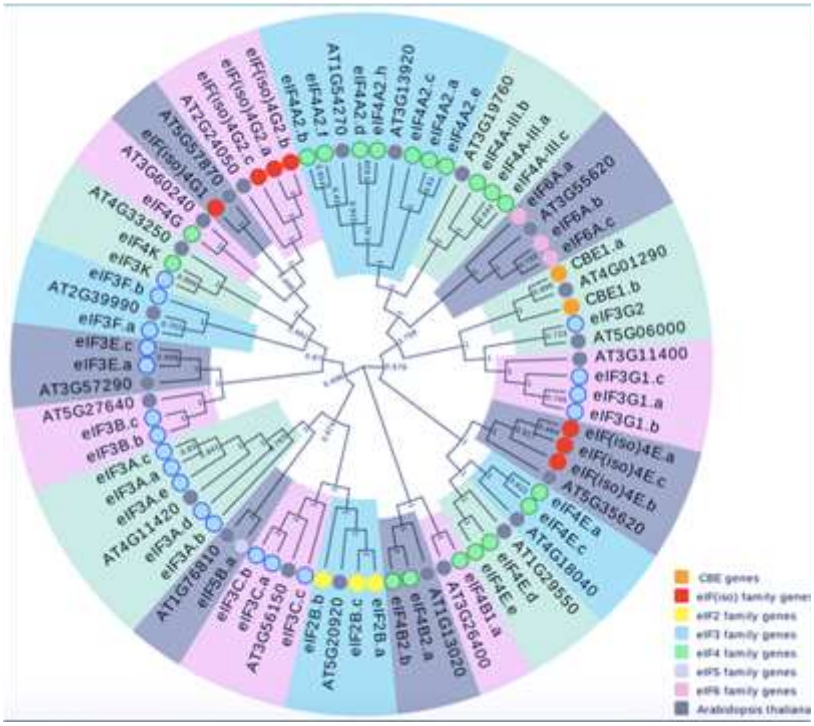

Figure 2*:* Phylogenetic analysis of 23 and 63 eIF family protein sequences from *Arabidopsis thaliana* and *Brassica rapa* respectively. Orange represents CBE genes, red represents class I (eIFiso family genes), yellow represents class II (eIF2 family genes), blue represents class III, green represents class IV (eIF4 family genes), light grey represents class V (eIF5 family genes), pink represents class VI (eIF6 family genes), and grey represents *A. thaliana* (Huang *et al*., 2022).

One method for generating PVY resistance is to overexpress eIF4E isoforms, which are targeted by potyviruses like PVY during translation start. A study found out that overexpressing the potato eIF4E isoform eIF4E1 conferred resistance to PVY in transgenic potato plants, (Duan *et al.,* 2005). This resistance was related to the disruption of the viral translation machinery caused by competition for eIF4E binding.

Another technique is to introduce mutations into eIF4E genes that disrupt their interaction with viral proteins, hence reducing viral replication. Zlobin and Taranov (2006) discovered natural eIF4E alleles that confer resistance to potyviruses. Subsequent research in potatoes found that specific eIF4E alleles with a lower affinity for PVY VPg (viral protein genome-linked) conferred resistance to PVY infection (Xu *et al*., 2017).

Aside from eIF4E, other components of the translation initiation complex, such as eIF4G, have been targeted for PVY resistance. Lebedeva *et al.* (2021) discovered mutations in the potato eIF4G gene that provide PVY resistance. These mutations affect the interaction between eIF4G and the viral protein VPg, reducing viral reproduction and dissemination.

2.2. Methods of Gene Editing

Gene editing refers to a collection of technologies that allow researchers to alter the expression of genetic information in organisms. Several strategies have been developed that target different stages within the flow of the central dogma of molecular biology, namely translation, transcription and the presence of the gene of interest.

2.2.1. Conventional methods

Prior to the development of CRISPR-Cas, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the most widely used gene editing techniques. These technologies utilized specific DNA recognition and binding properties of

specific proteins *viz*. modified homing nuclease (mega nuclease), zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) (Rajput *et al.*, 2021).

2.2.2. CRISPR/Cas9

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) gene editing technique, which uses RNA-guided nucleases, has been known for its effectiveness in targeted gene editing across a variety of crop kinds for the past six years (Rajput *et al.*, 2021). Below is a table of the crops that have undergone gene editing via CRISPR/Cas system.

Table 1: CRISPR/Cas9 system-mediated gene editing in crops.

Crop Used	Target Genes	Traits Edited	Reference
Nicotiana tabacum	BBL	Reduced nicotine production	Schachtsiek and Stehle, (2019)
Z. mays **(Maize)**	ARGOS8	Drought tolerance	Shi *et al.,* (2017)
T. aestivum **(Common wheat)**	Ms1	Male-sterile development	Okada *et al.,* (2019)
O. sativa **(Rice)**	Cyt P450 homeologs OsBADH2	Yield improvement	Usman *et al.,* (2020)
S. lycopersicum **(Tomato) &** ***N. benthamiana*** **(Benth)**	Coat protein (CP) Replicase (Rep)	Tomato yellow leaf curl virus (TYLCV) resistance	Tashkandi *et al.,* (2018)

CRISPR-Cas was first discovered in the genome of *Escherichia coli.* The short palindromic repeats that make up the CRISPR/Cas DNA fragment are separated by short, variable-length sequences known as proto-spacers. The DNA segments of invasive viruses and plasmids are identical to these spacers. The helicase and endonuclease enzyme Cas is associated with the proto-spacer segments that are separated by palindromic repeats. Proto-spacers adjacent motifs (PAMs) are short trinucleotides which are typically 5'-NGG-3' and sometimes 5'- NAG-3' and are closely linked to the Cas genes. They are found in the DNA sequence of invading

viruses or plasmids and are not present in the host bacterial genome (Zhang *et al*., 2019; Rajput *et al*., 2021). These elements of the CRISPR/Cas system are used by the CRISPR/Cas9 system to alter genes. The main components include the Cas9 which is a 160 kiloDalton endonuclease. Being a dual RNA-guided DNA endonuclease, it has two RNA segments that it uses to locate a particular DNA strand to cleave. Together, the two RNA segments are referred to as guide RNA (gRNA), of which one is the trans-activating CRISPR RNA (tracrRNA) and the other is the CRISPR RNA (crRNA). The CRISPR locus is where the crRNA is transcribed. The tracrRNA that holds the crRNA in the Cas9, acting as a scaffold. The process of using CRISPR/Cas9 for gene editing entails modifying the crRNA's sequence to match a target sequence (Rajput *et al.*, 2021).

Usually the failure of normal gene expression is linked to the emergence of indels. Therefore, in higher eukaryotic organisms like plants, the use of CRISPR to induce NHEJ is a practical strategy for gene silencing (Sinkunas *et al*., 2011). Because of the enhanced gRNA design tools, CRISPR-Cas9 also has the benefit of high accuracy and less off-target consequences. Furthermore, CRISPR/Cas9 modifies the organism's genome, resulting in permanent modifications. It has been established that plants that have undergone CRISPR-Cas9 mediated gene editing do not possess transgenic DNA material while maintaining the gene knockdown, therefore even though they express the desired changed trait, they are not regarded as genetically modified organisms (Rajput *et al*., 2021; Zhang *et al*., 2019; Sinkunas *et al*., 2011). This distinguishes CRISPR-Cas9 as a method with longer-lasting intended gene expression/gene knockdown with higher precision and predictability, while being less contentious in countries where genetically modified organisms are not sanctioned for cultivation (Schachtsiek and Stehle, 2019).

Schachtsiek and Stehle (2019) established a basic CRISPR//Cas9 method to lower the nicotine content of tobacco. They specifically targeted the berberine bridge enzyme-like (BBL) family flavoproteins, which catalyze the last stage of nicotine, anatabine, and anabasine oxidation. The BBL genes BBLa, BBLc, BBLd.2, BBLb, BBLd.1, and BBLe were specifically targeted for CRISPR Cas9-based knockdown. Their research was an adaptation of Lewis *et al.*, (2015), who repressed the BBL genes to lower nicotine levels without causing other alkaloids to accumulate more rapidly. The CRISPR-Cas9 single guide RNA (sgRNA) construct consisted of the 20 base pair target sequence between the ubiquitin 6-26 promoter from *A. thaliana* and the cassette was transferred to the binary vector pCas9- TPC (with the selectable bar-gene

marker). Gas chromatography-flame ionization detection (GC-FID) was used to measure the amount of nicotine present in the resulting transformed plants, and gas chromatography mass spectrometry was used to confirm the results. Some genotypes that had had their genes altered showed a 95% drop in nicotine concentration. Sanger sequencing was used to determine the molecular editing of the amplicons using vector-based cloning strategies based on PCR. While sequencing data for the other five genes confirmed the same base pair insertion of adenine, guanine, or thymine, this revealed the insertion of guanine or thymine for BBLa. The frameshift caused by each of these insertions effectively knocked out the gene.

2.3. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation began in the late nineteenth century with the discovery of crown gall disease in plants, which is caused by *A. tumefaciens*. In the 1970s, researchers discovered the importance of the tumor-inducing plasmid (Ti plasmid) carried by *A. tumefaciens*, which contains the transfer DNA (T-DNA) that causes genetic transformation (Gelvin, 2003). This finding paved the way for using Agrobacterium as a vector for plant genetic engineering.

The transformation process consists of multiple intricate steps. Initially, *A. tumefaciens* binds to damaged plant tissues, aided by bacterial surface proteins and phenolic chemicals generated by the plant. The bacteria then transfers the T-DNA region from the Ti plasmid to the plant cell nucleus. Virulence proteins and host factors help to facilitate this transmission. Once inside the nucleus, the T-DNA integrates into the host genome and regulates the expression of genes that cause tumor development and other phenotypic alterations (Tzfira and Citovsky, 2006).

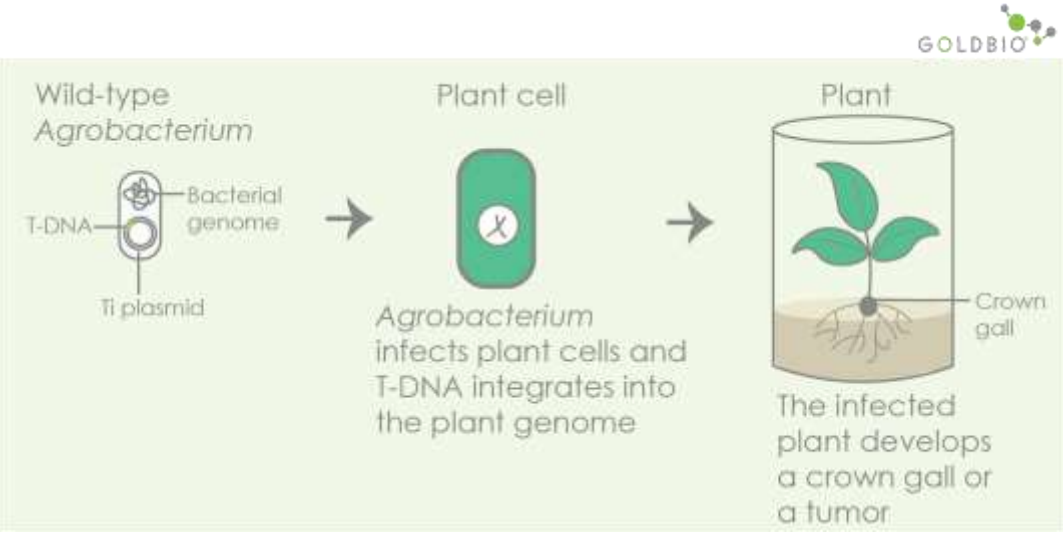

Figure 3*: Agrobacterium* infection in the host plants, the T-DNA within *Agrobacterium* integrates with the plant's genome causing the plant to develop crown gall disease (Image produced by GoldBio).

