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EGE UNIVERSITY

Graduate School of Applied and Natural Science



CHANGING LEAF SHAPE IN ORNAMENTALS BY GENOME EDITING CRISPR-CAS9 CHANGING LEAF SHAPE OF PETUNIA

MSc THESIS

Muhammad MOAZZAM

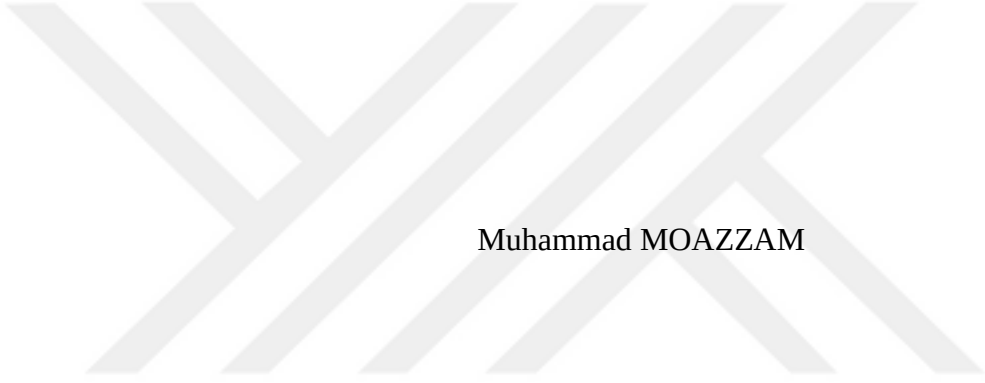
Department of Seed Science and Technology

İzmir

2020

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**CHANGING LEAF SHAPE IN ORNAMENTALS BY
GENOME EDITING CRISPR-CAS9 CHANGING LEAF
SHAPE OF PETUNIA**



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Erasmus Mundus Master Program in Plant Breeding Second Cycle Programme

İzmir
2020

Here we certify that this thesis entitled “**Changing Leaf Shape in Ornamentals by Genome Editing CRISPR-Cas9 Changing Leaf Shape of Petunia**” conducted, prepared and submitted for the degree of Master of Science by Muhammad MOAZZAM has been recommended for acceptance and approval for oral defence as it complies both with the “Ege University Graduate School policies” and “Ege University Graduate School of Natural and Applied Sciences' rules and regulations”. This study is fully adequate, in scope and quality, as a thesis for the degree of Master of Science and the Candidate was found successful and the thesis was approved by the thesis committee by unanimity voting on 16 / 09 / 2020.

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16 / 09 /2020

A handwritten signature in black ink, appearing to read 'Muhammad Moazzam', enclosed within a circular scribble.

Muhammad MOAZZAM

ÖZET**CRISPR-CAS9 GENOM EDITİNG İLE SÜS BİTKİLERİNDE
YAPRAK ŞEKLİNİN DEĞİŞTİRİLMESİ PETUNYANIN YAPRAK
ŞEKLİNİN DEĞİŞTİRİLMESİ**

MOAZZAM, Muhammad

Yüksek Lisans Tezi, Tohumluk Bilimi ve Teknolojisi Anabilim Dalı

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CRISPR-Cas9 teknolojisinin, bitkiler dâhil olmak üzere birçok canlı organizmada genom düzenlemesi için etkili bir yöntem olduğunu kanıtladı. Bu teknik hızlı, hassas, ucuz ve nispeten daha az karmaşık bir tekniktir ve bitkilerde genetik modifikasyonlar oluşturmak için yaygın olarak kullanılmaktadır. Bitkilerin tanımlanmasında ve sınıflandırılmasında yaprak şekli için en önemli fenotipik özelliklerden biridir. Pek çok üründe ve süs bitkisinde yaprak şekli ekonomik bir değere de sahiptir. Bu çalışma, yaprak şeklinden sorumlu gen veya genlerin etkin bir şekilde devre dışı bırakılması için optimize edilmiş bir CRISPR-Cas9 sistemi geliştirerek ve Petunyadaki yaprak şeklini değiştirmek amacıyla yapıldı. Arabidopsis, Domates, Tütün ve Petunya'da bulunan Knotted-1 geni, hücre bölünmesi ve uzaması yoluyla yaprak şeklini kontrol etmektedir. Bu çalışma, kültürü yapılan mevcut hibrit petunya bitkilerinden yeni petunya bitkileri rejenere etmek amacıyla Ghent Üniversitesi'ndeki Bitki in vitro biyoteknoloji laboratuvarında gerçekleştirildi. Solanaceae familyasında yaprak şeklinden sorumlu genler için gen devre dışı bırakma çalışmaları da yapıldı. Amplifikasyon primerleri tasarlandı ve PCR protokolü geliştirildi ve optimize edildi. CRISPR-Cas9 için oligoları tasarlamak amacıyla genin dizisi gerekiyordu. Gen dizilimi için farklı uzunluklardaki fragmanları çoğaltmak amacıyla birçok primer tasarlandı. Bitki bir melez olduğundan, Kn1 geninin her iki allelini ayrı ayrı dizmek için agrobacterium kullanılan PCR klonlama prosedürü gerçekleştirildi. Sonuçlar, MS

ortamında gövde kesimleri ile Petunya'nın filiz rejenerasyonunun çok verimli olduğunu ve %95'lik bir başarı oranı sağladığını, buna karşılık, yaprak kültürü ve agrobacterium ortak kültürü yoluyla rejenerasyonun farklı deneylerde farklı sonuçlar sağladığını ve başarılı kabul edilemeyeceğini gösterdi. Yaprakların toplam %90'ının kallus büyümesi göstermiş olmasına karşın %0'ı sürgün büyümesi sağladı ve Agrobacterium transformasyonu GFP ekspresyonu da %0 olarak gerçekleştiği gözlemlendi. Gen sekanslaması, genin her iki allelinin de yaprak infiltrasyonu ile agrobacterium ekspresyonu için uygun bir kısıtlama enzim bölgesi olan spesifik bir gRNA'nın seçilmesini zorlaştıran birçok tek nükleik polimorfik bölgeye (SNP'ler) sahip olduğunu gösterdi. Kendilenmiş hatlarda CRISPR-Cas gen devre dışı bırakılması üzerinde daha fazla çalışma yapılmalı ve heterozigot gen dizilemesini optimize etmek ve doğru bir CRISPR-Cas9 yapısı oluşturmak için daha fazla deney yapılması gerekmektedir.

Anahtar kelimeler: CRISPR-Cas9, yaprak şekli, gene knock-out, gene klonlama, rejenerasyon, co-culture, PCR klonlama, agrobacterium transformasyonu.

ABSTRACT**CHANGING LEAF SHAPE IN ORNAMENTALS BY GENOME
EDITING CRISPR-CAS9 CHANGING LEAF SHAPE OF PETUNIA**

Moazzam, MUHAMMAD

Master Thesis, Department of Seed Science and Technology

Supervisor: Prof. Dr. Eftal DUZYAMAN

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CRISPR-Cas9 has proved to be an efficient method for genome editing across many living organisms including plants. It is quicker, precise, cheaper, and relatively less complicated technique and has been widely used to induce genetic modifications in plants. Leaf shape is one of the most important phenotypic traits for plant identification and classification. In many crops and ornamentals, leaf shape holds an integral economic value as well. This study was conducted to alter the leaf shape in Petunia by developing an optimized *CRISPR-Cas9 system to efficiently knock-out gene(s)* responsible for the leaf shape. Knotted-1 gene controls leaf shape by cell division and elongation and it is present in Arabidopsis, Tomato, Tobacco and in Petunia as well. Experiment was performed at Plant *in-vitro* biotechnology lab, Ghent University, to regenerate petunia plants from available hybrid petunia cultured plants. Gene knock-out studies were also done for the genes responsible for the leaf shape in Solanaceae family. Amplification primers were designed, and PCR protocol was developed and optimized. To design the oligos for CRISPR-Cas9, sequence of the gene was required. Several primers were designed to amplify fragments of different lengths for gene sequencing. As the plant was hybrid, *PCR cloning procedure using agrobacterium* has been followed to sequence both alleles of the Kn1 gene separately. Results showed that shoot regeneration of Petunia by stem cuttings in MS media has been very efficient providing an overall 95% success rate while the regeneration through leaf culture as well as agrobacterium co-culture has not been

successful providing varying results in different experiments. An overall 90% of leaves showed callus growth but 0% shoot growth and 0% Agrobacterium transformation GFP expression was observed. The gene sequencing showed that both alleles of the gene had many single nucleic polymorphic sites (SNPs) which made it difficult to select a specific gRNA with a suitable restriction enzyme site, for agrobacterium expression by leaf infiltration. More studies should be conducted on CRISPR-Cas gene knock-out in inbred lines and further experimentation is required to optimize heterozygous gene sequencing and to establish an accurate CRISPR-Cas9 construct.

Keywords: CRISPR-Cas9, leaf shape, gene knock-out, gene cloning, regeneration, co-culture, PCR cloning, agrobacterium transformation.

PREFACE

The shape of plant, leaf, flower, or fruit has a significant importance in ornamentals. Throughout the history plant breeders and growers have implied many techniques to alter these characteristics. Most of the time they used conventional breeding. However, with the advent of the molecular tools and research, bringing changes in the phenotype of a plant by inducing specific genetic changes has been widely adapted. There are countless possibilities where genetic modifications can be of great use to develop new aesthetically appealing traits for ornamental plants. Petunia is one of the largest bedding plants around the world. It is available in a wide range of colors and color combinations. It's mainly used as an ornamental and holds a great aesthetically pleasing potential. In gardening, it is preferred because of its easy cultivation and range of colors. Apart from gardening, petunia is also one of the most important model plants, due its simple genome, ease in breeding, short life cycle and easy genetics study. Just like *Arabidopsis thaliana*, petunia has also been used by plant scientists to study various plant functions at genetic and cellular level.

In this experiment, essential leaf shape genes were targeted in Petunia, by transient expression of CRISPR/Cas9 endonuclease, and consequently plants with different leaf shapes will be created. A precise phenotyping will not only provide a measure of diversity among various leaf forms but will also signify the role of genes controlling leaf shape formation. Leaf shape consists of a unique order of cell multiplication and cell enlargement which requires different levels of genetic synchronization. With classical genetics, several leaf shape genes have been recognized, especially in *Arabidopsis*. With the recent advances in genome editing, there are handful of possibilities to create ornamentals with novel traits such as leaf shape. Literature review allowed to choose target gene(s) with significant effect on controlling leaf shape. The main goal has been to knock-out / modify such gene(s). Such genetic transformation of Petunia will give a proof of concept and will lay down the basis for gene editing in ornamental plants and will also be useful for plant breeders to breed plants with more economic value.