In a study on soybeans, researchers successfully used *Agrobacterium* to insert a gene conferring glyphosate resistance into soybean plants. This achievement allowed for effective weed control without harming the crop, showcasing the practical utility of A*grobacterium*-mediated transformation in agriculture (Hilder *et al*., 1987). Additionally, a landmark study demonstrated the efficient transformation of rice using *A. tumefaciens* by introducing a hygromycin resistance gene into Japanese rice varieties, achieving high-frequency transformation. This significant advancement proved that monocots, previously thought to be less amenable to *Agrobacterium* transformation, could indeed be effectively transformed (Hiei *et al*., 1994).

Fillati *et al*., (1987) successfully introduced a gene for kanamycin resistance into tomato plants using Agrobacterium. The resulting transgenic plants exhibited this resistance, providing an early example of using Agrobacterium to genetically modify vegetables (Fillatti *et al*., 1987). In another study, Sharma *et al*., (2009) enhanced disease resistance in tomatoes by incorporating genes that express pathogenesis-related proteins. The genetically modified tomato plants demonstrated increased resistance to fungal diseases, illustrating the effectiveness of Agrobacterium-mediated transformation for crop protection.

The creation of golden rice, a genetically engineered rice variety enriched with provitamin A, represents a significant milestone in plant biotechnology. Researchers successfully inserted

genes responsible for beta-carotene production into the rice endosperm using Agrobacterium-mediated transformation (Beyer *et al*., 2002), addressing vitamin A deficiency in populations that rely heavily on rice as a staple food.

2.3.1 Agroinfiltration

The primary methods for introducing genes into plant cells are direct delivery via biolistics and indirect delivery through *Agrobacterium tumefaciens* (Rivera *et al*., 2012). Agroinfiltration has been used to introduce DNA from various organisms into plant cells for many applications beyond studying plant-virus interactions (Vaghchhipawal *et al*., 2011). The most common agroinfiltration technique is "syringe agroinfiltration," which uses a needleless syringe to inject *Agrobacterium* into plant leaves (Santi *et al*., 2008). This method has been adapted for various plant species) and offers several key advantages (Wroblewski *et al*., 2005. It is a straightforward technique that does not require specialized equipment and can infiltrate an entire leaf with a single DNA construct or introduce multiple constructs into different parts of the same leaf, enabling multiple experiments on one leaf (Vaghchhipawal *et al*., 2011). These benefits have made syringe infiltration the preferred method for transient gene expression in various applications, including plant pathogen interactions, responses to abiotic stress, functional analysis of plant genes through transient silencing assays, protein localization and function studies, and protein-protein interaction analyses (Vaghchhipawal *et al*., 2011).

2.4. Virgin A Mutant

The tobacco cultivar Virgin A Mutant (VAM) is known to possess the recessive potyvirus resistance gene va, with varying resistance levels observed in VAM plants inoculated with Japanese *potato virus Y* (PVY) isolates. The development of the VAM breaking strain in tobacco represents a significant advancement in PVY resistance. This mutation in the Virgin A cultivar induces a unique hypersensitive response to PVY infection, which inhibits virus replication and spread within the plant. This literature review explores the production of the VAM breaking strain and its significance for PVY resistance in tobacco (Masuta *et al*., 1999).

The creation of the VAM breaking strain involved traditional breeding methods combined with molecular characterization to identify and select plants with the desired resistance trait. The mutation responsible for the resistance was identified in the wild-type tobacco cultivar, Virgin A, which exhibited a hypersensitive response to PVY infection. Researchers used conventional

breeding techniques to cross the VAM cultivar with commercial tobacco varieties, followed by extensive screening and selection of progeny that were resistant to PVY (Masuta *et al*., 1999).

Molecular analysis of the VAM breaking strain identified genomic alterations that contribute to the resistance phenotype. Further studies pinpointed specific genes and regulatory factors involved in the hypersensitive response and defense mechanisms activated by PVY infection (Masuta *et al*., 1999). The Virgin A mutation was found to enhance recognition and activation of these defense pathways.

The development of the VAM breaking strain has significant implications for controlling PVY and enhancing tobacco production. By utilizing natural genetic variation and the hypersensitive response to viral infection, researchers have established long-lasting and environmentally sustainable PVY resistance without relying on chemicals. Incorporating the Virgin A mutation into commercial tobacco cultivars offers a promising strategy for reducing production losses and improving crop health in regions where PVY is prevalent (Acosta-Leal and Xiong, 2008).

Moreover, the molecular insights gained from studying the VAM breaking strain are valuable for understanding plant-virus interactions and innate immune responses. Identifying key genes and signaling pathways involved in PVY resistance can guide the development of new breeding strategies and biotechnological approaches to enhance viral resistance (Acosta-Leal and Xiong, 2008).

2.5. Tissue Culture

Plant tissue culture is the cultivation of plant cells, tissues, and organs in a sterile environment using a nutrient-rich media. The process begins with by obtaining of plant tissue from a healthy plant, which is subsequently sterilized to kill microbes. The sterilized tissue is then placed in a culture medium that contains the required nutrients, vitamins, and growth regulators. The culture media contains all of the nutrients required for the growth and development of plant cells (Hasnain *et al.*, 2022).

One of the primary benefits of plant tissue culture is the capacity to generate a large number of plants from a single tissue sample. This technique, known as micropropagation, is used to create disease-free, genetically identical plants. It has significantly contributed to the

production of high-yielding and disease-resistant plants, resulting in improved agricultural and horticultural practices (Hasnain *et al.*, 2022).

2.5.1. Callus Induction

Callus regeneration is an important process in tissue culture that involves inducing undifferentiated cells, known as callus, to develop into shoots and roots. This technique is particularly valuable because it enables the production of numerous identical plants from a small starting material. Callus regeneration can be achieved through two methods: organogenesis, where shoots and roots grow directly from callus tissue, and embryogenesis, where embryos form from callus tissue (Long *et al*., 1958).

In plant transformation protocols involving *Agrobacterium tumefaciens*, callus induction plays a crucial role, especially when working with dicotyledonous plants. This process aims to prompt plant cells to revert to a pluripotent, undifferentiated state, resulting in the formation of callus tissue. This callus tissue can then be further regenerated into complete plants carrying the desired transgene (Qi *et al*., 2023). Various *Agrobacterium*-mediated plant transformation studies utilize callus induction, as demonstrated in the examples below.

In rice, the standard transformation protocol involves infecting scutellum-derived calli from mature seeds with *A. tumefaciens*. After cocultivation, the calli are placed on media with antibiotics to eliminate *A. tumefaciens* and select for cells that have incorporated the transgene. These genetically transformed calli are then induced to differentiate into full plants using regeneration protocols. This method was effectively employed by Hiei *et al*., (1994) to introduce new genes into rice.

Similarly, in wheat, transformation via *Agrobacterium* involves inducing callus from explants such as immature embryos, followed by infection with *A. tumefaciens*. Ishida *et al*., (2015) significantly improved wheat transformation by optimizing callus induction and regeneration protocols, enhancing the efficiency of wheat transformation for genetic engineering purposes.

In maize, immature embryos or other explants susceptible to *Agrobacterium* infection are used. After infection, the explants are cultured to form callus tissue, which is then selected for antibiotic or herbicide resistance conferred by the transgene. Regenerating these selected calli

into plants allows for the propagation of transformed maize. Frame *et al*., (2002) outlined a protocol that enhances maize transformation efficiency through optimized callus induction and regeneration methods.

Callus regeneration in tissue culture is primarily used for producing transgenic plants, which have foreign genes inserted into their genome. This technique has been extensively used in tobacco to introduce desirable traits such as disease resistance or increased yield. Callus regeneration enables the efficient production of large numbers of transgenic plants, which can then be screened for the desired traits (Efferth, 2019).

In addition to transgenic plant production, callus regeneration has been employed in tissue culture for genetic transformation studies. Researchers can explore the effects of various hormones, growth regulators, and stressors on plant growth and development by modifying the conditions that allow callus regeneration to occur. This has resulted in a better knowledge of the mechanisms behind callus regeneration and its potential uses in agricultural biotechnology (Ikeuchi *et al.,* 2013).

2.6. Determination of alteration

Modifying a desirable trait is the primary objective of gene editing. Researchers evaluate properties of the modified gene to ascertain whether gene editing was successful (Wang *et al*., 2009; Schachtsiek and Stehle, 2019). To make sure that no off-targets may have occurred, it is essential to analyze even more variables in addition to identifying the intended modifications. This evaluation guarantees the integrity of the edited organism's genome and the specificity of the gene editing tool, which in turn makes sure the other essential processes necessary for the organism's survival and development (Tashkandi *et al*., 2018; *Shi et al*., 2017). The criteria used to evaluate genome-edited plants may be molecular, biochemical, or morphological in nature.

The most precise method for identifying any nucleotide alterations in a gene is DNA sequencing. When comparing altered organisms to non-edited organisms, Sanger sequencing has proven to be an effective method for precisely identifying a gene's sequence (Gupta and Li, 2021). Sanger sequencing is an effective method for identifying indels since it can detect single nucleotide changes (Schachtsiek and Stehle, 2019).

The edited gene segment of the genome is amplified using the polymerase chain reaction and subsequently sequenced, as opposed to sequencing the full genome to find specific sites of gene editing (Schachtsiek and Stehle, 2019). Furthermore, as standard PCR might not be sensitive enough to identify single nucleotide alterations, the modified version of the PCR enables higher sensitivity in the amplification of target genes, allowing for the identification of indels (Gupta *et al*., 2021). According to Zhang *et al.*, (2019), the majority of mutations in wheat crops were deletions, with 88% of these being larger than 4 bp and 35% larger than 50 bp. These latter deletions were found to be detectable by conventional PCR, while the deletions of ~4 bp, which correspond to two-thirds of NHEJ mutations, fell into this range. In the absence of gene sequencing and/or 45 deep sequencing, Gupta *et al.*, (2021) found that a nested PCR using two sets of efficiently designed primers was a suitable, repeatable, and reasonably priced method of genotyping deletion mutations ranging from 3 to 50 bp.

2.6.1. Universal Rice Primers

Sets of PCR primers known as universal rice primers (URP) are derived from DNA repeat sequences found in the rice genome and are highly effective in DNA fingerprinting when used in PCR methods with relatively high annealing temperatures. URPs can fingerprint and differentiate both bacterial and eukaryotic genomes at inter- and intraspecies levels (Kang *et al*., 2002).

URPs offer several advantages for examining plant genome differentiation. They target conserved sections of the rice genome, often shared by related plant species, which allows for the amplification of homologous regions across diverse plant taxa and facilitates comparative genomic studies (Kang *et al*., 2002). Furthermore, URPs are cost-effective and efficient, enabling high-throughput analysis of multiple plant genomes simultaneously. Their versatility allows them to be used with various molecular methods, such as sequencing, restriction fragment length polymorphism, and polymerase chain reaction (Kang *et al*., 2002).

Pairwise combinations of URPs in URP-PCR were employed by Topu and Tiryaki (2022) to determine genetic linkages in a host of *V. sativa*. Three primary clades were identified in the dendrogram produced by the study for the 24 *V. sativa* varieties. Mishra *et al.*, (2015) utilized URPs to identify genotypic variability in *Rhizoctonia solani* isolated from rice, maize and greengram.

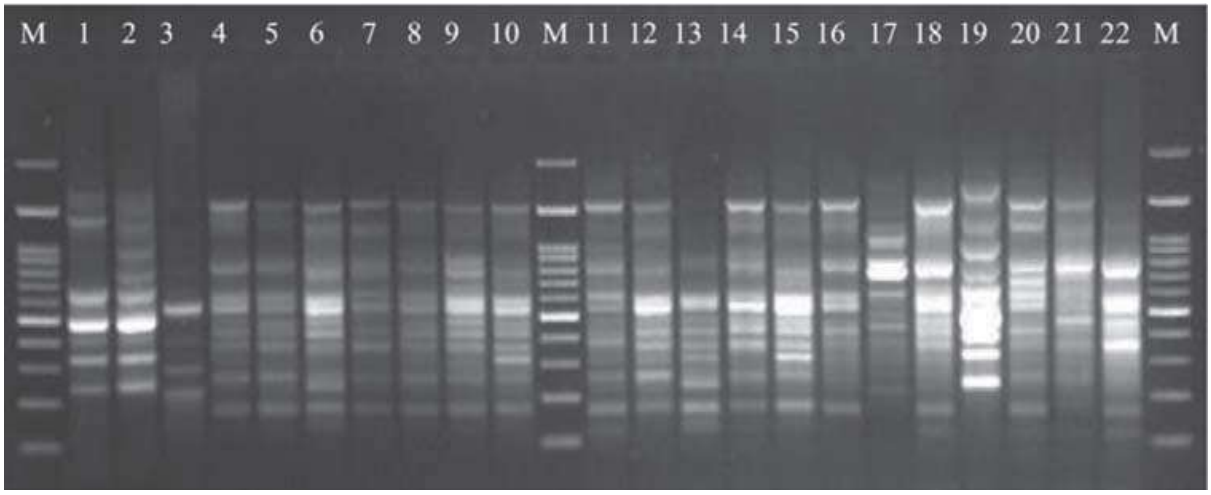

Figure 4: URP profile of twenty two isolates of *Rhizoctonia solani* amplified with URP13R. Lane 1-10: rice, 11-20: maize and 21-22: greengram isolates, M=1Kb Ladder (Mishra *et al*., 2015).

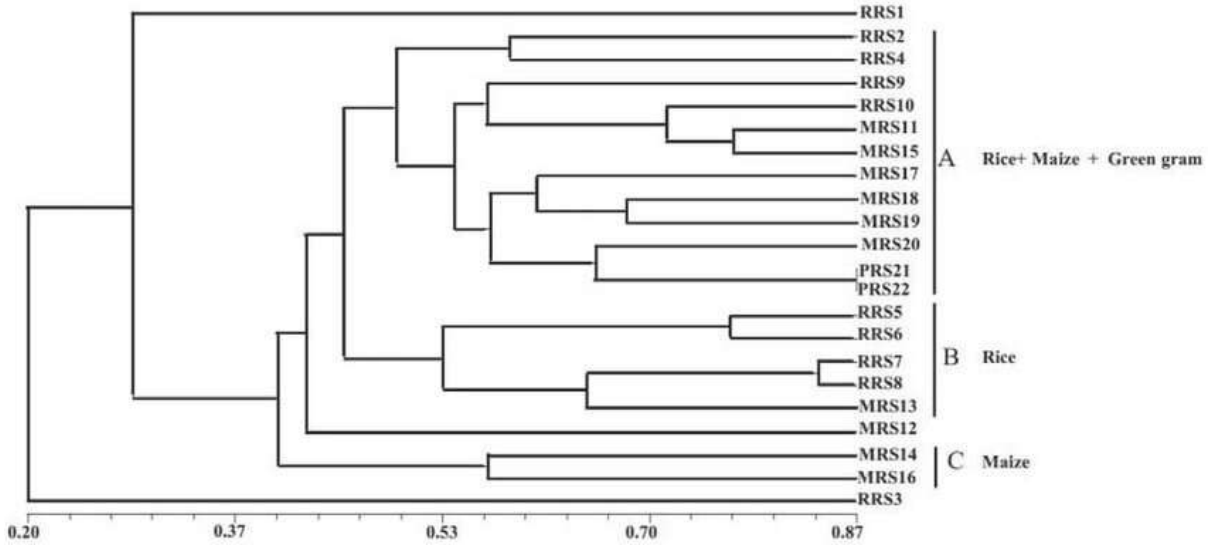

Figure 5: Dendrogram generated from the four URPs representing genetic relatedness among 22 isolates of *Rhizoctonia solani* originated from rice (RRS1-10), maize (MRS11-20) and greengram (PRS21-22) (Mishra *et al* ., 2015).

3. MATERIALS AND METHOD

3.1. Introduction

The Tobacco research board molecular department aims to improve tobacco varieties by studying the molecular mechanisms underlying gene regulation. The laboratory is ISO 17025 accredited therefore is competent to carry out cutting edge research. This chapter highlights the materials and procedures employed in the laboratory in the development of gene-edited tobacco plants via CRISPR/Cas9 and the *Agrobacterium* - mediated transformation. In addition, the chapter will detail the methods of sampling, data collection and the subsequent analysis used.

The chapter explores the research design which guides the methods to effectively and empirically meet the study's aims and objectives. This is followed by the research strategy which details the laboratory and bioinformatics procedures and/or protocols.

3.2. Guide RNA sequence design

The guide RNA (gRNA) design and eIF4e gene sequence were determined using bioinformatics databases and tools. In the determination of the eIF4e gene, the National Centre for Biotechnology Information (NCBI) gene database was utilized with the search query set for Eukaryotic translation initiation factor 4e. The selection of the Eukaryotic translation initiation factor gene was based on the organism of origin, *Nicotiana tabacum* (common tobacco).

This gene sequence was then used in generating primer sequences for gRNA synthesis. The CRISPR/Cas9 cleavage target sequence was produced using the web tool CRISPOR. Target selection criteria included the strand's orientation, its guanine/cytosine concentration, its specificity, efficiency, and chances to induce out-of-frame deletion. The CRISPOR online tool was also used to choose the forward and reverse oligonucleotides for the PCR assembly. The generated gRNA sequences were then sent to Inqaba Biotechnical Industries in Pretoria, South Africa for synthesis.

3.3. gRNA DNA template and pChimera Ligation

A glycerol stock of chemically competent *E.coli* containing the pChimera (Addgene plasmid # 61476 ; http://n2t.net/addgene:61476 ; RRID:Addgene_61476) plasmid was thawed on ice and 20 µl added to 1980 µl of SOC media. The inoculate mixture was incubated at 37 °C shaking at 300 rpm, with interval optical density assessment at 600 nm. Plasmid extraction was carried out on the inoculant when its OD 600 reached 0.3 (24-h culture). Extraction of the plasmid was done by following the Kit-free Alkaline Lysis Plasmid Miniprep by Addgene.

A total of 10 µL of each gRNA oligo was mixed with 30 µL of nuclease free water, the mix was incubated for 5 min at 95 °C then cooled at room temperature for 10 min. The pChimera plasmid was linearized at 880 bp where the restriction enzyme BbsI is specific, a point following the gRNA scaffold. The restriction digest was carried out using the one-step New England BioLabs Digest protocols. The key reagents and steps in the New England BioLabs Digest and Dephosphorylation protocol are outlined in table 2

Table 2: BbsI Restriction Digest protocol as prescribed by the manufacturer.

Component	Volume
DNA	1 µg
10X NE Buffer r2.1	5 µL
BbsI	1 µL
Shrimp Alkaline Phosphatase	1 µL
rSAP Reaction buffer (10X)	2 µL
Nuclease-free water	To 50 µL

The mixture was thoroughly mixed via pipetting and incubated at 37 °C for 15 min and Shrimp Alkaline Phosphatase was deactivated by incubating the mixture at 65 °C for 5 min.

The linearized pChimera plasmid and the annealed gRNA oligos were ligated via the New England BioLabs T4 DNA Ligase Master Mix.

Table 3: New England BioLabs T4 DNA Ligase Master Mix reagents.

Component	Volume
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
T4 DNA Ligase	1 µL
T4 DNA Ligase Buffer (10X)*	1 µL
Nuclease-free water	To 20 µL

Reagents were mixed by pipette and incubated at room temperature for 10 min.

The pChimera plasmid containing gRNA DNA template was utilized for transformation of competent *E.coli* NEB5µ cells using heat shock method. The competent cells were thawed on ice and 5 µL of plasmid DNA was added on to the competent cells. The cells were then incubated on ice for 30 min and heat shock was subsequently done by placing in 42°C water bath for exactly 30 secs. The cells were placed on ice for 2 min. Pre-warmed SOC medium (1950 µL) was then added to the cells where after the cells were shaken at 200 rpm and incubated at 37°C for 1 hour to induce outgrowth. The cells were spread plated in the 1:10 and 1:100 dilutions of the outgrowth cultures on warm selective or screening plates spiked with ampicillin antibiotic. The plates were then incubated at 37°C for 12-16 h.

3.3.1. Colony PCR

In order to confirm the successful transformation of the cells with the plasmid, a colony PCR test was conducted using the M13 forward and gRNA reverse primers. Single colonies from transformed plates were taken and aliquoted into 100 µL sterile water. A total of 20 µL of these cells were then aliquoted into small PCR tubes. This was then heated at 96 °C for 5 min as a prerequisite for hot-start colony PCR.

Table 6: The table below shows the PCR master mix reagents for confirmation of *E.coli NEB5µ* cells transformation using pChimera.

Table 4: Hot start PCR reaction mixture.

Component	**Volume**
Water, nuclease free	3.2 µL
Platinum II hot-start PCR Master mix(2X)	10 µL
10 Mm SS42 forward primer	0.4 µL
10 Mm gRNA reverse primer	0.4 µL
Template DNA	2 µL
Platinum GC enhancer	4 µL

After mixing all the reagents the PCR tubes were then transferred to a thermal cycler for the Polymerase Chain Reaction.