IZMIR

16/09/2020

Muhammad MOAZZAM

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Definition</u>
2,4,5-T	Trichlorophenoxyacetic acid
2,4-D	Dichlorophenoxyacetic acid
2-iP	isopentenyl-adenine
ARF	Auxin response factor
ARP	Asymmetric rough phantastica
AS2	Asymmetric leaves2
BAP	Benzylamino purine
Bp	Base pairs
CIM	Callus induction media
CIN	Cinninata
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeat
CUC	Cup shaped cotyledon
DKW	Driver and Kuniyuki woody plant medium
DPA	Development PcG Apex
DSB	Double stranded break
GIF1	Angustifolia interacting factor
GRF5	Growth regulating factor5

LIST OF ABBREVIATIONS (continued)

<u>Abbreviation</u>	<u>Definition</u>
gRNA	Guide Ribonucleic acid
HDR	Homology directed repair
IBA	Indole-3-butyric acid
Kanadi	KAN
kinetin	furfurylamino purine
Knotted-1	Kn1
LB	Lysogeny broth
LGN-R	Liguleless narrow-reference
MAW	Maewest
MH	Schenk and Hildebrandt
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NOA	Naphthoxy acetic acid
ns	Narrow sheath
OD	Optical density

LIST OF ABBREVIATIONS (continued)

<u>Abbreviation</u>	<u>Definition</u>
PAM	Protospacer adjacent motif
p-CPA	Para-chllorophenoxyacetic acid
PCR	Polymerase chain reaction
PGRs	Plant growth hormones
PIN-1	Pin formed-1
PRS	Pressed flower
RDR6	RNA-dependent RNA polymerase6
RE	Restriction enzyme
rpm	Rounds per minute
SIM	Shoot induction media
SNP	Single nucleotide polymorphism
Temp.	Temperature
VW	Vacin and Went
WPM	Woody plant medium
YAB	Yabby
YUC	Yucca

1. INTRODUCTION

The area of biological research has expanded immensely with the new advances in technology, inventions, and discoveries. Various fields of biological research have gotten faster, cheaper, and more reliable. In this era of ever-changing environment, increased demand of food, diseases, and the struggle to survive, CRISPR-Cas finds quite a lot of applications in research (Noman et al., 2016). There have been many areas of research where scientists had restrictions in the past, but now with this new tool, the potential of genome editing in humans, plants, and animals has been greatly accelerated (Li et al., 2019; Wang, 2020). Because of its excellent precision and accuracy CRISPR-Cas has found to be extensively used in many aspects of genome editing. Disease resistance, gene knockout, trait enhancement, drought resistance, stress resistance, causing and curing mutations, curing genetic disorders, producing desired characters and many other such goals can be achieved by CRISPR-Cas application. It has given scientist the confidence to provide rapid solutions to the modern problems (Mao et al., 2013; Ma et al., 2016).

In explanation CRISPR can be too complex or too simple depending on the details provided. As mentioned before, CRISPR was first studied in *E. coli*. There are two main segments found in the CRISPR; first, there are the repeats, these are short sections of DNA which are between 20 to 30 nucleotides in length which are called palindromes. A palindrome is a chain of nucleotides that repeat the same left to right on both strands of DNA. These palindromes are identical and in between there are DNA pieces called “spacer DNA” (Semenova et al., 2011). The spacer DNA has been interesting because it is not homogeneous, and each portion of spacer DNA is unrepeatd (Garneau et al., 2010). Later scientists discovered that the spacer DNA is perfectly similar with viral DNA and mainly with the DNA of a bacteriophage (viruses that attach bacteria) (Bolotin et al., 2005). They also identified several genes linked with CRISPR, so these are the CRISPR-linked genes (also called cas-genes). Helicases are the cas-proteins which are encoded by these cas-genes. These proteins uncoil and cut the DNA. So, the idea is that this is essentially an immune system of bacterial organisms, a defensive strategy to fight the bacteriophages (van der Oost et al., 2014). So, when a bacteriophage inserts its

DNA into *E. coli* normally it should take over the cell mechanisms and kill it. However, since the cell has CRISPR system, the cas-proteins are encoded and the CRISPR RNA is transcribed. The cell deactivates viral DNA before it starts the infection (Rollie et al., 2015). If a new virus attacks the bacteria, the bacteria encodes another type of protein called cas1. This cas1 takes the viral DNA, breaks it, and more importantly copies it to the CRISPR system of the bacterial genome as another spacer DNA. So, all these spacer DNAs are essentially the history of old viral infections (Fagerlund et al., 2017).

Scientists worked the same way and used CRISPR to cause precise mutation, knock-out or to insert a gene. This CRISPR system was first discovered in the laboratory of J. Doudna and E. Charpentier when they were studying *Streptococcus pyogenes*. They created a CRISPR-cas9 system with their own guide RNA (gRNA) specific to the part of DNA of their interest (Doudna et Charpentier, 2014). The gRNA has two parts, 1st is the CRISPR part which has the RNA direction of where to make the cut and 2nd is the protein which makes the cuts in the DNA (Jinek et al., 2012). There is also another sequence called protospacer adjacent motif (PAM) which helps protein to find exact position on the DNA where it is designed to cut. PAM is always next to the targeted sequence. It consists of a short sequence of 3 or more nucleotides and it often starts as NGG, NRG, NGCG (and there are many other types of PAM) where N can be any nucleotide (Kleinstiver et al., 2015). The guide RNA scans the whole DNA strand and once it finds the matching PAM sequence it makes a double stranded cut into the DNA (Jinek et al., 2012). So, once the cut is made, the DNA breaks apart and the cell tries to repair it which causes mutations. So, this way a gene can be silenced/knocked-out (Schiml et al., 2014). The other way around is when a gene is to be added to a DNA strand. In that case a host nucleotide sequence is provided to cell which fits in a gene is inserted (Ding et al., 2016; Song et al., 2016b).

Figure 1.1 explains that the gRNA along with Cas9 sits on the target DNA at the PAM site and cuts the DNA into a double stranded break (DSB). This break is then restored by the cell in two ways. In case of gene knock-out, the cell repairs

it by non-homologous end joining (NHEJ) method by introducing indels (Kleinstiver et al., 2015).

While in case of gene knock-in the introducing DNA donor template is provided to the cell and is repaired by homology directed repair (HDR / HR repair) method. Both methods are used among scientific community for various goals of gene knock-out or gene insertion (Kleinstiver et al., 2015; Ding et al., 2016).

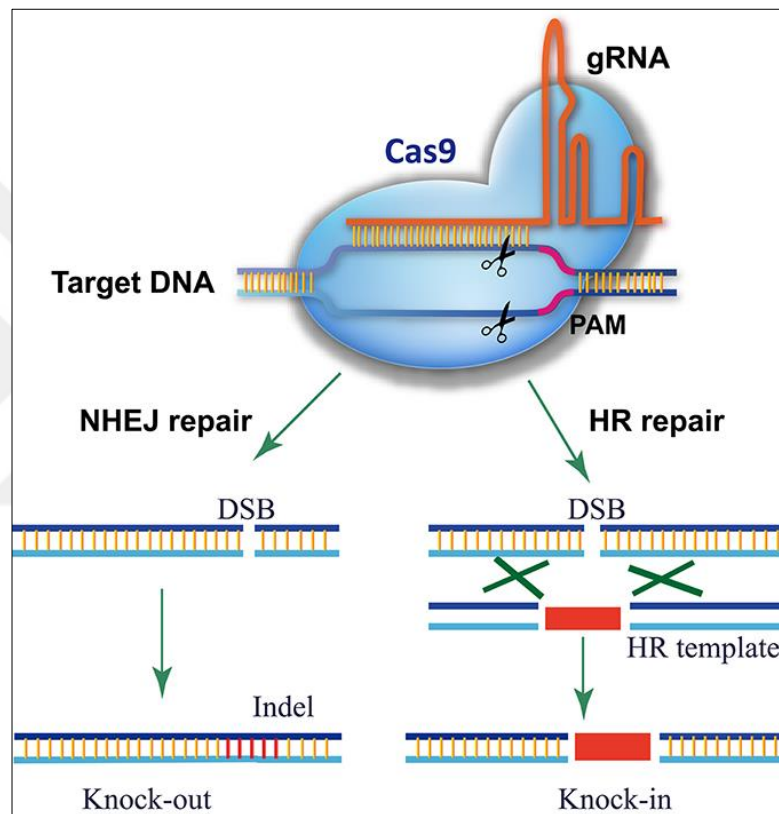


Figure 1.1. Visual demonstration of working of Cas9. Source: (Ding et al., 2016).

Leaf shape has always been an important aspect in plant science. In many crops leaf shape holds economical value, ornamental essence and/or just the visual appearance of the leaf is undoubtedly an easier way to identify plants. The mechanism of defining leaf shape is still not well known. The available literature and research suggest that leaf shape is relatively a very complex trait and it has different controlling factors among different groups of plants (Tsukaya, 2006). Figure 1.2 is an example of leaf shapes present in different groups of plants. The

shape of the leaf can be modified by natural mutation, evolution, or it can also be done by artificial means i.e. genetic mutation, controlled breeding or even by changing the growth environment of plant (Mohan Jain, 2006). Modifying leaf shape is interesting for scientists as well as growers to take economic benefit, make plant visually more appealing, make plants better adept according to climate, or for scientific research to understand the mechanisms (Lowe, 2017).



Figure 1.2. Different forms of leaf shape among different plants. Source: (Hines, 2017).

The history of Petunia traces back to the early years of 19th century. It belongs to the family Solanaceae the same family of Tomato and Tobacco. The most famous of it is *Petunia hybrida* which resulted in a cross between *P. integrifolia* and *P. axillaris*. It is well known as a bedding plant is famous for its flowering colors (Bombarely et al., 2016). There has been quite a lot of research on petunia flowers and it has been produced in many colors now. Flowers are shaped like trumpet. The leaves of petunia are oval shaped and have smooth margins. The size of leaf in an adult petunia plant range from 5cm to 10cm. The leaves lack a central petiole therefore leaves are sessile (Peter, 2015). The appearance of the leaves seems rough, not like having a waxy surface. Figure 1.2 shows different available colors of Petunia and the leaf shape.

Since Petunia is a potential candidate for being used as a model plant. There is less data available on the genetics of petunia controlling leaf shape and so this is interesting to discover which are the factors that determine the leaf shape in petunia. (Vandenbussche et al., 2016).

As Plant / leaf / flower / fruit shape plays a key role in ornamentals. Many techniques have been developed to alter these characteristics. Some of these techniques are related to conventional breeding while others include molecular tools in plant biology. Considering these possibilities, genetic modifications can be of great use to develop new aesthetically appealing traits for ornamental plants. In this experiment essential leaf shape genes were targeted in Petunia, by transient expression of CRISPR/Cas9 endonuclease, and consequently plants with different leaf shapes will be created. A precise phenotyping will not only provide a measure of diversity among various leaf forms but will also signify the role of genes controlling leaf shape formation. Leaf shape consists of a unique order of cell multiplication and cell enlargement which requires different levels of genetic synchronization. With classical genetics, several leaf shape genes have been recognized, especially in *Arabidopsis thaliana*. With the recent advances in genome editing, there are handful of possibilities to create ornamentals with novel traits such as leaf shape. Literature review allowed to choose target gene(s) with significant effect on controlling leaf shape. Sequence of those genes was determined, and CRISPR/Cas constructs was designed accordingly. The main goal is to knock-out / modify those gene(s). Such genetic transformation of Petunia will give a proof of concept and will lay down the basis for gene editing in ornamental plants and will also be useful for plant breeders to breed plants with more economic value.

2. LITERATURE REVIEW

2.1 History of CRISPR

The system of CRISPR-Cas had been discovered in *Escherichia coli* in early 1987 while analyzing its DNA (Ishino et al., 2018). Biological life is characterized by biomolecules which are based on chain of nucleic bases called DNA. These DNA structures define the core biological attributes and features of an organism. DNA is transcribed and translated to make proteins which are involved in almost all the cellular processes. Changes in genomic sequences can be positive or negative and can occur naturally or can be caused artificially (Singh et al., 2016). These CRISPR regions were soon discovered in many other organisms i.e. bacteria and archaea, defined as their defense mechanism (Han et She, 2017). The CRISPR regions did not catch the attention of scientific community until recent 5 to 10 years when it came out to be a revolutionary technology of precise site-specific genome editing (Han et She, 2017; Ishino et al., 2018). Since then, this novel technology has gained interest by both manufacturers and scholars (Sherkow, 2015). It is very simple, accurate, always expanding technique that is used these days for genomic alterations in both animals and plants (Song et al., 2016a). Genomic alterations include gene knockout, gene insertions, mutation, gene multiplication and CRISPR proves to be an effective tool for these purposes (Endo et al., 2015).

2.2 CRISPR Under Controversies

However, with its great accuracy, lower cost and precise working CRISPR has also been under the discussion either its GMO or what are the ethical challenges (Polcz et Lewis, 2016). CRISPR on one hand has been proving to be quite helpful in solving food and medicinal problems but on the other hand just like any new scientific innovation; CRISPR is also under major concern of scientific community. The main concern here arises that previous genome editing practices were trackable and could be distinguished (Butler, 2016). But because CRISPR can make changes even in few nucleotides, it looks like natural mutation and around the globe food regulation authorities have shown agitation regarding

this (Ledford, 2019). As in Europe, GMOs are defined as any organism either plant or animal in which the DNA is manipulated in such a way this change in the genetic material of the organism does not occur by nature (Plan et al., 2010). GMOs are banned in Europe and here is the major challenge for food regulation authorities in Europe of how they would identify if a change in an organism is natural or caused by CRISPR. On other hand in countries like USA, GMOs are allowed but their worry is about the unethical uses of CRISPR (Shew et al., 2018). A clear example comes from the usage of CRISPR in altering human genome in China and their research was not accepted for publication by Nature and other major journals (Jiang et Stevens, 2015). Despite being helpful CRIPSR also poses major threats to the ethical values of various countries, people, cultures, and beliefs. The story from China is a good example of this because people from many different backgrounds do not appreciate using CRISPR on humans. However, these controversies are mainly about animals and humans, using CRISPR in plants is still not a big issue (Hefferon and Herring, 2017; Benston, 2017).

2.3 Model Plant

Plant scientists focused on model plants as it becomes easier for research specially for molecular research. For a long period of time Arabidopsis has been model plant because of its small genome, early maturity, short life cycle and easy transformation. Various experiments have been conducted on this plant (Meinke et al., 1998; Koornneef and Meinke, 2010). In the past when sequencing was relatively expensive, there were not many options to look for other mother plants. Arabidopsis was then everyone's choice because of its above described characteristics. Arabidopsis has also been a good host for the study of functional genomics, genetics interactions, mutations and over all genetic studies (Meinke et al., 1998; Cheng et al., 2017). With the recent advancements in bioinformatics, Arabidopsis has been adapted for modern research. However, since the Next Generation Sequencing (NGS) has become cheaper and other technologies have also become easily available even for individual teams of researchers, scientists have become confident for other model plants (Goodwin et al., 2016). Petunia is an interesting case in this discussion because of its various characteristics and it has the potential to be a model plant (van der Krol and Immink, 2016).

2.4 Introduction of Petunia / Importance of Petunia / Petunia as Model Plant

The growth of Petunia is fast, and it can give almost 4 generations in a year which is very suitable for the laboratory research. Petunia grows very well at a slightly higher temperature range of 23-35 °C under long day conditions of 16h day light. Cold temperature might cause delay in flowering. The plant adjusts easily to the growth medium and is quite easy to grow in different types of growth medias (Deborah and Kristine, 2018). Multiplying this plant is also an easy task as it can be multiplied either by seeds or even by asexual means. The seeds of this plant prefer well-drained sandy. The pH range of 6.0-7.5 is preferred (Marie and Julie, 2020). The plant is cross-pollinating as well as self-pollinating. So, it is easy to pollinate this plant and to get seeds from it. Asexually, the plant can easily be reproduced by cuttings and this is extensively done in horticulture industry. This is very important to conserve the genetic structure of the plant and to obtain plant with same characteristics generation after generation. In the laboratory, this plant can be sub-cultured either by stem cutting or by leaf cuttings. The plant regrows by forming callus tissues (Izhar and Zelcer, 1984). Petunia also finds equal importance in laboratory research while dealing with genomics and genetic modifications. Moreover, the genome of petunia has also been published at Solgenomics (https://solgenomics.net/organism/Petunia_inflata/genome) (Knapp and Zamir, 2016). This plant is very easy for the transformation. Agrobacterium mediated transformation has been conducted in many plants and petunia is one of the examples. So, the ease of multiplication, quick growth, easy transformation, sequenced genome and short life cycle makes petunia a good candidate for being a model plant (Vandenbussche et al., 2016).

2.5 Leaf Shape

There are roughly 310,000 – 420,000 species of the plants which are known to science and have been given scientific names. With this huge diversity of plants, leaf shape is probably the first visual element to recognize a plant species (Du et al., 2007). The leaf shape is essentially one of the most important features for a plant for its overall growth success. These huge number of plant species have

been divided in different divisions based on their characteristics. The first ones are called Bryophytes, which are simple plants with simple leaves. They do not have vascular transport system. They do not produce flowers and do not have lignin in their cell walls. These are important in maintaining the soil structure and avoid soil erosion (Vanderpoorten and Goffinet, 2009). The second division is called Pteridophytes. These have real compound leaves and show active vascular system. They have sporangia for reproduction but do not produce flowers (Mirek et al., 2002). The next division is called Spermatophyta. These have green photosynthetic leaves and this division is divided into two major subdivisions called angiosperms and gymnosperms (Rothwell and Serbet, 1994). Gymnosperms show needle shaped leaves which are thick, have waxy coating and have submerged stomata. They produce seeds but no fruit (Chamberlain, 1935). The last and the most advanced of all the divisions is the angiosperms. Angiosperms show mainly two types of leaves i.e. monocotyledonous and dicotyledonous. Monocots have long and thin leaves showing parallel leaf veins while dicots show branched structure of leaf veins. Angiosperms produce seeds in an enclosed fruit (Eames, 1961). Among this whole classification and grouping based on leaf shape, petunia falls under the category of dicot angiosperms. Because of its flower production, branchy leaf venation, fruit and seed formation (Sink, 2013).

2.6 Factors Affecting Leaf Shape

Just like any other physical feature of living organisms, leaf shape is also mainly defined by the genetic make-up of an organism. The different kinds of leaf shapes have been developed by the plants over the course of evolution. The major reason behind this change of leaf shape over the course of evolution is to be able to better adjust in the environment and this is mainly a survival change adopted by the plants (Tsukaya, 2018). However, although genetics of a plant describes major feature of leaf shape of a plant, it is not the only parameter to decide leaf shape. There are many other factors that alter the leaf shape (Tsukaya, 2006). An increase or decrease in light can cause the plant to develop larger or smaller leaves (depending on the species) (Njoku, 1956). Similarly, temperature plays very important role in the morphogenesis of leaf shape as the plant may require more

or a smaller number of stomata according to the temperature (Njoku, 1957). Leaf shape is also affected by the nutrition of the plant. If the plant is under drought stress, salinity stress, disease attack or if some essential nutrient(s) is/are less available to plant, the plant may respond to the stress and it may cause a change in the leaf shape. The change in leaf shape due to such stresses is not huge and the leaf shape still more or less remains same as the original one (Farris, 1984; Brodribb and Holbrook, 2005). Genetic mutations can also be a cause in the change of the leaf shape. This kind of change depends on the type of mutation and can be quite a lot different or can cause just minor change. Inter and intra-species crosses can also develop new leaf shapes. Plant breeders have created huge number of ornamental plants by doing such changes and making the plants visually more attractive (Ashby, 1948).

2.7 Genetics of Leaf Shape

The major contribution factor to the shape of the leaf is a plant species is certainly its genetics. The genetics that is preserved by the plant generation after generation and which gives a plant its identity. There are several genes which are involved in controlling the morphogenesis of leaf shape. By studying the genetics of leaf shape it can be said that determining the leaf shape is not a simple trait but rather it is very complex and as discussed above, it is dependent on quite a lot of number of factors (Chitwood et al., 2014). Considering the genetics of plants, Cup shaped cotyledon (CUC) 1, 2 and 3 genes play an predominant role in defining the leaf margins in *Arabidopsis*. The expression of CUC2 effectively makes the leaf margins serrated. On the other hand, the expression of a microRNA called miR164 works antagonistically of CUC2. If the miR164 is expressed too much, it makes the leaf margins smooth. The equilibrium among the expression of both genes determines the final form factor of the leaves in *Arabidopsis* (Nikovics et al., 2006). Some other genes i.e. Pin formed-1 (PIN1), Asymmetric rough phantastica (ARP), Clavata (CLC), Wuschel (WUS) genes are found to be involved in controlling the leaf initiation in different plant species. PIN1 is found to control auxin efflux and ARP influences the differentiation of stem cells (Okada et al., 1991; Waites et al., 1998). CLC helps the plant in maintaining the cell size and WUS manages the identity of the floral and shoot meristem (Clark et

al., 1995; Laux et al., 1996). In *Zea mays* proximodistal leaf outgrowth is being controlled by Liguleless narrow-reference (LGN-R) and Knotted1 (Kn1) genes. Here LGN-R plays its role to create the boundary of the sheath and Kn1 helps the plant to maintain the identity of the stem cells (Ramirez et al., 2009; Moon et al., 2013). On the other hand, the dorsoventral leaf outgrowth in *Arabidopsis* is influenced by gene like RNA-dependent RNA polymerase6 (RDR6 / DCL4), Auxin response factor (ARF), Kanadi (KAN), Asymmetric leaves2 (AS2) etc. DCL4, ARF and KAN control the polarity specifications of the leaf (Eshed et al., 2001; Pekker et al., 2005; Fahlgren et al., 2006) while AS2 plays its role in maintaining the patterns of venation and the development of the lamina (Xu et al., 2003). The Maewest (MAW) gene in *Petunia x hybrida* is responsible for the fusion of the organ and lateral expansion (Vandenbussche et al., 2009). In *Arabidopsis*, the mediolateral outgrowth patterning of the leaf is controlled by Yucca (YUC), Narrow sheath (ns), Yabby (YAB), Pressed flower (PRS), genes. YUC controls the vascular development and the patterning of the flower (Cheng et al., 2006). The expansion of the leaf is being controlled by ns gene, while the expansion of the lamina and specification of the polarity of the leaf is controlled by YAB gene (Kumaran et al., 2002; Nardmann et al., 2004). PRS gene mainly deals with the proliferations of the margin cells (Matsumoto and Okada, 2001). The expansion of the leaf and the maturation of the leaf is controlled by Cincinnata (CIN) (Nath et al., 2003), Angustifolia interacting factor (GIF1) and Growth regulating factor5 (GRF5) genes in *Arabidopsis*. All these genes play their role in the proliferation and expansion of the cells (Kim et Kende, 2004; Horiguchi et al., 2005). The margins of the leaf, whether it would be lobbed, serrated or smooth is controlled by Development PcG Apex (DPA) and Pin-formed1 (PIN1) genes. The DPA deals with the initiation and the development of the organs and leaf while the PIN1 controls the auxin efflux in *arabidopsis* (Hay et al., 2006; Julia et al., 2012). These evidences come from different studies and in different plants. The expression of these genes highly fluctuates among species and so to see the effect of a specific gene in a plant, a specific study must be conducted.

2.8 Genetics of Leaf Shape in Petunia

As discussed above that petunia has the potential to be a model plant. There is a lot of genetic data available related to different traits of the plant. Anyhow, it is almost impossible to completely study an organism. Many researches are still working on Arabidopsis and are making new discoveries. This opens a vast opportunity of research and findings for petunia plant. A lot of literature has been cited about the flowers of petunia i.e. flower shape, color, size etc. However, leaf shape of the petunia has not been studied much since it does not contribute to the market value of the plant so genetics of the leaf shape of petunia finds a great research potential (Vandenbussche et al., 2016). The leaves of petunia are oval shaped with smooth margins and no waxy appearance. To look for the genes controlling the leaf shape in petunia, the genes in closely related relatives such as tobacco and tomato were studied. Knotted1 (Kn1) which is a homeobox gene controls several different traits and is influential on the leaf shape. It was studied that under-expression of this gene in Tomato disturbed the normal phenotype of leaf and several different phenotypes were produced. Moreover, in the same study, it showed different results in Arabidopsis (Hareven et al., 1996). In another study in Tobacco plant it was found that over-expression of the same gene group deformed the leaf shape (Nishimura et al., 2000). In another study the Kn1 gene was over expressed in tobacco to observe its effects on the senescence of the leaves. It was found that the over expression delayed the senescence time and proved that these homeobox genes play important role in the leaf morphology (Ori et al., 1999). In another study the expression of Kn1 was compared among the leaves of tomato and pea. The results showed varying results suggesting that the genes control different aspects of leaf growth in both species (Hofer et al., 2001). Another study on maize suggests that Kn1 homeobox may help the researchers to further understand the mechanism of cell division control as well as a dominant mutation in Kn1 showed its role in controlling the cells in the veins of the leaves (Vollbrecht et al., 1991; Smith et al., 1992). As a summary of the above examples, another study discusses about the role of Kn1 gene in the determination of the proximal end of the leaf (Bolduc et al., 2012). From these studies, it can be concluded that the disruption of the gene produces different phenotypical traits depending upon the species and the nature of the disruption.