Table 5: Cycling conditions used for colony PCR.

Cycle Step	Temperature °C	Time (secs)

Initiation denaturation	90	120
Denaturation	90	15
Annealing	60	15
Extension	68	15
Hold	4	∞

The contents were incubated for 35 cycles, the PCR product was then transferred for gel electrophoresis using 1 % agarose gel for 100 volts with 2 amperes of current flowing. The electrophoresis ran for one hour and the gel image was analysed for an expected band at approximately 370 bp to determine if the primers had amplified a region containing the gRNA DNA template and part of the pChimera plasmid.

After confirmation of the presence of the plasmid the colonies were inoculated in fresh Luria Bertani (LB) media with 100 µg/ml ampicillin antibiotic. The inoculate mixture was incubated at 37 °C shaking at 250 rpm. Plasmid extraction was carried out on the inoculant after overnight shaking. Extraction of the plasmid followed Kit-free Alkaline Lysis Plasmid Miniprep (Addgene).

3.4. Extraction of pCas9-TPC plasmid

A glycerol stock of *E.coli* containing the pCas9-TPC plasmid was thawed on ice and 20 µl added to 10 ml of LB Media containing 50 mg/ml spectinomycin antibiotic. The McCartney jars containing the inoculant were incubated in an incubator-shaker at 37 ºC and 250 rpm overnight. The plasmid was subsequently extracted the following day using the Kit-free Alkaline Lysis Plasmid Miniprep by Addgene.

3.4.1. Recombination of pChimera and pCas9-TPC

The gRNA vector and pCas 9 - TPC plasmid were digested separately using AvrII enzyme which produced a double cut with sticky ends. The restriction digest was carried out using the

one-step New England BioLabs Digest protocols. The following are the key reagents and steps in the New England BioLabs Digest and Dephosphorylation protocol.

Table 6: AvrII restriction digest protocol as prescribed by manufacturer

Component	Volume
DNA	1 µg
10X rCutSmart Buffer	5 µL
AvrII enzyme	1 µL
Nuclease-free Water	to 50 µL

The mixture was thoroughly mixed via pipetting and incubated at 37 °C for 15 min. The sgRNA construct was ligated into the pCas9-TPC backbone via the New England BioLabs T4 DNA Ligase Master Mix.

Table 7: T4 DNA ligase master mix reagents for Cas 9 vector and gRNA construct ligation

Component	Volume
pCas9-TPC vector backbone	50 ng (0.020 pmol)
gRNA construct	37.5 ng (0.060 pmol)
T4 DNA Ligase	1 µL

T4 DNA Ligase Buffer (10X)*	1 µL
Nuclease-free water	To 20 µL

The resulting plasmids were transformed into chemically competent *E.coli* NEB5µ cells and plated on LB agar supplemented with streptomycin. The resulting colonies that grew were aliquoted to microtubes containing 100 µL of sterile water and 20 µL of these cells were heated at 96 ºC for 5 min as a prerequisite for hot-start colony PCR under the following conditions;

Table 8: PCR master mix reagents for confirmation of recombination of pChimera and pCas-TPC.

Component	Volume
Platinum GC enhancer	4 µL
Platinum II hot-start PCR Master mix(2X)	3.2 µL
10 Mm SS42 forward primer	0.4 µL
10 Mm gRNA reverse primer	0.4 µL
Template DNA	2 µL
Water, nuclease free	To 20 µL

After mixing all the reagents the PCR tubes were then transferred to a thermal cycler for the Polymerase Chain Reaction.

Table 9: Cycling conditions used for colony PCR in plasmid recombination confirmation.

Cycle Step	Temperature (°C)	Time (sec)	Cycles

Initial denaturation	94	10	1X
Denaturation	94	5	
Annealing	60	15	
			35X
Extension	68	15	
Final extension	68	600	1X
Hold	4	∞	1X

The PCR product was then transferred for gel electrophoresis using 1 % agarose gel for 100 volts with 2 amperes of current flowing. The electrophoresis ran for one hour and the gel image was analysed for an expected band at approximately 1kb to determine if the primers had amplified a region containing the sgRNA and part of the pCas9-TPC plasmid. The confirmed colonies that were positive to possess the pCas9-TPC plasmid with the sgRNA integrated were inoculated in LB media and incubated at 37°C with 250 rpm agitation overnight. The overnight inoculate had the plasmid extracted according to Addgene protocol.

3.5. *Agrobacterium*-mediated Transformation

The resulting vectors that were confirmed for the presence of the DNA insert were then introduced into *A. tumefaciens* using the heat-shock protocol according to Gold Biotechnology transformation. A total 1 μL of plasmid DNA was aliquoted into chilled microcentrifuge tubes on dry ice and 50 μL of cells were subsequently added to the microcentrifuge tube on ice and mixed gently by tapping 4 times. The tubes were kept on dry ice for 30 min. Thereafter, a water

bath was used for incubation of tubes at 37 ºC for 2 min. A total 950 µL of SOC media was immediately added after removal from the water bath to resuspend cells. Tubes were subsequently incubated at 30 ºC for 3 hours at 200rpm in a shaking incubator. The cells were then plated onto selective LB agar plates containing 25 mg/ml rifampicin and 50mg/ml spectinomycin. The plates were incubated for 3 days at 30 ºC.

3.5.1. Confirmation of *Agrobacterium* Transformation

Colony PCR was done to confirm successful transformation using pCas9-TPC plasmid. Colonies were aliquoted into 100 µL of sterile water and 20 µL of the mixture was heated at 96 ºC to use as the DNA template for colony PCR. SS43 reverse primer and gRNA forward primer were used to amplify targeted DNA.A total of 20 µL of transformed *Agrobacterium* cells were added to 10 ml of LB broth and incubated at 30 ºC at 200 rpm agitation overnight and resulting growth was used for Agroinfiltration.

3.6. Plant Transformation and Micropropagation

The ONC grown from seeds grown *in vitro* maintained for 8 weeks. The leaves were then transformed according to Gallois and Marinho (1995). The leaf discs were submerged in a liquid culture of transformed *A. tumefaciens* for 30 s and dabbed on sterile cotton wool. The leaf discs were co-cultured on Murashige and Skoog (MS) media supplemented with sucrose. After 24 h the leaves were covered with bacterial growth. The leaf discs were then transferred into callus-inducing media supplemented with plant preservative mixture (PPM) for 6 weeks. The callus was grown in shoot-inducing media and plantlets maintained for 8 weeks. The plantlets were then transferred into MS media for 3 weeks to induce root system development. The plantlets were then transferred into the greenhouse where they hardened and planted in a pine bark and vermiculite mixture.

3.7. Agroinfiltration and Sequencing

Agroinfiltration was assayed essentially as described by Yang *et al.,* (2000) with minor modifications. *A. tumefaciens* strain LBA 4404 cloned with Cas 9-TPC was cultured in 2ml LB supplemented with appropriate antibiotics kanamycin and rifampicin and then inoculated into 25 ml LB with 10 mM Mes, 20 µM acetosyringone as well as the antibiotics. The culture was grown overnight to log phase (OD6000.8) at 28 °C, pelleted by centrifugation and resuspended in MMA solution (10 mM Mes, 10 mM MgCl2, 100 µM acetosyringone) to a final

OD600 of 1.5. A total 100 µL of the bacterial suspension per spot was infiltrated into intercellular spaces of near fully expanded leaves using a 5 ml plastic syringe. After agroinfiltration, the treated plants were kept in dim light or dark at 23 °C under high humidity for 1-2 d.

Figure 6: Agroinfiltration of ONC tobacco plantlet using the transformed *Agrobacterium.*

Genomic DNA of the tobacco leaf was extracted using the modified cetyl trimethyl ammonium Bromide (CTAB) method. A total of 0.5 g of the leaf was ground using mortar and pestle and 0.1 g of the fine powder was placed in a 2 ml micro centrifuge tube. To the tube, 3% CTAB buffer (200 mM Tris-HCl (pH 8.0) was added to fill the tube and the tubes were vortexed vigorously. Thereafter the tubes were left in an incubator shaker for 1 h at 65 °C, with shaking at 120 rpm. The tubes were then placed on ice for 4 min and centrifuged at 14,700 g for 10 min. An equal volume of chloroform was added to the supernatant and centrifuged at 14,700 g for 10 min. The chloroform wash was repeated with an equal volume of the supernatant. Thereafter, the supernatant was precipitated for 1 h with 1% CTAB buffer (50 mm TRIS-Cl, pH 8.0; 1% CTAB) at ambient temperature. After centrifugation, the samples were dissolved in 1 M CsCl and precipitated with isopropanol. The pellet was resuspended in 70% ethanol,

centrifuged and left to dry completely at ambient temperature. The DNA was then dissolved in 50 µL nuclease-free ultra-pure water and placed in a water bath at 37 °C for at least 5 min.

Reverse and forward eIF4e primers were used in a polymerase chain reaction on the extracted plant DNA. A PCR purification kit was utilised to purify the PCR amplicons and remove PCR reaction residuals.

Table 10: Cycle sequencing master mix Preparation

Reagent	Volume (µL)
H_2O	4
5X Sequencing buffer	1
Reaction Mix	2
Primer	1
Template	2
Total Volume	10

Table 11: Sequencing cycling conditions

Temperature **(°C)**	Time	Cycles
96	4 min	
96	10 s	
57	5 s	40X
60	4 min	
4	∞	

Unincorporated dye terminators remaining in solution after cycle sequencing cause Dye blobs. There is need to carry out a purification process of the cycle sequencing reactions. The BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit and SAM^{TM} solution was used for the purification process. The plate was vortexed for 30 min then subsequently centrifuged for 2 min. The reaction plate was placed in the Applied Biosystems^{R} Genetic analyzer and run.

Table 12: List of primers used in this study

Oligo name	Sequence 5'- 3'	Orientation
M13	CACAGGAAACAGCTATGAC	reverse
SS42	TCCCAGGATTAGAATGATTAGG	forward
SS43	CGACTAAGGGTTTCTTATATGC	reverse
gRNA	TCCGAAATAGGGGGTAAAAT	forward
gRNA	AGATTCTGTTTCTTGCATTCCC	reverse

4. RESULTS

4.1. Introduction

This chapter outlines the results obtained during the experimentation of the research project. The results are given in a manner that follows the sequence in the previous chapter.

4.2. NCBI Search

The NCBI search for Eukaryotic translation initiation factor yielded gene results including some from *Nicotiana tabacum*. KF155696.1 was selected as the target due to its predominance in expression in plant material (both in vitro and ex vivo).

Figure 7: Selected Eukaryotic translation initiation factor 4E gene details, GenBank ID: KF155696.1.