2.9 Plant Tissue Culture Basics

The asexual propagation is interesting for researchers as well as for growers since it costs less, its quick, it maintains the genetic make-up and a huge number of plants can be produced in short time. This has resulted in development of many asexual propagation techniques i.e. cuttings, layering, budding, and grafting (Bidhan and Krishi, 2013). However, these techniques are more used in gardening, horticultural lands, plant breeding and other field related activities. In laboratories, maintaining a sterile plant culture is very important for the growers as these plants are subjected to various research experiments and any contaminant may interfere in the authenticity of the results or it may fail the experiment. The plant tissue culture lab must have growth containers (glassware, plasticware etc.), place to prepare, sterilize and store the media, growth chambers (controlled environment of light, intensity, temperature, and humidity), and other observatory facilities. The general procedure of producing a plant in tissue culture involves preparing growth media in growth container and sterilizing it, after solidification of the media transferring the plants into the media in a laminar flow to lower the risk of contamination. Using sterile forceps and scalpels are always advised. Closing the lid of the container properly and then putting it in a growth chamber according to the light, humidity, and temperature requirements of the plant (Bhojwani and Razdan, 1986). Plant hormones may and may not be added to the media (it is dependent on the purpose of the tissue culture and the plant which is grown). The three major hormones which are being used are Auxins, Cytokinins and Gibberellins. Auxins are normally responsible for the apical dominance, abscission, elongation of the internodes, rooting and tropism etc. For the *in-vitro* culture auxins are used for differentiation of the root cells and for the cell division. Most used auxins are Indole-3-butyric acid (IBA), Dichlorophenoxyacetic acid (2,4-D), Naphthoxy acetic acid (NOA), Naphthaleneacetic acid (NAA), Para-chlorophenoxyacetic acid (p-CPA), Trichlorophenoxyacetic acid (2,4,5-T) (Yu et al., 2017). Cytokinins control shoot differentiation, apical dominance modification, division of the cells etc. For the *in-vitro* culture it is used for the induction of differentiation and division of the cells of the callus. It is also used for the proliferation of the shoot. Most used cytokinins are isopentenyl-adenine (2-ip), Benzylamino purine (BAP) and furfurylamino purine (kinetin)

(Mok, 2019). Gibberellins are used rarely and there are more than 20 different kind of gibberellins. The most common one is GA₃ (Rizza et al., 2017).

A variety of growth medias have been used over the years for different plants under different conditions. The Murashige and Skoog (MS) media has been widely used and it is the most common growth media (Murashige and Skoog, 1962). Other than MS, there are many other growth medias including, SH media (Schenk and Hildebrandt) (Schenk and Hildebrandt, 1972), MMS (Modified MS), DKW (Driver and Kuniyuki woody plant medium) (DRIVER et KUNIYUKI, 1984), WPM (Woody plant medium) (Lloyd and McCown, 1980), B5 medium, VW (Vacin and Went) media (Vacin and Went, 1949) etc. The composition of some of these important medias is available in Table 2.1 (Phillips and Garda, 2019). These different kinds of media are mainly providing different nutrients to the plant. The media provides macronutrients which include Carbon, hydrogen and Oxygen as well as Potassium, Calcium, Nitrogen, Sulphur, Phosphorus etc. In micronutrients it provides Copper, Zinc, Iron, Manganese, Molybdenum, Iodine, Chlorine, Cobalt etc. The media also delivers essential amino acids, vitamins and other organic and inorganic compounds to the plant (I.M. et M., 2012).

A solidifying agent is another important constituent of the media. There are many solidifying agents available such as agar, gellan gum, agarose, micro agarose, gelatin etc. All the gelling agents come from different sources. Agar out of all the gelling agents is most used and it is obtained from seaweeds. Chemically agar is a polysaccharide. Agar is better in use compared to the other agents as it is easily dissolved in water, it gets solidified at 45 °C, the melting temperature ranges from 60-100 °C. Agar does not react with other substances added to the gel. Moreover, agar is stable under almost all the growth conditions and is not digested by the plant enzymes. So, these characteristics make agar much more useful and a main choice for plant growth media solidifying agent (Debergh, 1983). While discussing all these constituents of a growth media, pH is also of great importance. It provides stability to the nutrients, gel and helps the plant to grow in an optimum environment. It is adjusted before the sterilization of the media. Normally pH of a media ranges from 5.0 to 6.0 but it may vary in certain cases (George et al., 2008).

Table 2.1. Composition of growth medias

Basal medium	B5	MS	MMS	WPM	DKW
Vitamins and organics (mg L ⁻¹)					
Myo-inositol	100	100	100	100	100
Nicotinic acid	1	0.5	1	0.5	1
Pyridoxine HCl	1	0.5	1	–	0.5
Thiamine HCl	10	0.1	10	1.6	2
Glycine	–	2	–	–	20
L-Glutamine	–	–	–	–	250
Sucrose (g/L)	20	30	30	20	30
pH	5.5	5.8	5.8	5.6	5.5
Macronutrient components (mg L ⁻¹)					
KNO ₃	2500	1900	950	–	–
K ₂ SO ₄	–	–	–	990	1559
NH ₄ NO ₃	–	1650	825	400	1416
Ca (NO ₃) ₂ ·4H ₂ O	–	–	–	556	1948
NaH ₂ PO ₄ ·H ₂ O	150	–	–	–	–
(NH ₄) ₂ SO ₄	134	–	–	–	–
MgSO ₄ ·7H ₂ O	250	370	185	370	740
KH ₂ PO ₄	–	170	85	170	265
CaCl ₂ ·2H ₂ O	150	440	220	96	149
Macronutrient components (mg L ⁻¹)					
H ₃ BO ₃	3	6.2	6.2	6.2	4.8
KI	0.75	0.83	0.83	–	–
MnSO ₄ ·H ₂ O	10	16.9	16.9	22.3	33.5
ZnSO ₄ ·7H ₂ O	2	10.6	10.6	8.6	–
Zn (NO ₃) ₂ ·6H ₂ O	–	–	–	–	17
CuSO ₄ ·5H ₂ O	0.04	0.03	0.03	0.25	0.25
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25	0.25	0.39
CoCl ₂ ·6H ₂ O	0.03	0.03	0.03	–	–
NiSO ₄ ·6H ₂ O	–	–	–	–	0.01
FeSO ₄ ·7H ₂ O	27.8	27.8	27.8	27.8	33.8
Na ₂ EDTA	37.3	37.3	37.3	37.3	45.4

A fully grown plant has almost all its cells in specialized form. Which means each cell has grown into performing a specific function. This is a typical growth behavior of complex organisms that their cells grow and specialize into performing specific tasks. This is known as cell differentiation (Glover, 2000). When talking about tissue culture, there are two ways, first is to regenerate the plant by its shoot while second is called leaf initiation where a new plant is developed from a small section of the leaf. The first type of growth is easier as the plant's shoot has buds and it regrows through these buds forming a new plant again. On the other hand, the second case is different as it requires the cells of the leaf section to become least specialized and behave as meristematic cell.

Meristematic cells are the differentiated cells in the plant. Turning differentiated cells into least specialized cells is called dedifferentiation (Grafi, 2004). This process requires a balanced amount of PGRs (Plant growth hormones). These PGRs are provided to the plant from the growth media and the cells are directed to develop into roots or shoots depending on the purpose of the experiment and type of the hormones used. If it happens successfully, the plant produces callus and then it starts to grow into normal plant (Trigiano and Gray, 2004).

2.10 Agrobacterium Mediated Transformation and Regeneration in Petunia

Genetic transformations gained interest of scientists after the discovery *Agrobacterium tumefaciens* which a bacteria with an ability to transfer its vector to the plants cells (Fraley et al., 1983). Since petunia finds huge potential in becoming a model plant, it is also because it grows very well in variety of growth medias. It is very easy to transform by agrobacterium and regeneration can be carried out easily as well. An agrobacterium mediated transformation means that a gene of interest is isolated from the source plant, it is inserted in a vector which has the promoter so that the gene can be transcribed in the plant (Science, 1985). The vector is then inserted into a bacterium i.e. *Agrobacterium* by using different transformation methods i.e. heat shock, electroporation etc. The bacteria containing the transgenic vector are then allowed to divide in a growth media (LB media) (Gelvin, 2003). Then, the leaf sections are treated with this bacterial media for a while allowing the bacteria to transfer the vector to the leaf cells and then leaf sections are then grown on a co-culture medium in dark allowing the bacteria to transfer the vector to plant cells. The leaves are then transferred to another media which has antibiotic so that no bacteria grow after this stage and the transformed leaves stay sterile (Jones et al., 2005). The leaves are then grown like normal *in-vitro* plants and the gene transfer is observed later when the plants grow up.

Petunia performs very well in genetic transformation experiments and so it is an interesting plant for researchers for studying gene functions, discovering new genes, and dealing with much more complex genetic traits. The first case of

petunia transformation was recorded by (Horsch and Klee, 1986) and (Janssen and Gardner, 1990). It is notable that different cultivars of petunia perform differently in a regeneration experiment. Some grow very well while some show poor growth (Mitchell et al., 1980). Conner et al., (2009) well explains the method of transient gene expression and gene transfer by agrobacterium to Petunia.

2.11 Transient Gene Expression

While the gene transfer by agrobacterium is efficient, if the procedure of co-culture growth is followed, it takes a while to observe the results and to evaluate the success rate. However, transient expression of a gene into a plant is a rapid, easy, and cheaper way to check the construct of a gene. It is also called leaf infiltration. In this method, the gene construct is directly injected into the leaves and flowers of the plant and in a few days, the results can be evaluated. This method was very well performed on petunia to see the transient gene expression by agrobacterium (Shang et al., 2007). The Figure 2.1 (Nekrasov et al., 2013) shows the method of injecting the agrobacterium into the leaf of petunia.

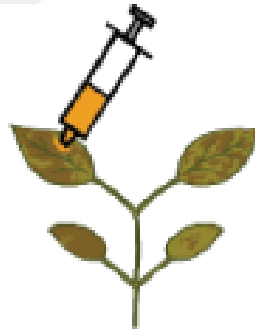


Figure 2.1. Agrobacterium infiltration.

3. MATERIALS

3.1 Trial Year and Place

The research project was conducted at Laboratory of *in-vitro* plant biotechnology at Bioscience engineering department of Ghent University, Ghent, Belgium. It was collaborated research between Laboratory of *in-vitro* plant biotechnology and Vlaams Instituut voor Biotechnologie (VIB) at Ghent, Belgium. The start date of the project was 2nd February 2020 till 31st July 2020.

3.2 Environment of the Place of Experiment

The whole experiment was conducted in a controlled environment in the lab. All the necessary equipment required for the experiment were available at the lab. For the tissue culture of petunia laminar flow, autoclave, glassware and plastic ware, chemicals for the media preparation, growth chamber, physical balance, pH meter, Umonium (and/or alcohol), fluorescence microscope were available. For the molecular work PCR machine, centrifuge, electrophoresis, UV transilluminator, DNA isolation kit, pipettes, -4 °C and -20 °C storage, chemicals for the molecular analysis, agrobacterium, cold plate, were available.

3.3 Plant Material, Chemicals, Kits, and Services Used

The petunia plant used for the experiment was taken from the *in-vitro* growth chamber of the Laboratory of *in-vitro* plant biotechnology. It was *Petunia hybrida* and was F6th generation of the cross. For the regeneration of the plant MS media was used in glass jars with little opening to allow gaseous exchange. For all the regeneration experiments, sterile environment was maintained, and plants were grown in growth chamber with 80% red and 20% blue light at temperature of 26 °C with 16h day and 8h night period.

The DNA isolation was carried out by using “[Stratec Molecular](#)” kit. For the PCR reactions the PCR buffer, dNTPS and Taq polymerase, loading dye was used by “[Promega](#)” company. The kit to isolate DNA from the gel was also used from

“[Promega DNA excision kit](#)”. The TAE buffer for gel preparation was prepared in the lab according to the following concentrations:

Table 3.1. Composition of the TAE Buffer

Chemical name	Concentration	Amount (for 10X concentration)
Tris	400 mM	24.2 g
Na ₂ EDTA.2H ₂ O	20 mM	3.7 g
NaAc.3H ₂ O	200 mM	13.6 g
Hac	296 mM	8.5 g
H ₂ O		Raise the final volume to 500 ml
pH of the final solution		Approx. 8.2

To clone the PCR products “[CloneJET PCR Cloning Kit](#)” was used from [ThermoFisher Scientific](#) company. The molecular markers were ordered from [IDT Technologies](#). For the sequencing of the genomic samples were sent to [LGC Biosearch Technologies](#) and [Eurofins Genomics](#). For the analysis of the genomic sequences [UGene software](#), [NCBI genomic tools](#), [Batchprimer3](#), [Clustal omega](#) were used.

4. METHOD

The whole project was divided into several different tasks and was carried out side by side to have good results and to perform the activities in-time. The division of the project is as follows:

- **Tissue culture of Petunia**
 - Shoot regeneration
 - Leaf regeneration
 - Agrobacterium Transformation for GFP Expression
 - Leaf Infiltration for GFP expression
- **CRISPR-Cas9 Protocol**
 - Identification the target gene
 - Designing molecular markers
 - Designing the gRNAs for infiltration protocol
 - PCR Reactions for DNA Fragment Amplification
 - PCR optimization for MG1 and MG2 primer pairs
 - PCR optimization for Kng11, Kng12 and Kng2 primer pairs
 - Gel Electrophoresis
 - DNA Excision from Gel
 - Sequencing of the amplified genomic fragments
 - PCR Cloning

4.1 Tissue Culture of Petunia

The petunia which was already available at the lab was taken and was used for multiplying to have more number of plants for the experiment. For the multiplication, shoot regeneration was performed first.

4.1.1 Shoot regeneration of petunia

The regeneration media was prepared according to the general media composition of media being followed in the lab. According to the composition, following ingredients were used:

Table 4.1. Ingredients for the shoot regeneration of petunia

Ingredient name	Amount (for 1 liter of media)
Distilled water	1025 ml
MS	4.405 g
Plant Agar	7 g
Sucrose	30 g
pH	5.8

1st Shoot Regeneration: Distilled water was taken in a container with a magnetic stirrer in it. MS media and sucrose were added and dissolved. The pH was raised to 5.8 by adding KOH (1M) drop by drop. After Adding these chemicals and adjusting the pH, the media was transferred to a stove and heated. While heating the media, plant agar was added, and the media was heated until it became clear (indication that plant agar was dissolved properly). 20 glass jars were prepared and 100ml media per jar was poured into each jar (Figure 4.1). Jars were autoclaved for 15 minutes at 120 °C. The jars were kept aside to cool down till the media became solid. 13 parent plants were taken from the growth chamber and in the laminar flow parent plants were taken out one by one. The leaves were removed, and branches of the parent plant were cut into small pieces. It was taken into consideration that each piece of shoot contains at least 2-3 buds so that the plant can regenerate. The size of these pieces ranged from 1cm to 3cm. 6 cuttings of shoot were placed into each jar.



Figure 4.1. Filling of the jars with MS media.

The jars were then closed and wrapped into wrapping paper. Each jar was tagged with the initials of the person who performed the experiment, the date of experiment, the media code, and the code of the variety. All the jars were placed in the growth chamber at 26 °C in 80% red and 20% blue light (Figure 4.2).

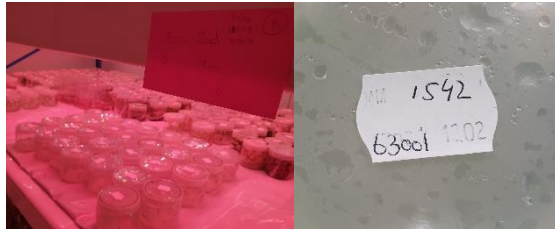


Figure 4.2. Tagging and placing the jars in growth chamber.

2nd, 3rd, and 4th Shoot regeneration: After almost 3 months of the 1st shoot regeneration, 2nd shoot regeneration was performed to further multiply the plants in 20 jars with 6 plants in each jar. After almost 2 months, 3rd shoot regeneration was performed with 20 jars and 6 plants in each jar, and the problem of hyperhidrosis was observed. Due to the problem of hyperhidrosis the 4th shoot regeneration was performed after almost a month by following same protocol for this shoot regeneration as the previous one with 10 jars and 6 plants in each jar.

4.1.2 Leaf regeneration of petunia

Leaf regeneration is an important part to follow the CRISPR-Cas9 methodology. The plant must be able to grow on growth media in order to transfer the CRISPR-construct into the plant through agrobacterium transformation and to observe the genetic change in the plant.

1st Leaf Initiation: The leaf initiation of petunia was performed to be sure under which circumstances the plants grow best. The same protocol was to be used to initiate leaf growth after the CRISPR/Cas9-construct transfer. For leaf initiation 500ml growth media was prepared according to MS media composition given in Table 3. The media was prepared in a 500ml glass bottle and autoclaved. Laminar flow and all the necessary materials were disinfected. Four hormones were used according to the following concentrations:

Table 4.2. Concentration of hormones for the leaf initiation of petunia

Name of Hormone	Concentration	Amount added per 10ml
BA	10 μ M	22.5 μ l
TDZ	2 μ M	4.4 μ l
2iP	10 μ M	26 μ l
Z	10 μ M	22 μ l

For each hormone, there were 1 blank and 8 test samples. The hormones were added to each petri dish of 10ml according to above concentration and then 10ml media was added. It was taken care that the media was neither too hot nor too cold (Approx. 50-55 °C). Each petri dish was slowly moved in circles to mix the media and hormones. After solidification, small slices (4-7mm) of petunia leaves were cut and placed onto the solidified media upside down (stomata facing lid of the petri dish). 5 slices of leaf were placed in each petri dish. Thus 36 petri dishes were prepared. The plant was almost 2 months old when the leaves were taken from it. All the petri dishes were then wrapped and settled in white light at 22 °C in the growth chamber for growth.

2nd Leaf initiation: Leaves of petunia were initiated on a new media. This media was prepared to first force the leaves to make callus by placing on callus induction media (CIM) and then these leaves were to be transferred to the shoot induction media (SIM) for faster shoot growth. CIM was prepared according to concentrations provided in Table 4.3.

Table 4.3. Composition of Callus Induction Media (CIM)

Ingredient	For 1 liter	For 0.4 liter
Water	1025 ml	410
MS media	4.405 g	1.762
Plant agar	5 g	2
IBA	2 mg	0.8
2iP	0.5 mg	0.2
Sucrose	30 g	12
pH	5.8	5.8

The media was prepared with the same protocol as it was followed before. According to the composition given in table 5, 800µl of IBA and 200µl of 2iP was added to 400ml of media. With the same procedure the media was autoclaved and then it was poured into the 10ml Petri dishes. 10ml media was poured in each Petri dish and it was left to dry in laminar flow. Petunia plants were brought from the *in-vitro* growth chamber and the leaves were cut into small pieces of almost 5mm². The plants were almost 3 months old when the leaves were taken for this experiment. It was made sure that the leaves had cuts on almost all the sides to promote callus formation. The Petri dishes were closed and were wrapped. The Petri dishes were then put in with white light at temperature of almost 22°C. The dishes were left there for almost a week and callous growth was observed on daily basis. Once callus was visible on all the leaves, the leaves were transferred to SIM. SIM was prepared according to the composition provided in the Table 4.4.

Table 4.4. Composition of Shoot Induction Media (SIM)

Ingredient	For 1 liter	For 0.4 liter
Water (ml)	1025	410
MS (g)	4.405	1.762
Plant agar (g)	5	2
IBA (mg)	0.2	0.08
Sucrose (g)	30	12
pH	5.8	5.8

The media was prepared with the same protocol as explained before and was autoclaved. This media was then taken to the laminar flow to be poured into the petri dishes. Before pouring media into petri dishes, same four hormones given in table 4 were added to petri dishes. For each hormone, there were 1 blank and 8 test samples so total 36 petri dishes were prepared. When the media was around 50-55 °C hot, 10ml media was added to each petri dish. Each petri dish was then slowly moved in circles to allow the hormone to get mixed into the media. After media was solidified the petri dishes with CIM were disinfected and were unwrapped in the laminar flow. Leaves that had formed callus were carefully transferred to the petri dishes with SIM. The SIM petri dishes were then wrapped

and were put in growth chamber with white light and 22 °C temperature. 2 petri dishes from each type of hormone were put in another growth chamber with white light and 26°C temperature to check if the callus grows faster at higher temperatures.

3rd Leaf Initiation: New generation of in-silico designed cytokinin oxidase inhibitors had been used on petunia for the development of shoot regeneration. A different MS mod3.B media (2.63g/L) was used with sucrose (30g/L) and agar (7 g/L). The pH was set to be 5.8. The media was autoclaved. The cytokinin oxidase inhibitors poured into each petri dish according to the concentrations given in the Table 4.5. The media was cooled down to almost 50-55°C. 10ml media was poured in each petri dish and gentle shaking was done to mix the hormones into media. After solidification 5mm² of leaf sections of petunia were cut and transferred the petri plates (5 leaf sections per plate). The plant was almost 2 months old when the leaves were taken. Three replications were applied for each concentration treatment of the hormones.

Table 4.5. Concentration of the cytokinin oxidase inhibitors

					concentration	0	0.5	0.5	0.5
					Hormone	control	CPPU	JN1	JN4
					Molar Mass	0	248	419	354
					stock concentration	1	1	1	1
					to add (μl)	0.0	1.2	2.1	1.8
concentration (μM)	Cytokinin hormone	stock concentration	Molar Mass	to add (μl)					
0	control	1	0	0.0		1	2	3	4
5	2iP	1	220	11.0		5	6	7	8
5	BA	1	226	11.3		9	10	11	12

Lower left part of table (blue colored) shows the concentrations and used amount of the hormones, upper right part of the table (red colored) shows the concentrations of the new hormones, lower right part of the table (green colored) shows the number of petri dish from 1-12. Three replications of each mixture were performed.

4.1.3 Agrobacterium transformation for GFP expression

The agrobacterium transformation had been a crucial step in the experiment as it was one of the most important tests to see how the plant reacts to the foreign DNA and what is the success rate. To carry out the transformation, to grow the agrobacterium before the transformation liquid YEB media was prepared according to the composition given in Table 4.6. The media was then autoclaved.

Table 4.6. Composition of YEB media

Ingredient	Amount per 1 liter of distilled water
Yeast Extract	1 g
Beef Extract	5 g
Pepton	5 g
Sucrose	5 g
MgSO ₄ ·7H ₂ O	500 mg
pH	7.2

25ml of the YEB media was taken in 50ml sterile Falcon tube. Two of such tubes were prepared, one for the bacterial growth while one as control. To make Agrobacterium virulent 20mg/l acetosyringone (100 μ M) was added to the media in the falcon tube. Under sterile environment of vertical laminar flow, a very little quantity of bacteria was added to one of the falcon tubes with a sterile spatula from already grown bacterial colony. The lid of the falcon tube was fixed with a paper tape in order to keep it a little bit open but not too open that it falls off. The falcon tube was then placed on the shaker for overnight at 28°C at 200rpm.