4.2.1. CRISPOR search

The target DNA generated by CRISPOR had the following sequence;

Table 13: gRNA primers for target sequence selected with PAM sequence upstream of 5` gene sequence.

gRNA	Forward Primer	Reverse Primer	Off-targets
gRNA 1	TCCGAAATAGGGGGTAAAAT	AGATTCTGTTTCTTGCATTCCC	0-0-0-0-1

4.3. gRNA synthesis

The forward and reverse gRNA oligos were annealed and the product was run on 1% agarose gel with a 50bp ladder for size estimation. The product was 130-150bp in size.

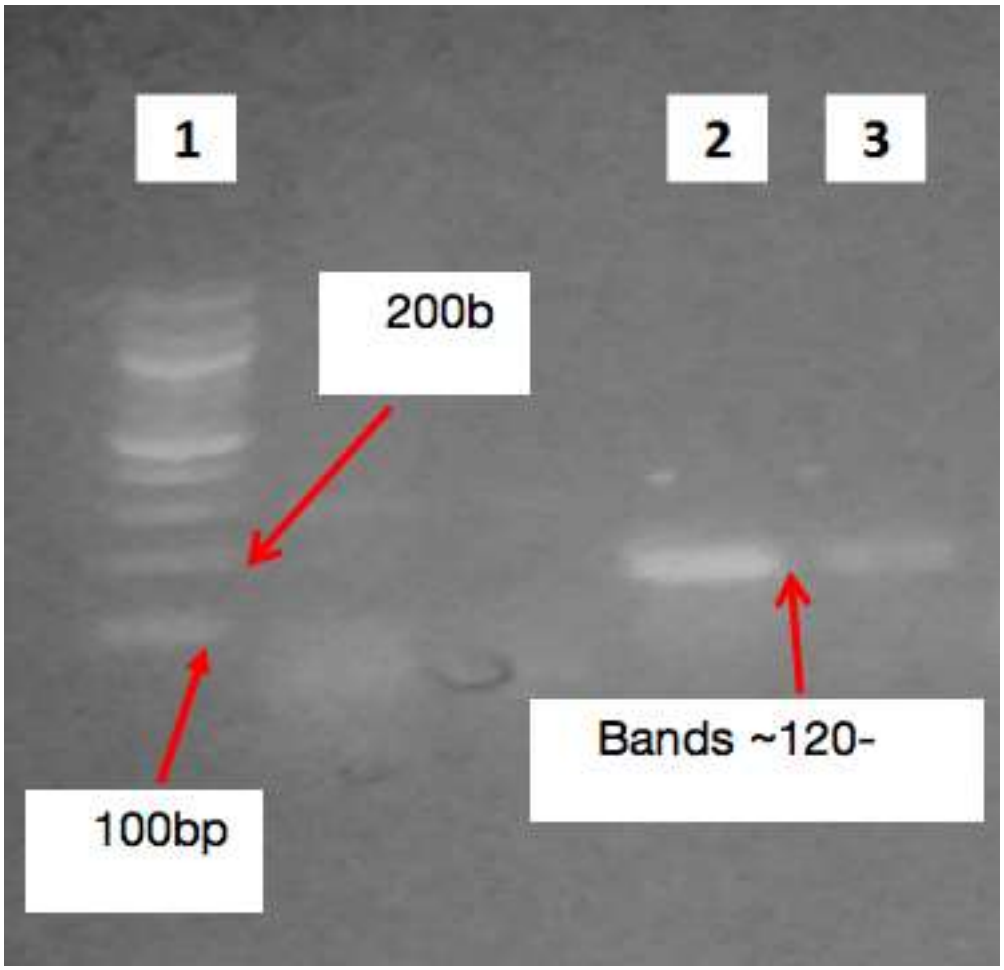

Figure 8: UV Gel image showing the gRNA DNA template. Lane 1 consists of the 100bp ladder, Lane 2 and Lane 3 contain of the synthesized gRNA DNA template oligo. The bands of the produced oligos were consistently situated at 120 bp.

4.3.1. Confirmation of gRNA DNA template in pChimera and *E.coli* transformation

The transformed *E.coli* grew on LB media that contained 100 µg/mL of ampicillin while in the non-transformed *E.coli* no colonies developed.

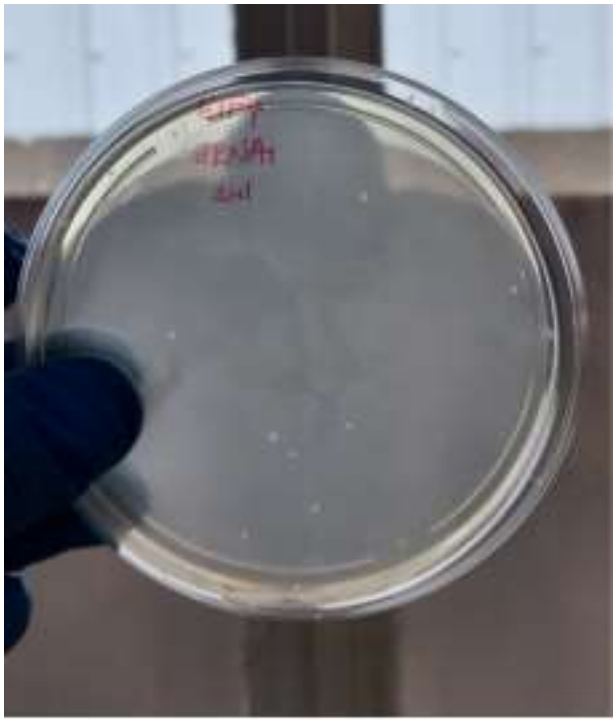

Figure 9*: Escherichia coli* colonies that grew on selective media containing ampicillin.

Transformation efficiency

Transformation Efficiency = Number of colonies / (dilution / µg of plasmid)

1 µl of (10 pg/µl) Cas 9 TPC was transformed into 25 µl of chemically competent cells. 975 µl of Recovery Medium into the tube. 10 µl of this was diluted in 990 µl of Recovery Medium and plated 50 µl of the diluted medium.

On the next day, 250 colonies were counted on the plate.

Based on this information :

- Colonies = 100
- µg of DNA = 0.00001 (or 10 pg = 0.00001 ug)
- Dilution = 10/1000 x 50/1000 = 0.0005

Therefore, the transformation efficiency of competent cells:

TE = 250/(0.00001/0.0005) = 2.0×10^{10} cfu/µg

The colony PCR confirmation produced a band of approximately 370bp.

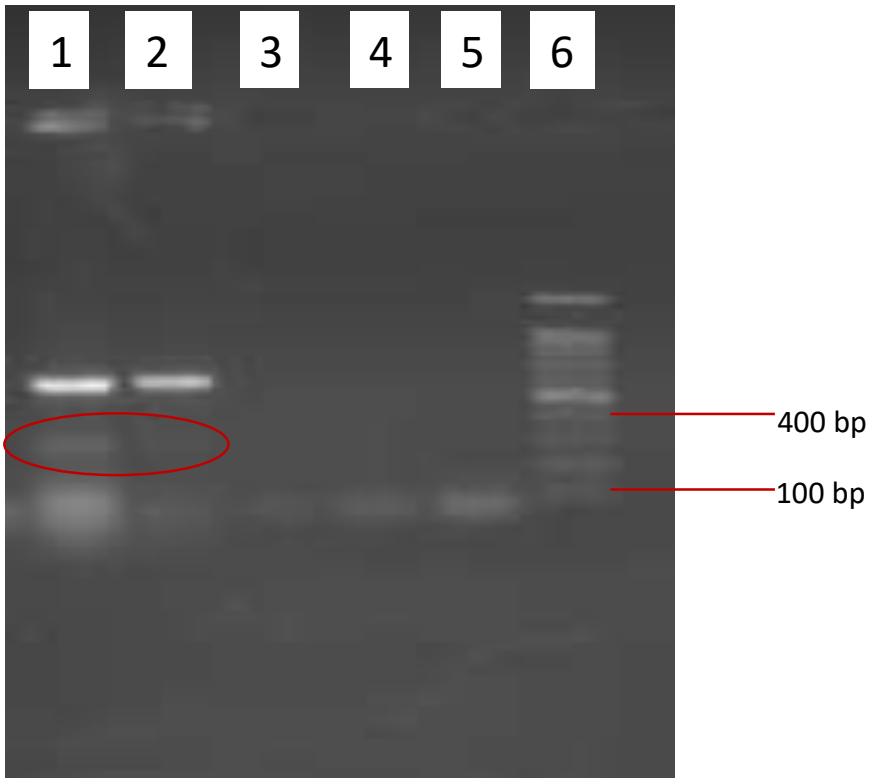

Figure 10: UV gel for insert confirmation in pChimera. Lane 1 - Colony amplified using ss42 forward and gRNA reverse primers; Lane 2 - Colony amplified using ss42 forward and gRNA reverse primers; Lane 3 and 4- Non-transformed pChimera with no bands; Lane 5- Negative control (ultrapure water).

4.3.2. Confirmation of gRNA DNA construct in Cas9-TPC and *E.coli* transformation

The transformed *E.coli* grew on LB media that contained 50µg/mL of spectinomycin while the native (non-transformed) *E.coli* failed to develop colonies on the antibiotic-containing media.

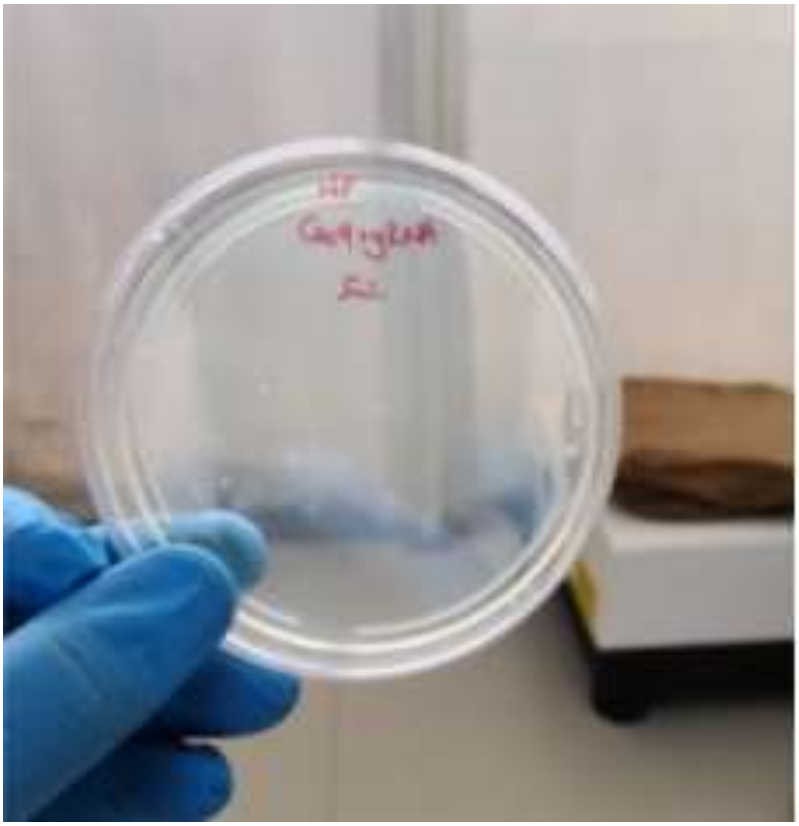

Figure 11*: Escherichia coli* transformed cells that grew on selective media containing spectinomycin.