Next day, petri dishes of CIM were prepared as explained in the section 4.1.2. The petri plates were left in the laminar flow to solidify. Liquid MS media was also prepared according to the section 4.1.1. For liquid MS, all the constituents remain the same and only the agar is not added. Optical density (OD) of the previous day's bacterial culture was measured by the OD meter where 1 OD = $8 \cdot 10^8$ bacteria per ml. 25ml of the liquid MS was taken in a falcon tube and

20mg/l acetosyringone was added to it. According to the calculated OD, enough YEB media containing bacteria was added to the liquid MS so that the OD become 10^7 bacteria per ml. The plant was almost 2.5 months old when the leaves were taken and the leaves were cut in small pieces of almost 5mm^2 and almost 30-40 leaves were added to the falcon tube with liquid MS and bacteria. The falcon tube was put on shaker at 10rpm. After almost 30 minutes the leaves were taken out of the media and were dried on a sterile household paper. Now 10 leaves were added to each petri plate of CIM. The plates were covered with aluminum foil and were incubated in the growth chamber at 22°C for 2 days. After two days, the leaves were transferred to new CIM petri plates in which 400mg/l Ticarcillin was added before solidification. This was done to kill the agrobacterium.

After almost a week when the plant started to make callus. SIM petri plates were prepared with 400 mg/l Ticarcillin to continue to suppress Agrobacterium and 50 mg/l Kanamycin to select only the transformed callus. The leaves producing callus were carefully transferred to these SIM plates under sterile environment. The plates were then sealed and were put in growth chamber for shoot growth.

4.1.4 Leaf infiltration for GFP expression

A faster way to see if the plant responds to the foreign DNA and how well is the success rate is to inject the GFP containing agrobacterium directly into the leaves and flower of the plant. This method is efficient and quick. It was performed to check how well petunia responds to the GFP gene incorporated into its genome by agrobacterium vector and results could be used to check the expression of the CRISPR-Cas9-construct expression when ready. For injecting the bacteria directly into the leaves. The bacteria were grown in YEB media the same way as they were grown for agrobacterium transformation in section 4.1.4. The same composition of the YEB media was used. After overnight growth of the bacteria in YEB media, the OD was calculated. The falcon tube was centrifuged suspend the bacteria at the bottom. The supernatant YEB media was removed and only bacteria were remaining in the falcon tube.

For this kind of experiment, another media is used as bacteria cannot be injected in YEB media because plants react to the media. So, MES buffer was prepared by taking 10 mM MES and 10 mM MgCl_2 . The pH was maintained at 5.7. The buffer was stored at 4 °C. Quantity of the MES buffer was calculated and added to the falcon tube containing bacteria that the OD became 1. To make the bacteria virulent 150 μM acetosyringone was mixed in the solution and it was incubated for 3 hours at room temperature before injecting (Wydro et al., 2006). A syringe was taken and 5ml of the bacterial solution of MES was filled in it. A very small hole was carefully made on the backside of the leaf. The syringe was used without needle and was pressed against the hole. On the other side of the leaf, the head of the syringe was pressed by fingertip creating a little pressure on the leaf. Care was taken to not to put too much pressure so that the leaf gets damaged. The bacterial MES solution was slowly injected into the leaf (Figure 4.3). The leaf was marked with a marker. Several leaves were also injected and marked.

After two days of the injection, the leaves were examined under the UV fluorescence microscope from Leica company model MZFLIII to observe the expression of the GFP. Progress C10 plus cameras was used to capture the pictures.



Figure 4.3. Agrobacterium infiltration in Petunia.

4.2 CRISPR-Cas9 Protocol

Following the CRISPR-Cas9 method involves various aspects to be careful about. One little mistake can make the whole experiment go wrong. To successfully carry out the experiment, following steps were taken.

4.2.1 Identification the target gene

Various genes were studied, and literature was considered to select a confident gene. The primary goal was to target a gene which after being knocked-out, produces serrated leaves in the plants. Petunia leaves are normally smooth and do not have serrations. For an ornamentally aesthetic value, making serrated petunia could have been an interesting subject. For this CUC2, miR164 and AS1 genes were thoroughly studied in Arabidopsis. WUSCHEL and PHAN genes were also considered. The secondary goal was to target such a gene which after being knocked-out, changes the leaf shape from its regular form. So, after looking for genes which control serrations in leaves, another search was done to look for genes which control leaf shape in any aspect. The manipulation of such genes could be done to achieve the target of changing the leaf shape of Petunia. Another homeobox gene Knotted-1 was found to be involved in determining various aspects of leaf shape. The gene was also verified in Petunia by NCBI BLAST. The sequence of the gene was fetched from Solgenomics.org and exons and introns were highlighted as given below:

Exons; Intron; and Start & End Codons are highlighted, respectively.

ATG GAGAATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAACCTCTTTTATGGTGGAAATTCAGTCTTGACCTCCAAATCTTCTATTT
 ATGATGATCAATCTCAAGTAGTGGCACCCCAATAGTGAAGACAGAAAGGAGGAGCAGCAGTAACTCTCATCATCATCATACATTTCAATACC
 CTTCCGATAATTCGTAGTGGTTCATCATCAACATCAGACGGTTCATAATCATCATCACGAAAGTGAATTTCTTGGTGAAGTTGGGGCTTAAAGC
 CAAGATTCTTGGCCATCTCAGTGTTCAGTCTTTTGGATGCTTACATGGACGTGCAAAAGGTATAAACACATTTTCTCAGATGTATATATAACAAA
 CATACTCAGAAATACGGAGTGGTTCCTTTATTTATTTTGTGCTTTTAAACATACCATAAGGAATTAATTGACTTGGAAAAAATAATTTCTTGGT
 CTCCTGACITTTCTCAGATATTATATCAATTTTATTGCACTATCATTTGAAAATAACTACCAATAAATACTCATGTAAAGTGTACTTAATTATCTATA
 TGGAAAAATAAGAAAGTCGAACATGTTAGACGAAGTTAAATATGTGTAAATAATAITATTACAGTTGTGAGTATATATTTTTCATAGGTGGGAGCA
 CCGCTGAAAGTGATGGCGAGGCTATCCACTTTACGGCAAGAGTTGAGATGAGGCGAGGCTTCATTGACCGGAAAAGATGTGCCAAGGACCCA
 GAACCTTGATCAATTCATGGTCACTTTTAACTCTCGTAATATATTAATAATCAACATCCATTGTAGAATCGTGTGCATTAGGCCCTT
 AGGATTTCTTGGTTATAAAAAATGTATATTGAAATCAACATTTTGTCTCTTTCACATTACCAACTATATTGAAAAGGTTGATTTCTTGATGATATCC
 AGGAAGCTTATTATGACATGTAGTGAAGTATCGAGAGGAGCTTACAAGGCCCTACAAGAGCCATGGATTTCATGCGGAACATTGAGACTCAGC
 TTAATATGCTTGGAAATGGGCTGTTCGAGCTTTCAACTCTGGTACTTCTCTTCTCTCATTTGATATTATGAGTCCGTTTGGGTGAGCTTAAAGT
 GACTTATAAGCTTATAAGCTTATAATATATGTTTGGCAATCTCAAAATTTTGAACCTATAAGCCATAAGTTGCTTAAAGCCACTTTTGAAGT
 TAGGAAATCTCACTCTCAAAAGTAGCTTTTAACTGACTTTTGTACTTATAAGCCACTTAAAATAAGCAGCTTTAAGCCACCCAAACAGCTC
 TATGTATCTCTCTCATTTAGTATTATGATCATGCTTTTGTGAAAACATTTTCTTATCTGTCTTAAACGGAGGATAGGAATTTAAATTTCTATT
 CTGTTTAAACCAATGACATGCTGCCACAAAAATGTTAAAGTACTTTTGATACATGTCAATTTCACTTCTTAACTCGAGTGTAAAGAAATGCAAG
 AATGGTGAGTGGAAATAGTGAGAACTGTTCTCATGTAATGCAAGCAATGGAAAGATGCAAGAAGATAAAATAAGGGACTGATTAAGATTCC
 AAATATAGGTGGTCAATATAAGAAACAGAAAAAGGAGAAAAACAAACAACTAAGGCCATAAGGGCTATGATTGAACTGTGTTCTGCGGTTCATATGTA
 TAAATACTGTTGGTTCATTCAATAATGTCCTTGTGCAATGCTATTCTCTTCCAAGACAGCTTGACCCAAATATCATTTCCACTTTCCGAT
 GCITTTCTGTGTAGAGAGAGAAGACCTTTTATTGGAGTACGAAAATCTACTTACGAGTGTACAGGAGAGTAATAGTATAAACTAGGAGTATTTTG
 TTATAAAGAAAAAGAGGATTTTGTGATATGTACAAATAAACAAATGGAGAGTGCCAGAGACAAACAAATCTTTCAGATATAAACTGTTAGG
 TGTGCCATTTTATCTAGCATGTTGATTAAACAGTAGTATAAAAAATGAAGAAAGTAATTTGATATTGTTGGTCTAAACCAAGATATAAAATTTT
 ATGTGTAAGCAATTTATTTCAATTAAGGTAATGAAAAATTTAACCTTTTCACTTCTTTTGTGACACCAAAAAATGTGCCATATAAATTTGAGATTA
 AAAAGTAGTACTAATTAATAACGATGTCCTTGTGCTCTTAACTTCAAGTTTCAAGTTTCAAGTTTCAAGTTTCAAGTTTCAAGTTTCAAGTTTCAAGTTT
 ACATACGGACATCAATAAATTTTACATCGTGCCTCTCTTAAACCAATTTATCTGACACTCAGAATAGATCTCTTGAAGTGTACAGTACTTGCCA
 AACCTTCAACAAACTAAATGTTGGTCTTCTGCTCTTCTTCAAGTGCACCTCTATGTTATGGAATCGTTTCAAAATTTCTGCTCTCTTCTCTCT
 TAAAGAGAAAAATAGAATGTGATTTCTAGAGTTAAACCTATACCGCATGTAAATAGGTAATGTAAGTATTAATATGTATATACGTCATACGTGC
 ATCTACCACTAGATATAAACCGTATAGAATGTTGGTGTCTTTTCCAAAGTTGATCGAGTGTCTTTTATGTTGCTTATGTTGGTAGCTAGTAGGCATTGGGA
 ATCTAGTAATAACACCTGGGTGCTATTCACACAGTTTCTACCATGTAGACTGAACATGCTTGTAGGTAATCACTTTGTTCACTTTATGTGATTTT
 TTTTTCGTTTATTAGAAAAAATTTGATTTTAAATTTGAAAAATTTTAACTTCACTCTCTCTTTTACACTTAATGAGATGATCTATAA
 CTGTACAAAAATCTTACTCTCTCTGCTTCCACTTTAGATGAACCCCTTTGACCTTGCAGCGAGTCTAATAGAGAAATTTTAAACCTTGTGGTCTAA
 AATAAGTTATAGATATTGTTGGTCTTAAACATCATTTAGGATGATAAAAGTGAATTTCTTTCTTAAATTTAGAAAGTGGTCTATCTTTTAAAC
 AGACTAAAAATGAATAGGTTTCATTTTGAACCGTGGGAGTAAACCTTATTTATGCAAGTTTCAAAAGTAGTCTTTCGACGCTTGAAGGCTTC
 AATATCAAGAAAGATCTTTTGAACATAGTGGTAACATTTTACTACCTCAATAAATTTTAAATTTCTGCTGAGTCAAAATGAGTTCACACAAA
 TTAATTTCTGCTGAGTCAAAATGAATTCACACAAATTAATACGGAGGGAACATTCAGCAGAAATGTGTGCTTGTGAGTTGGTTTCTCTCTATTTGT
 CAGTTATATAGCATAATTTGTTCTTAACTGGCTTCTATGTGTAGTATTACTGGCTTCTTGTGTAATTTTCACTTCTGCGTCTCTAGACATGTTT
 TTTGTAAGAGCTGCTTGGTTGTACATTTTCTTTTAAAGTTTTCAGTCAATGGAGAACGGGGAAGAGGATTAAGAAATGGTGTGTAGAGACTGG
 ATAAATAAAGTGCAATCTAACAGTTTAACTATTAATGAAATGGTGCATTTGTTCAAAAAATATGATATTAAGAGGAGGTCCTATCTAATTTGTT
 TTAACCTATGTTAGGACCATATTATGCTGCGGCTCCAATTGACAAGCATGATGCACAAAGGTTGTTGAGTTTCCACATTTGGCTTTTAGGTGGT
 GAGTCTCTTATATGGGTATGGGCAATCTTATTTTATGCTAACTTTTGGGTTGAGTTAGACTCGGTATTAATTTTGTGCTATTAAGAAATTTT
 AACGCACAGATGTAATATGGATTCTAATTTGCTAGGAGTGTCTGATTTAGCTTAAATTCATCAAAAGTTATCCAACAGAACCATCCGAGGAAAAAG
 TGGACATGAAAAATGTTGAGCAAAAAGGATCTTATACCAATATATTTACAGTACCACATTTCTTAAAGCTCATCTGTTAAGTTCACTATTCA
 ACCCTCTTTTCTTCTTCTTGTGGCATCTGAACCTGTGTTTATGTTAGAAATTTGCACGAGGAGAGAAAGTAAAGATATTGGCAGAGCATGGAAAC
 AAGTTTGTAGTTCAAAATGGGTGATTATAGTTTAAACCATTAATCAATTAATTAATATGTATCTTAACTTATTGCAACATTTGAAGTTTCGAGATGAG
 CCAGTAGAACCTATCAATCATATTAGACTTTAGTGATTAATTTTACCGGACTCTTGGGAGAGAAATAAAATATAAAATTTGAACATCATCATCAACA
 ACACACAGAGTGTGATGGAGGTTGACTTACCTCTACATTGAGTGAGGCGGTTCCGAAAACTTTGAACATCATCTCCGTGGATAATTCCGTTACTA
 CTAGCTATGAGTACTTTTCTTTCTTTTGTGCTGGGGGGGGGGGGGGGGGGGGGAGGAGTTTGAATTATAGCAGTCATAGATTCTGAAGGATA
 ATTACCACTATCTTTGTTGATCAATATTGAACAGAGGATAATAAGTTGAAGGTTTGGATCATCAGAAGAGGACCAAGACAAACAGTGGTGGAGA
 AACTGAACCTACCAGAGATTGATCCAGGAGCAGAAAGACAGAGAACTGAAGAACATTTGTTGAAAAATACAGTGGCTACTTAAGTAGTCTCAAC
 AAGAATTTTCCAAGAAAGAAAGAAAAAGGAAATGCCCCAAGATGCTAGGCAGAAAGTTGCTCAGTTGGTGGGAGTTGCACATAAAATGGCCATAC
 CCATCGGTACAAACTCTACTTAATTAAGCACTACTCATTATATTATGAGCTTAATTTATAAATAAATGCAAGAAATTTTACCCGATCAGTGCA
 ATTTAAATGATTATAGCAATTTATGTTCAAAATTTAAGGTTTGGATATTAATTTGATCCGAAGAATTACCTGATATTATAAATCTCATAGTGTATAT
 TTTAAAGTGGTCTGACGCAAAACATGACTCTATTTTCACTTAATATTATTTGAGTTTACCTTACATACAGTTATAGTACATAAAGTAAAGGAT
 ATATGTCATATAAAATGATTGCTGTAACTAAGGTAAGTTAACTGTTACAACGTTCTTAAAAATATTATTTAGTAAATACTCTTAAAAATGTTAG
 TGTGTATAAGTAATATCCATTTTCTGAGGTTAATTAAGTTGTGACAAGCTTTCAGGAGTCAAGAGAAGGTGGCAATTTGGCTGAATCAACAGGTT
 TGGACCAAAACAAATAAATAACTGGTTCATTAACCAAGGAAAGACACTGGAAGCTTCAGAAGACATGCGAGTTTATGGTGTATGGATGGTCTTC
 ATCATCATCTCAAAATGCAGCTCTCATATTGAAGTCAATTATATGGGAGAAGGTCCTTTTCGTTTGGGATAA