The PCR confirmation produced a band of approximately 1kb.

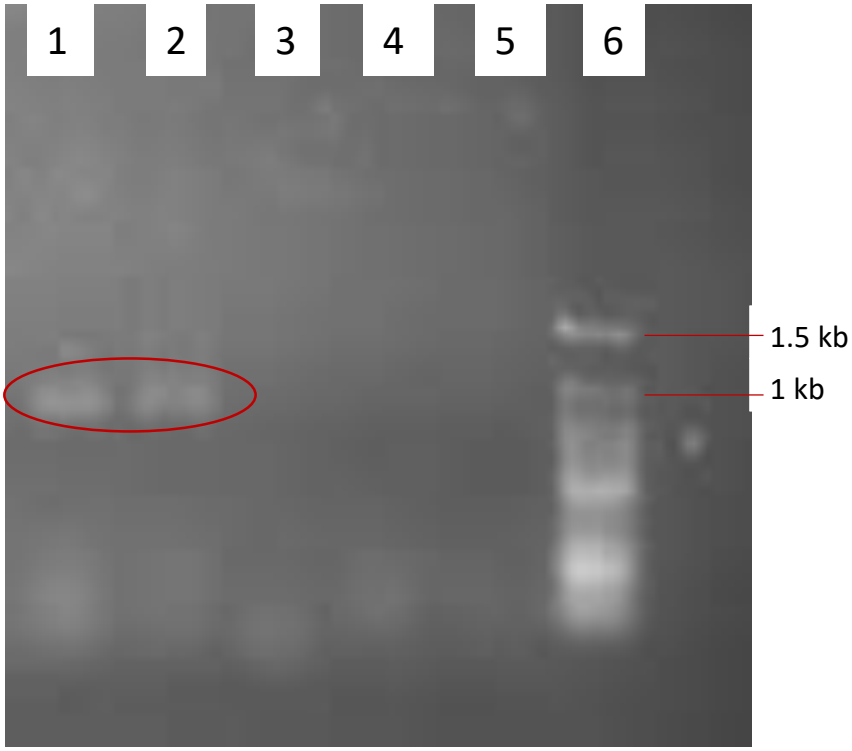

Figure 12: Lane 1 and 2 – colonies producing approximately 1kb band sizes due to amplification with ss43 reverse and gRNA forward; Lane 3 and 4 – non transformed *E.coli*; Lane 5 - Negative control (ultrapure water).

4.3.2. Confirmation of *Agrobacterium* transformation using pCas9-TPC plasmid

The transformed *Agrobacterium*'s grew on the LB media spiked with 50µg/mL of spectinomycin and 25µg/mL rifampicin.

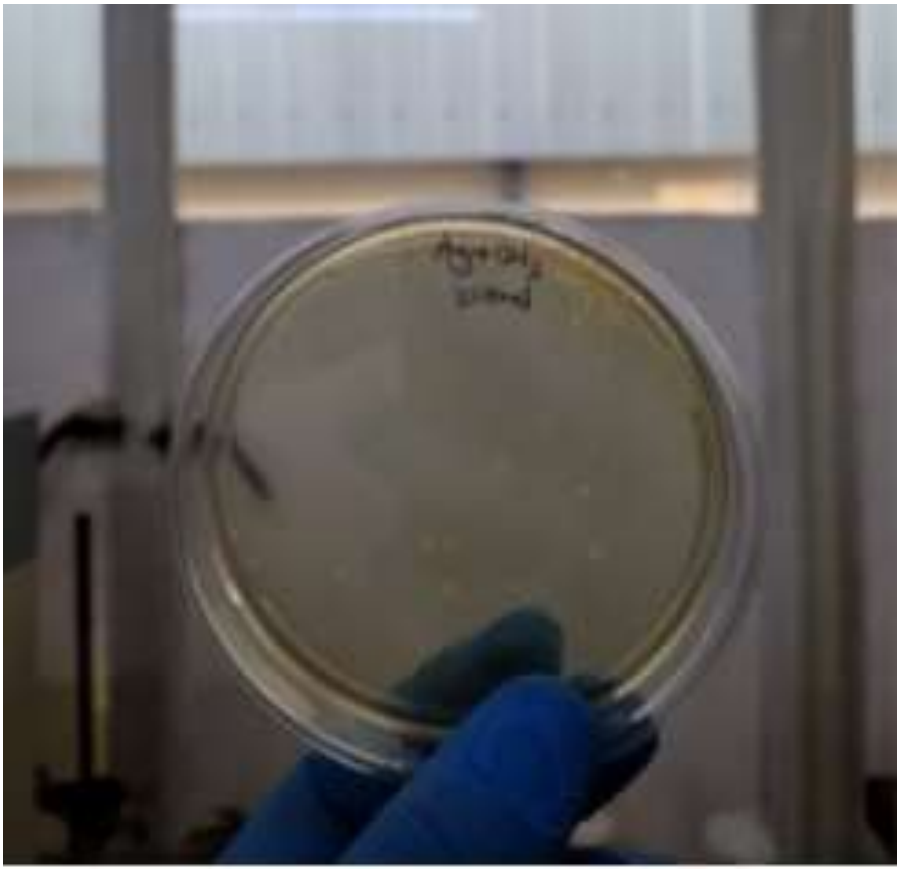

Figure 13*: Agrobacterium tumefaciens* cells that grew on selective media containing spectinomycin and rifampicin.

Transformation efficiency

Transformation Efficiency = Number of colonies / (dilution / µg of plasmid)

1 µl of (10 pg/µl) Cas 9 TPC was transformed into 25 µl of chemically competent cells. 975 µl of Recovery Medium into the tube. 10 µl of this was diluted in 990 µl of Recovery Medium and plated 50 µl of the diluted medium.

On the next day, 250 colonies were counted on the plate.

Based on this information:

- Colonies = 250
- µg of DNA = 0.00001 (or 10 pg = 0.00001 ug)
- Dilution = 10/1000 x 50/1000 = 0.0005

Therefore, the transformation efficiency of competent cells:

TE = 250/(0.00001/0.0005) = 5.0×10^{10} cfu/μg

The PCR confirmation produced a band which corresponded with the positive colony control with a 100bp ladder.

Figure 14*:* Lane 1- positive control *Escherichia coli* containing Cas9-TPC plasmid; Lane 2 – non transformed *Agrobacterium* producing no bands; Lane 3 & 4 – *Agrobacterium* colonies producing band sizes that correspond with the control.

4.4. Agroinfiltration

Figure 15: Tobacco variety ONC 2 days after agroinfiltration.

4.4.1. Transformation of Tobacco Leaf Discs and Plantlet development

Leaf discs that were grown in MS media after submerging in LB broth containing an overnight culture of Cas9-TPC/DNA template transformed *Agrobacterium* displayed bacterial growth around the edges of the discs after 48 hour co-culture.

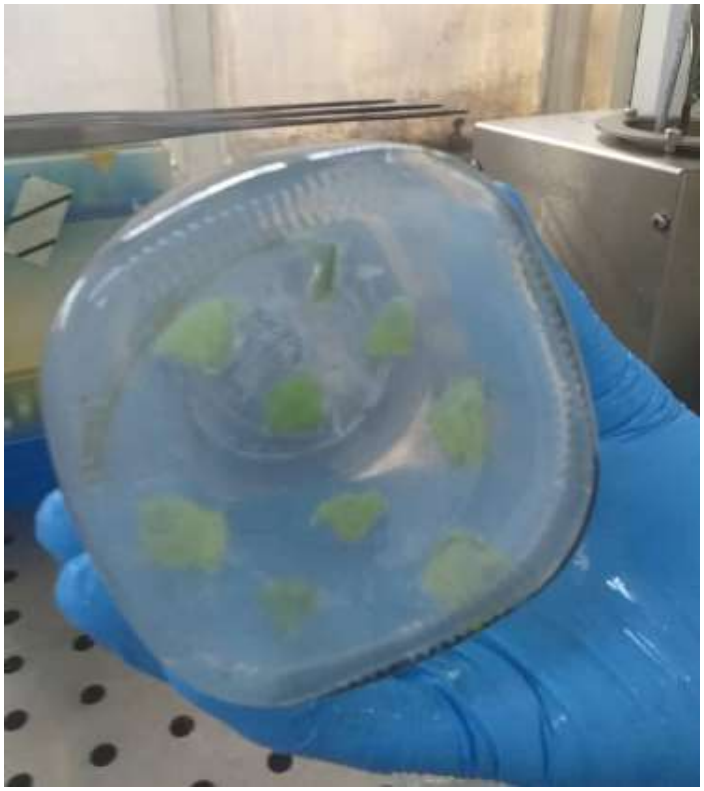

Figure 16*:* Tobacco leaf discs after 48 hours of co-culture with transformed *Agrobacterium*. The bacteria grew around the discs, indicating *Agrobacterium* proliferation enabling the *Agrobacterium* mediated plant transformation.

The leaf discs were transferred to callus-inducing media after co-culture on MS media with the transformed *Agrobacterium*. The leaf discs began curling and blistering after 10 d. The resulting blisters formed into a callus that increased in size over 4 weeks.

The callus was transferred to shoot-inducing media where organogenesis commenced after 9 days with the formation of a stem-like structure. Over the next 6 weeks the stem increased in size and developed leaf-like structures (Figure 17).

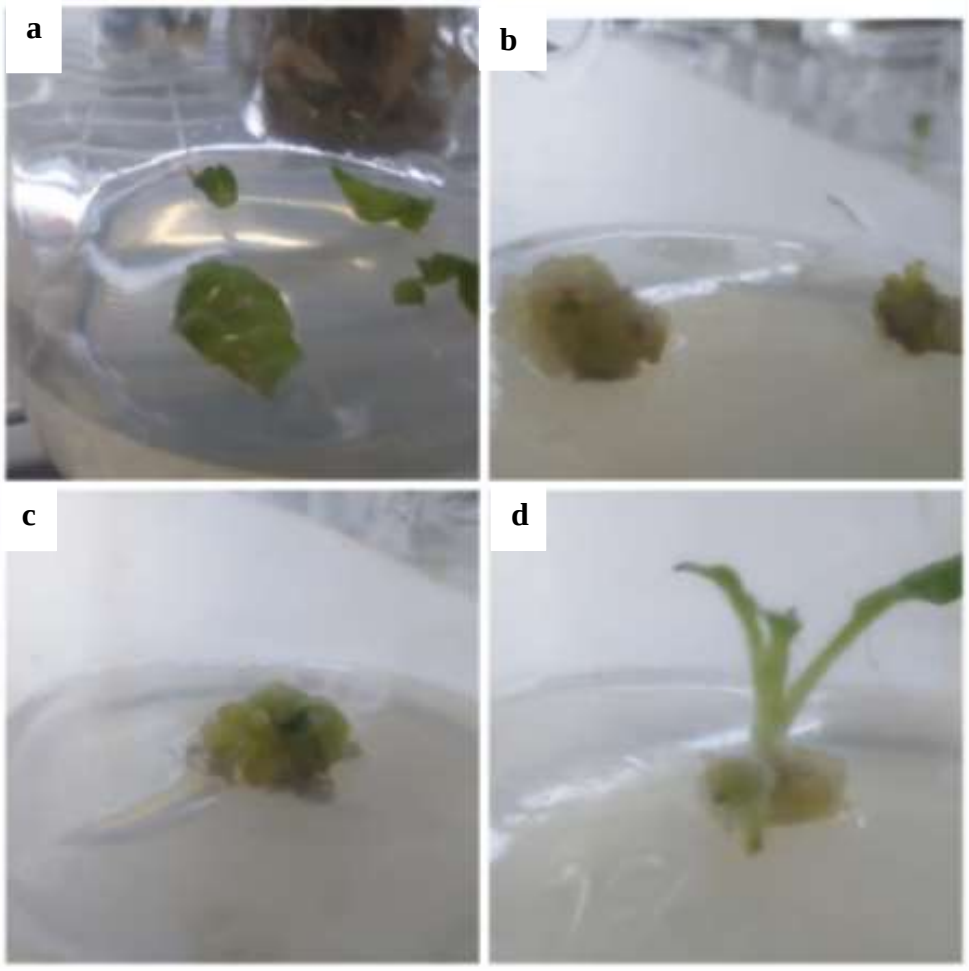

Figure 17: The stages of callus development and organogenesis. a: Leaf discs displaying curling and blistering, notable signs of callus formation onset after 10 d. b: Tobacco callus from leaf discs displayed an increase in size after 7 weeks. c: Callus developing a green stem-like structure after 10 days in shoot-inducing media. d: Shoot-like structure developing multiple stems and leaves after 8 weeks.

4.4.2. In vitro rooting and ex vitro growth of plantlets

The callus- was transferred to MS media where the plantlet developed dense root systems in 3 weeks. After hardening the plants were transferred to the greenhouse and planted in sterile soilless media. The plants displayed vigorous leaf development after 5 d.

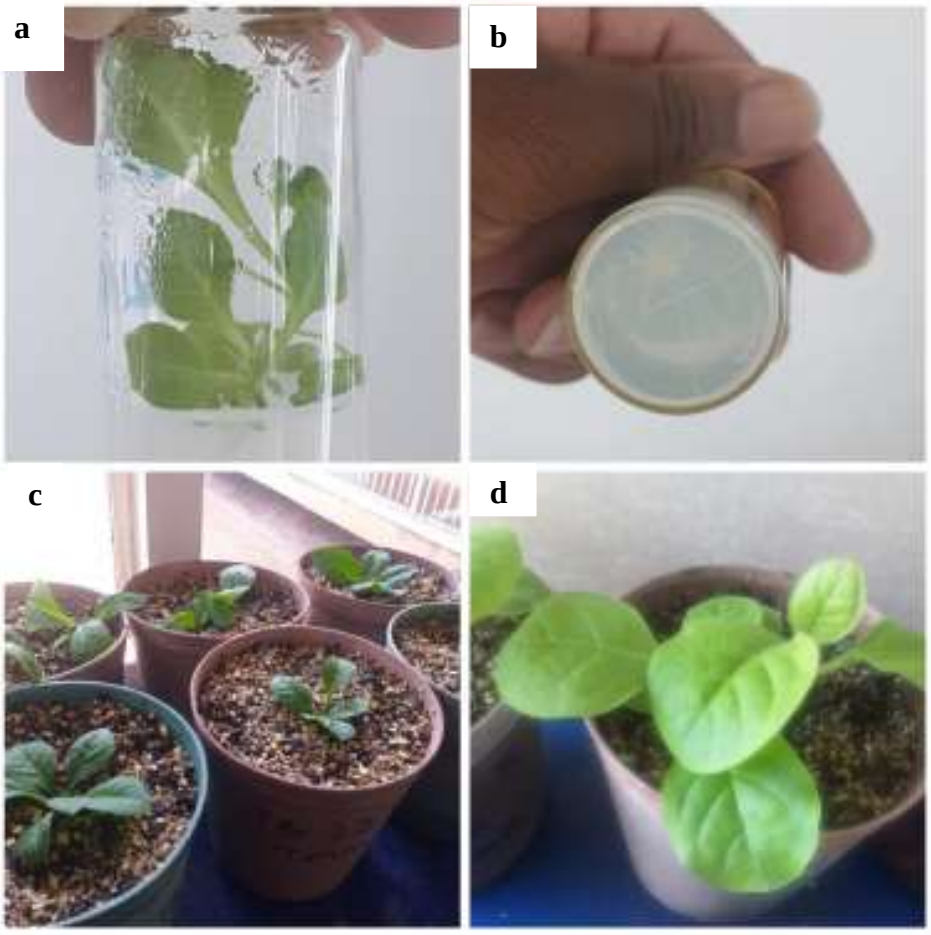

Figure 18*:* Development of Tobacco from *in vitro* rooting to greenhouse planting. a: Edited ONC displaying stem and leaf development after 1 week of *in vitro* culture in MS media. b: Edited ONC displaying root development after 2 weeks. c: Non- edited ONC in the back row 4 d after planting and edited ONC in the front row after the same number of days after planting. d: Non-edited ONC after 3 weeks after planting.

4.5. Sequencing analysis

Table 14: Description of the mutation within the ONC tobacco edited line

Variety	Sequences	Description
ONC wild-type	**ACTTGCTGGGTGAAAGGGGGCC**	**1 nt Del at 3`**
ONC transformed	**ACTTGCTGGGTCCATCGGGG - C**	

The sequences obtained were used to generate a phylogenetic tree along with those of TB22 and VAM. The transformed and wild type ONC variety displayed a close relatedness indicating a mutation present in the gene.

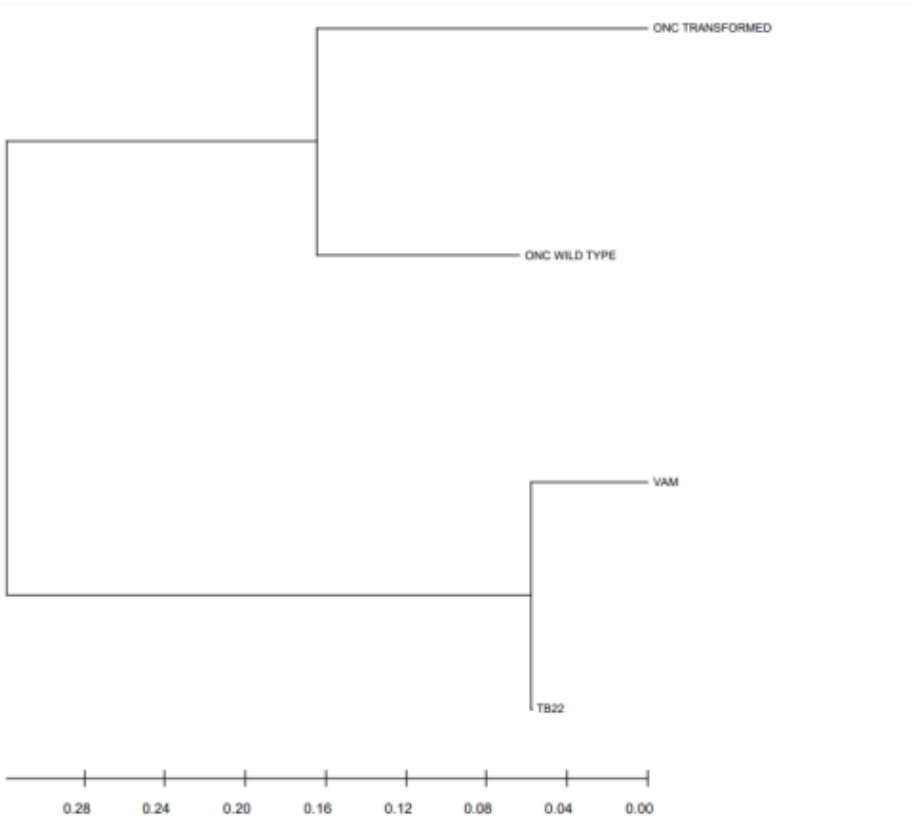

Figure 19: Phylogenetic analysis of 3 parental lines, edited ONC,TB22 and VAM after sequencing of the eIF4e region.

The dendrogram consisted of the edited and unedited ONC and an out-group (Irish potato). The tobacco varieties showed a high similarity and were both unrelated to the outgroup.

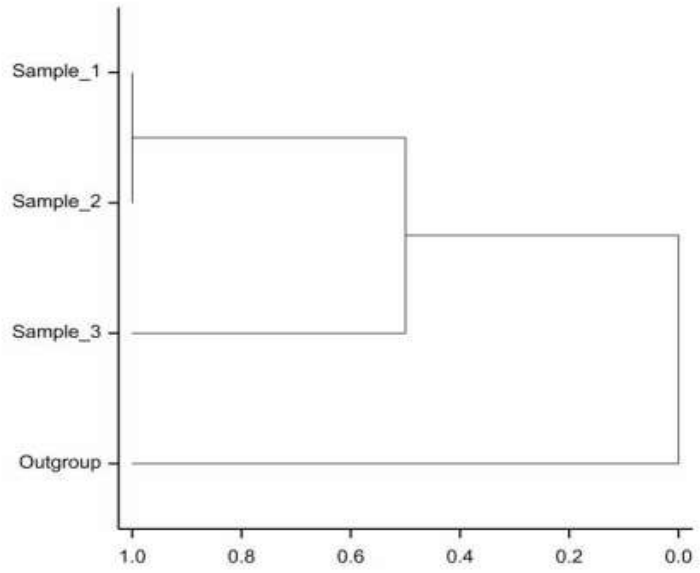

Figure 20: Dendrogram illustrating of parental line of edited ONC, unedited ONC and the out-group based on the results of URP 17 reverse primers.

Sample 1 and 2 - Unedited ONC; Sample 3 – Edited ONC; Outgroup – Irish Potato

5. DISCUSSION

According to Brown *et al,.* (2015) the Gene database of the National Centre for Biotechnology Information (NCBI) is a resource that offers useful gene-specific data including related publications, gene products, phenotypic consequences and related pathways amongst other details. According to the NCBI database, the KF155696.1 gene is a protein-coding sequence in common tobacco with the gene product being eIF4E. Reducing the damage caused by viral infections can be achieved in part by breeding plants for resistance and it is essential to identify the host genes that cause virus resistance in order to do this. According to Udagawa *et al.,* (2021) the eIF4 gene is responsible for producing a protein called eIF4E, which is a key component of the plant's translation initiation complex. Mutations in the genes that encode these factors reduce susceptibility to the viruses.