It was important to check whether the first exon has any peptide sequence because if peptide sequence is present, that part of the exon does not play any role in the expression of the gene. [SignalP 5.0](#) was used to check the peptide sequence. Figure shows the results of the peptide sequence check.

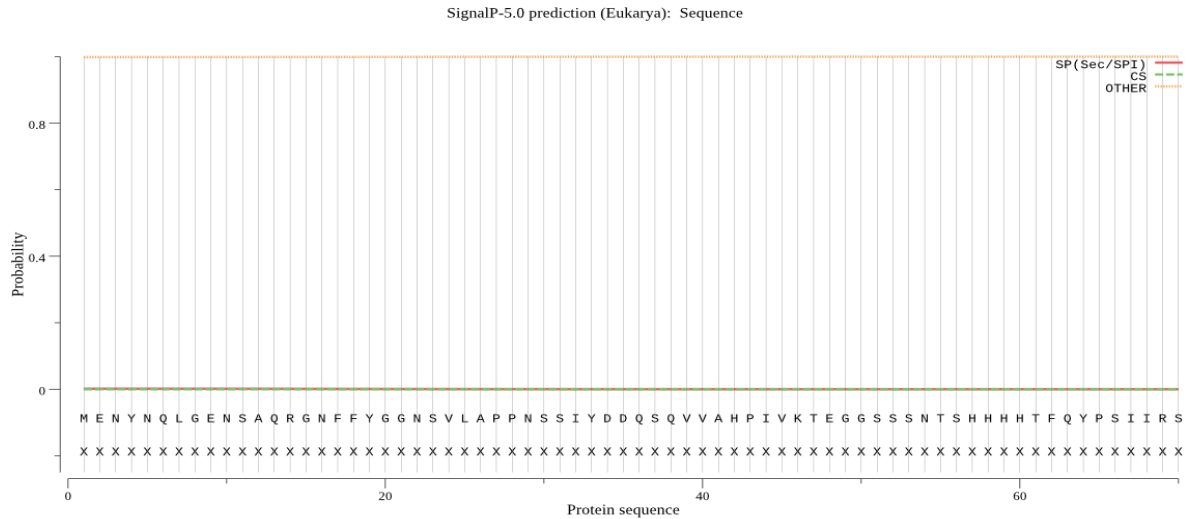


Figure 4.4. Peptide sequence check for the target Kn1 gene in Petunia.

4.2.2 Designing molecular markers

To check whether the downloaded sequences of Kn1 gene was present in the petunia plant being used in the lab and to amplify first exon of the sequence, molecular markers were designed. While making the primers it was considered that the primers are of length between 18-24 nucleotides, they do not make primer dimers or hair-pin structures, the G-C content is between 40-60%, the temperature of primers is between 50-60°C, there are no repeats or runs of base-pairs (Chuang et al., 2013). [NCBI primer designing tools](#) was used for designing the primers.

1st Set of Primers: To amplify the first exon of the Kn1 gene in petunia, the sequence of the first exon was loaded into the NCBI primer designing tool and required parameters were set. The tool suggested 10 possible primer pairs out of which two were shortlisted as provided in Table 4.7.

Table 4.7. 1st set of primer pairs for the Exon1 of the Kn1 gene of Petunia

Primer name	Sequence (5'→3')	Template strand	Length	Temp.	GC%	Self-complementarity	Self-3' complementarity
MG1	ACTCAGCTCAAAGAGGTAACCTT	Forward	22	58.43	40.9	4	1
	AAGCATCCAAAAGACTGGAACA	Reverse	22	57.03	40.9	4	3
Product length	306 bp						
MG2	GGGGAGAACTCAGCTCAAAGAGG	Forward	24	60.14	45.8	4	0
	CCATGTAAGCATCCAAAAGACTGG	Reverse	23	62.5	56.5	5	1
Product length	319 bp						

Following is the visual representation of the 1st pair of primers in the downloaded sequence of Exon1:

MG1 primer pair is represented as **Exon** and **Primer** respectively on the following sequence:

ATGGAGAATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAACCTTCTTTATGGTGGAAATTCAGTTCCTGCACCTCCAAATCTTCTATTTATGATGATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACAGAAAGGAGGCAGCAGCAGTAATACTTCTCATCATCATCATACATTTCAATACGCTTCGATAATTCTGAGTGGTTCATCAACATCAGACGGTTCATAATCATCATCAGGAAAGTGAAAATCTTCTGGTGAAGTTGTGGGCTTAAAGCCAAAGATTCTTGCCCATCTCTCAGTGTCCAGTCTTTGGATGCTTACATGGACTGTCAAAAG

MG2 primer pair is represented as **Exon** and **Primer** respectively on the following sequence:

ATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAACCTTCTTTATGGTGGAAATTCAGTTCCTGCACCTCCAAATCTTCTATTTATGATGATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACAGAAAGGAGGCAGCAGCAGTAATACTTCTCATCATCATCATACATTTCAATACGCTTCGATAATTCTGAGTGGTTCATCAACATCAGACGGTTCATAATCATCATCAGGAAAGTGAAAATCTTCTGGTGAAGTTGTGGGCTTAAAGCCAAAGATTCTTGCCCATCTCTCAGTGTCCAGTCTTTGGATGCTTACATGGACTGTCAAAAG

2nd Set of Primers: Another set of primer pair was designed to amplify the sequence of the Kn1 gene prior and after to the Exon1. This was important in order to amplify a larger sequence to be able to later highlight and cut the gRNAs from the exon1. Further details of why these new primer pairs were designed can be found in section 4.2.3. To design these primers, a larger sequence of Kn1 was downloaded from solgenomics.com and was annotated as shown below:

Exon1

TGTATGAATAAAAGCTAAGATAAAATATGGAAGAAAGGAAAGTAGAATGAAAAAGTAACAAAATAGAAGAGAAGTAGAAAGCAACAAACAA AAGTAACATATAAAGGAATATGAATACCTCACCACATACAAAGGAGAAATATGCCTGCCTATCTACTACTCTTCTATCCAAATCTTCGGCCTCCATCT TCTCTTAAATAGGAACATAATATAATTTTTCATAAAAAAATCAATACATGCAATTATTTGTCACTATAAGTAATTTAAGTAATTTGATGATCGAGA TAAATATGCTAACATGTTTTACATGTTAAACCATGTTTAAATGTGAAACGCTTTATATATTTTTGTTAGTACTTAATCCCATTTGGTATATAGGTAA AAATAATAAACAATCTGTTGTCATATGTTAAAACTATACTAAGCTGTAATTAATAATTTTGTCTGGACATATTAAATTTTCAATAATATTAACCTT AAAAGTCAAGAAATTTGTTGATTTAGCCAAAGAAAAATATGGAAGGAAGAACTCCCAAGAAAAAGAAAAAGAGGAGCTTAATGCCCCCGTGAA AGCAATGGTAAAAAGCAAGGGGAAAAAGTTGGAGGAGAAAAAAGAAAGAGAGAGAAGAAAGTTCCCTTTAACATTTGAGTAGGATCATAGGG GAAATAGTAGTAAACCATAAATTAATATATTTAATTTCCAAATACAAATATTTATTTATCTCTCAACGAAGAAGCTCTTGTACCCACGAGGCGGC TACCAGCAGTGGCCACAAGCTTCTCTTTCCCAATATGTTTCTGTTCAAAGGCCACCATCTGTGATGATCTCTACCCCTTTATCCTCTAATGATAATTT CATGCCCTCATCTTTAATTAAGATACAAAACTTCTCACTTCAACTTCTCCTTATCTAGTACACATCCAATTCATTACTTTTACCAATAAAAAAAGAG ATCTCCAAGTGTGAATTAACITTTGCTACTTCTAGTCTAGTTACTAGTTTATCCTATATTTGTTGGGATAGTTGAAGCATAATAAGTCTTGGCTTTCT TTAGGGTTTCTTACTAGTTCAACTGTTTATTATCTTCTGATTTAGGGTTGCTTTATTTTAAATTAATACTACTAGTAACATATTTTGAGGAAAAATGGAG AATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAACCTTCTTTATGGTGGAAATTCAGTTCCTGCACCTCCAAATCTTCTATTTATGATG ATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACAGAAAGGAGGCAGCAGCAGTAATACTTCTCATCATCATCATACATTTCAATACCCCTTCGA TAATTCTGAGTGGTTCATCAACATCAGACGGTTCATAATCATCATCAGGAAAGTGAAAATCTTCTGGTGAAGTTGTGGGCTTAAAGCCAAAGATT CTTCGCCCATCTCAGTGTTCAGTCTTTTGGATGCTTACATGGACTGTCAAAAGGTATAAACACTTTTTTCTCAGATGTATATAACAAACATACAT CAGAAATCAGGAGTGGTTCCTTTATTTATTTTGTCTTTTAAACATACCATTAAGGAATTAATTGACTTGGAAAAAATAATTCTTCTGCTTCGGT ACTTTCTTCAGATATTTATGAAATTTTATTCACATATCATTTGAAATAACTACCAATAAATACTCATGTAAAGTGTACTTAATTATCTATATGGAA AATAAGAAAGTCAACATGTTAGACGAAGTTAAATATGTGTAATATATTTACAGTTGTGAGTATATTTTTTCATAGGTGGGAGCACC GCC TGAAGTGATGGCAGGCTATCCACTTTAGCGGAAGAGTTTGAGATGAGGCAGGAGCTTCATGACCGGAAAAAGATGTTGCCAAGGACCCAGAAGT TGATCAATTCATGGTCAGTTTTTTTACACTTTTTTAAATCTCGTAATATATTTAAAT

The primer pairs were designed to amplify a larger sequence. Same characteristics of a good primer were considered while designing these new primers. The details of the primer pairs are given in Table 4.8.