According to the CRISPOR manual, the sequence retrieved was within the eIF4 gene and of the forward strand. The forward strand sequence was selected over reverse as the use of the forward keeps all sequences in one direction making position information easier to calculate and display in related tools such as genome browsers.

The specificity of the target is a score that predicts the likelihood of the resulting RNA guide leading to off-target cleavage. According to Haeussler *et al.,* (2016) a CRISPOR specificity score equal or greater than 50 (100 being the best) results in less off-targeting and subsequently potentially less unpredictable genomic alterations. The specificity score obtained for the given gRNA sequence was 99 and according to Haeussler *et al*., (2016) it would therefore be an appropriate gRNA for a predictable and specific cleavage. This would allow greater ease in the tracking of the edit via sequencing techniques.

The efficiency of the gRNA sequence was scored 66 out of 100 (100 being the best), this score determines the efficacy of the gRNA's cleavage of the target site. This score is based on the Doench design rule (Doench *et al*., 2016) which was found to be the most effective scoring for guides expressed *in vitro* with a T7 promoter (Haeussler *et al*., 2016). The score of 66, therefore, indicates a theoretically efficacious gRNA sequence.
The out-of-frame shift score is a prediction of how likely a gRNA sequence will result in an out-of-frame deletion and lead to gene knockouts (Haeussler *et al*., 2016). This is a critical

score as it determines the capacity of shutting down the proper expression of the gene being edited (Bae *et al*., 2014).

According to the gRNA DNA template sequence retrieved from CRISPOR, the DNA template was hypothesized to be approximately 120bp in length. The band produced was estimated to be approximately 130 – 140bp. Upon enquiry, a common rule of thumb states that a PCR product within a 10% difference of the expected band was deemed acceptable (Schiml *et al*., 2014). In addition, the use of ethidium bromide (EtBr) has been cited to reduce the migration rate of DNA band by 15% resulting in larger observable bands as compared to the expected (Lee *et al*., 2012). The gel electrophoresis protocol used in experimentation utilized EtBr in the gel preparation and would offer a rationale as to why the band (~130 – 140) was obtained. Given the band size being close to the expected band (within 10%) after migration in gel consisting of EtBr, it was therefore deemed that the gRNA DNA template was successfully synthesized.

The process of CRISPR/ Cas 9 gene editing strongly depends on the ability to clone the gRNA DNA template in the first plasmid which is pChimera. This plasmid contains the chimeric sgRNA scaffold which is a short synthetic RNA composed of a scaffold sequence. This scaffold sequencing is necessary for Cas-binding. The gRNA DNA template was then ligated into dephosphorylated linear pChimera after BbsI restriction digestion of the plasmid.

The growth of *E.coli* colonies on ampicillin-enriched LB media indicated that the *E.coli* had taken up the pChimera plasmid (Schiml *et al*., 2014). The determination of whether the pChimera had taken up the gRNA DNA template via PCR confirmation resulted in a band of approximately 350-400bp. The expected band size is 370bp according to Schmil *et al.,* (2014) and the negative control showing no band length of the same size. The presence of the 370-400bp band indicated successful integration of the gRNA DNA template into the pChimera. The pChimera plasmid contains a scaffold (tracrRNA) which is responsible for binding to the Cas 9 protein and therefore necessary to be part of the gRNA construct to be transferred to pCas9-TPC plasmid (Rajput *et al*., 2021).

The gRNA was extracted from the plasmid via AvrII restriction digest and ligated into dephosphorylated linear pCas9- TPC.

The gRNA DNA with the scaffold was transferred into the second plasmid which was pCas9-TPC. This plasmid vector was of utmost importance because it carries the Cas 9 gene which is responsible for the formation of the Cas 9 endonuclease. The Cas 9 enzymes is directed by the gRNA to cleave the DNA at a specific site and allow natural DNA repair hence knocking off the gene on interest. The successful growth of transformed *Agrobacterium* colonies in LB media enriched with spectinomycin indicated the successful uptake of the plasmid. PCR confirmation of insert uptake resulted in 1kb bands being obtained. This was comparable to the positive result stated by the plasmid editing protocol by Schiml *et al.,* (2014). The two results indicate that the binary vector with the gRNA DNA template was successfully transformed into *Agrobacterium*.

The plant transformation produced bacteria that circled the leaf structures after 24 h incubation. The morphology of the bacteria was similar to the *Agrobacterium* colonies that were produced after pCas9-TPC transformation. The leaf discs that were then grown on callus inducing media containing antibiotic and plant preserving mixture began blistering and lifting off the media and growing without any bacterial growth. The blistering leaves then developed in to pale green globular structure identified as a callus according to Piyatrakul *et al.,* (2012). The calli were transferred to shooting-inducing media and subsequently to regular plant media where the relevant organogenesis resulted in plantlets.

This appearance is similar to that described by Gallois and Marinho (1995) as successful co-culturing of the leaf discs and transformed *Agrobacteria*. The formation of callus occurred as reported by Husin *et al.,* (2008) who used the auxin and cytokinin combination of 1-Naphthaleneacetic acid (2.0 mg/L) with kinetin (0.2 mg/L). The combination of auxins and cytokinins to induce calli formation was reported by Ikeuchi *et al.,* (2013), where the artificial combinations of these two growth factors in specific ratios, Husin *et al*., (2008) and Ali *et al.,* (2007) stating that the ratio requires a higher ration of auxins compared to cytokinins. This ratio of plant growth factors mimics the plant response during organ injury with the hormone combination inducing the transcription of the shoot re-differentiation defective2 *(SRD2)* and root initiation defective2 *(RID2)* genes with a combined activity that is linked with high cellular proliferative activity (Ikeuchi *et al*., 2013). The action of benzyl amino purine (BAP) has been well documented and has an overall effect of promoting the shooting and leaf formation of plants (Amelia & Wulandari, 2020).

Agroinfiltration technique was utilised due to its ability to allow for the transient expression of genes within a few days thereby enabling quick analysis of the plant genome. The suspension is gently pressed into the leaf, allowing the bacteria to infiltrate the intercellular spaces of the leaf tissue. The *Agrobacterium* cells spread throughout the intercellular spaces of the leaf tissue and recognize specific plant-derived signals, such as phenolic compounds and plant hormones released in response to the plant's wound or at the site of infiltration. These signals trigger the expression of a set of genes called the Vir genes, which are essential for the transfer of the bacterial DNA from *Agrobacterium* to the plant cell (Kapila *et al.,* 1997). In this study's case, the pCas9-TPC plasmid is integrated with the plant genome and express the CRISPR/Cas9 system.

The presence of the pCas9-TPC in the transformed ONC was carried out in the same manner as the pCas9-TPC identification in *Agrobacterium*. The PCR confirmation generated a 1kb band which was in accordance with the expected results in the protocol according to Schmil *et al.,* (2016). These findings indicate that the tobacco leaf was successfully transformed and contained the pCas9-TPC which possessed the gRNA DNA template.

The sequencing results presented in the table 13 compare the sequences from wild-type and transformed variety of ONC. Comparing the sequences of the wild-type and transformed varieties shows a clear deletion of a single nucleotide at the 3' end of the sequence. A single nucleotide deletion can lead to a frameshift mutation if it occurs within the coding sequence. This frameshift alters the reading frame of the gene, potentially resulting in a completely different and often non-functional protein. In the given sequences, the deletion is at the 3' end, and it is not clear if it is within the coding region. If it is, the consequences could significantly mean the disruption of the eIF4e gene thereby inducing PVY tolerance.

The URP17 fingerprinting generated a dendrogram that showed the edited and unedited parental line ONC had very limited differences. The edited and unedited ONC were closer in relation as compared to the out-group (maize). According to Iqbal *et al*., (2021) DNA fingerprinting allows for crop variety compatibility for breeding capability, with closely related varieties displaying better breeding compatibility and the generation of more stable offspring.

The phylogenetic tree generated represents the evolutionary relationships between different tobacco samples based on the sequencing of the eIF4e region. The close relationship between

ONC Transformed and ONC Wild Type implies that the modification in the ONC Transformed sample altered the eIF4e region to some extent. This desired alteration gives a greenlight for transplantation of transgenic plantlets and subsequent assessment of PVY tolerance. The separation of VAM and TB22 from the ONC samples suggests that these groups possess mutations within the eIF4e gene which give rise to their evolutionary differences.

6. CONCLUSION

In conclusion, CRISPR-CAS9 mediated mutagenesis of the eIF4 gene in tobacco holds great promise for the induction of PVY tolerance in this economically important crop. This technology offers a precise and targeted approach to disrupt the function of the eIF4 gene, leading to resistance against PVY. With further research and development, CRISPR/CAS9 mediated mutagenesis can be a powerful tool in the fight against viral diseases in crops, helping to ensure food security and sustainable agriculture.

The use of genome editing technology has revolutionized the field of genetics and has opened up new possibilities in crop improvement. One such technique that has gained widespread attention is CRISPR-CAS9 mediated mutagenesis. This powerful tool allows scientists to make precise changes in the DNA of an organism, offering a way to introduce beneficial traits and improve crop performance.

6.1. Limitations

The main limitations to this project were time and limited reagents. The limitation of time disallowed the increased in vitro multiplication cycles limiting the number of plants used in the greenhouse trials. In addition, limited time did not allow the full maturation of plants and transgenic status assessment of T1, T2 and T3 generations as well as possible induction of PVY tolerance in the progeny.

6.2. Recommendations

Recommendations would include the sequencing of the eIF4 gene in the edited ONC and comparison via BLAST against the native eIF4 gene. The comparison would allow the determination of specific frameshifts.

To increase the efficiency of cloning the gRNA in plasmids, the sgRNA can initially be cloned into a plasmid like which confers resistance to an antibiotic besides ampicillin then restrictions enzymes are utilized to digest the sgRNA and antibiotic resistance marker as a construct. The forementioned is then cloned into pChimera as usual but this time two antibiotics wills be used to select successfully transformed bacterial colonies and this ultimately increases efficiency in selection. The newly cloned plasmid can be named and deposited on Addgene to assist researchers making use of plasmids in CRISPR/Cas 9 technology.

The potential for off-target consequences is one unwanted outcome. To make sure that gRNAs only target the intended gene, attention must be taken in their creation. It is also advised to use methods like whole-genome sequencing to ensure the absence of off-target effects.

Another drawback is the possibility of unforeseen consequences on plant development and growth. Therefore, it is advised to perform rigorous phenotypic evaluations to make sure the modified plants don't have any unfavorable impacts. Finally, it is advised to carry out field testing to assess how well the altered plants defend against PVY infection in actual environmental circumstances. Greater collaboration between research institutions with the sharing of information, resources and credit would foster a greater environment for research and produce higher-quality output.

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APPENDICES

Appendix 1: Plasmid extraction protocol used during experimentation

https://www.addgene.org/protocols/purify-plasmid-dna/

Appendix 2: Extracted plasmid concentrations

pChimera

pCAS9-TPC

Appendix 3: Execution of project practical aspects.