Table 4.8. 2nd set of primer pairs for the Exon1 of the Kn1 gene of Petunia

Primer Name	Sequence	Template strand	Length	Temp.	Self-complementarity	Self-3' complementarity
Kn11	CCGTGAAAGCAATGGTAAAAAG	Forward	22	60.08	3	0
	CAAAAGACTGGAACACTGAGGATG	Reverse	24	61.5	3	0
Product length	914 bp					
Kn12	GTTCAAAGCCACCATCTTGTAG	Forward	23	59.29	3	1
	CTCATCTCAAACCTCTTGCCGTAA	Reverse	23	60.01	3	3
Product length	1080 bp					
Kn2	TCTTCGGCCTCCATCTTCTCT	Forward	21	60.34	4	0
	AACTGAGGATGGGCAAGAATC	Reverse	22	61.36	3	3
Product length	1301 bp					

The visual representation of the Exon, primer pair Kn11, Kn12 and Kn2 is presented below:

TGTATGAATAAAAGCTAAGATAAAATATGGAAGAAAGGAAAGTAGAATGAAAAAGTAACAAAATAGAAGAGAAGTAGAAAGCAACAACAACAA
AAGTAACATATAAAGGAATATGAATACCTCACCACATACAAAGGAGAATATGCCTGCCTATCTACTACTCTTCTATCCAAA
TCTCTTAAATAGGAACATAATATATTTTTCATAAAAAAATCAATACATGCAATTATATTTGTCACTATAAGTATTAAAGTAATTGATGATCGAGA
TAAATATGCTAACATGTTTTACATGTTAAACCATGTTTATAATGTGAAACGCTTTATATATTTTGTAGTACTTAATCCCAATTGGTATATAGGTAA
AAATAATAACAATCTGTTGTCATATGTTAAACTATACTAAGCTGTAATTAATAATTTTGGCTGGACATATTAATTTTCAATAATATTAACCT
AAAAGTCAAGAAATTGTTGATTTAGCCAAAGAAAAATATGGAAGGAAGAACTCCCAAGAAAAAGAAAAAGAGGAGCTTAATGCCCC
ACCAATGGTAAAAAGCAAGGGGAAAAAAGTTGGAGGAGAAAAAAGAGAGAAGAAGTTCCTTTAACATTGAGTAGGATCATAGGG
GAAATAGTAGTAAACCATAAATTTAATATATTTAATTTTCCAATACAAATATTTAATTTATTCTCTCAACGAAAGAACGCTTGTACCCACGGAGCCGC
TACCAGCAGTGGCCACAAGCTTCTCTTCCCAATATGTTTCTTGTTCAAAGGCCACCATCTTGTAGATCTCTACCCCTTTTATCCTCTAATGATAATTT
CATGCCTCATCTTTAATTAAGATACAAAACTTCTCACTTCAACTTCTCCTTATCTAGTACACATCCAATTCAATTACTTTTACCAATAAAAAAAGAG
ATCTCCAAGTGTGAATTAACCTTTGCTACTTCTAGTTCTAGTTACTAGTTTATCCTATATTTGTTGGGATAGTTGAAGCATAATAAGTCTTGGCTTCT
TTAGGGTTTCTTACTAGTTCAACTGTTTATTATCTTCTTGATTAGGGTTGTCTTTATTTAATTACTACTAGTAACATATTTTGGAGAAAAATGGAG
AATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAACCTTCTTTATGGTGGAAATTCAGTCTTGCACCTCCAAATTCCTTATTTATGATG
ATCAATCTCAAGTAGTGGCACACCCAATAGTGAACACAGAGGAGGAGCAGCAGTAATCTCTCATCATCATCATATTCATACCTCCATACCTTCGA
TAATTCGTAGTGGTCATCATCAACATCAGACGGTTCATAATCATCATCACGAAAGTGAAAAATCTCTGGTGAAGTTGTGGGCTTAAAGGCCAAGAT
TCTTGGCCATCTCTCAGTGTTCAGTCTTTGGATGCTTACATGGACTGTCAAAAGGTATAAACACTTTTCTCAGATGTATATATAACAACATAC
CAGAATACGGAGTGGTTCCTTTATTTATTTTGTCTTTTAAACATACCATAAGGAATTAATTGACTTGGAAAAAATAATTTCTCGTCCGTCCGT
ACTTCTTCAGATATTATGAAATTTTATGCACTATCATTTGAAATAAATACCAATAAATACTCATGTTAAGTGTACTTAATTATCTATATGGAA
AATAAGAAAGTCGAACATGTTAGACGAAGTTAAATATGTGTAATATATTATTACAGTTGTGAGTATATATTTTTCATAGGTGGGAGCACCGCC
TGAAGTGAAGGAGGCTATCCACTTACGGCAAGAGTTTGAGATGAGGACGCGAGCTTCATTGACCGAAAAGATGTTGCCAAGGACCCAGAAT
TGATCAATTCATGGTCAGTTTTTTTACACTTTTTTAAATCTCGTAATATATTTAAAT

4.2.3 Designing the gRNAs for infiltration protocol

Choosing a gRNA also has some parameters to consider such as gRNA should be present in the targeted exon, it must have a restriction enzyme (RE) site, it should be 20bp long, it must not create a hairpin / looping structure, it must not have off-targets with 0 or 1 mismatch, it must have NGG (where N can be A, T, G or C) as the Protospacer adjacent motif (PAM) site. The most important point to be considered while performing the infiltration protocol is to be careful about the restriction sites. gRNAs have been explained in section 1 of this document. The

idea is that such gRNAs are identified in the targeted sequence of the gene which contain an RE site. A restriction site is a short nucleic sequence which is recognized by a specific RE and the RE cuts the DNA from that specific part. There are many restriction enzymes (REs) available in the market and most of them are available at [NEB BioLabs Inc.](#) (Vincze et al., 2003). For choosing a good gRNA which contains an RE of interest several online tools were used such as [Crispor](#), [Chopchop](#), [CCTop](#). By analyzing the results from each of these online tools, Crispor (<http://crispor.tefor.net/crispor.py>) was found to be most useful which also provides the information of the RE sites. The results of this search can be found at [results of the gRNA analysis at Crispor](#) (Table 4.9). The idea is to select such gRNA which contain an RE site and there is no other similar RE site at least 200bp before or after the selected gRNA. When the CRISPR-Cas9 construct is ready to be delivered to plant by infiltration method explained in section 4.1.4. The Cas9 cuts the DNA at the 3bp 5' of the PAM site and the DNA tries to repair itself, if the DNA repairs itself correctly, the Cas9 cuts again and the process continues and the DNA makes a mistake (insertion or deletion while repairing) and Cas9 can no longer recognize the restriction site. The RE site must be present somewhere at 3 bp 5' of the PAM, so that when the mistaken repair happens the RE site also gets changed and the RE can no longer cut the DNA from that part.

After two days of the infiltration, the DNA is isolated from the injected cells. The DNA is then digested by the respective RE so that all the unaltered DNA fragments are digested and only the Cas9 repaired sites (which are different than the original ones) are left. Later the fragment is amplified by the primers (section 4.2.2), the amplified PCR products are cloned by PCR cloning method (section 4.2.8) and are sent for sequencing (section 4.2.7). The sequences are then compared and the changes in the targeted site are observed.

Table 4.9. Details of the shortlisted gRNAs

	Position on Strand	gRNA Sequence	PAM	Off targets with 0, 1, 2, 3 & 4 mismatches	Restriction Enzymes
1	290/reverse	CTGAGGATGGGCAAGAATCT	TGG	0-0-0-3-34	Hinfi
2	230/forward	TGGTCATCATCAACATCAGA	CGG	0 - 1 - 16 - 18 - 85	Hpy188I

The visual representation of the gRNA1 and gRNA2, their RE sites, and corresponding markers (to later amplify them by PCR) is following:

Exon; gRNA1 sequence; PAM; Kng11; Kng12; HinfI Restriction site (5'.....G[↓]ANTC.....3')

*Following whole sequence does not contain any HinfI Restriction site other than the one in the targeted gRNA region

[illegible]

Exon; gRNA2 sequence; PAM; Kng2; *Hpy188I* Restriction site (5'.....TCN[↓]GA.....3')

*Following whole sequence does not contain any Hpy188I Restriction site other than the one in the targeted gRNA region

GAAAATTGATTTCACAGAAATTAATTAAC TCGATTGTTTAAACAACAACAACAAAAATCCAATGTAAAGCTCGCGGAGAATGAGATGGACGC
AGAACTTCTCTATTAATTTGAAAGACAGAAAGATTATTTTGTAGATGCTCAITTTAAAGAAACATTTTACAAAGTCGATTCCAGCGAAATGTAT
GACATAAAAGCTAAGTAATAAATGTGAAGAAAGGAAAGTAGAATGAAAAAGAACAAATAGAGAAAGATGAGAAAGCAACCAACAAAGAAAT
ACACATATAAAGGAATATGAATACTTCACCACATACAAAGGAGAATATGCTCGCTCATCTACTACTCTCTATCCAAA **CTCTGGGCTTCATCTCTCT**
TAAATAGGAACAATAATATATTTTTCATATAAAAAATCAATACATGCAATTAATTGTCATATAAGTATTAATGAATTTGATGATGCAGATATAA
TATGCTAACACATGTTTTCACATGTATTAACCATGTTTATAATAGTGAAACGCTCTTATATATTTTGTAGTACTTATACCCAGTTGGTATATAGGTAATAAT
AATAAACAACTCTGTGTCAATATGTTTAAACATATACTAACGTGTAATTAATAATTTTGTGCACATTAATTAATTTTTCATATAATTAACATCTAAAA
CTCAAGAAATTTGTTGATTAGCAAGAAAAAATGTGAAGGAAGAACTCCCAAGAAAAAGAAAGGAGGCTTAATGCCGCCCGGTGAAGGCA
ATGGTAAAAAGCAAGGGGAAAAAGTTGGAGGATAGAAAAAAGAAAGAGAGAAAGATTCCTTTAACATTAGAGTAGGATATAGGGGAAA
TAGTAGTAACACCAATTTTAAATATTTAAATTTCCAATACAAATATTTATTTCTCAACGAAGAACGCTCTTGTCACCCACGAGCCGCTACCA
GCAGTGGCCACAAGTCTCTCTTCCCAATATGTTTCTGTTCGTAAGGACCACCATCTGTAGATCTCAACCTTATATCTCTAATGATAATTTATCTC
CTCATCTTTAATAAGATACAAAAACTCTCTCACTTCAACTCTCTCTATCTAGTACATGCACCAATCAITACTTTTACCAATAAAATAAGAGATCTC
CAAGTGTGAATTAACCTTTGCTACTCTAGTCTCTAGTTACTAGTTTCTCTATATGTTGGGATAGTGGAAGCATATAAGTCTGTGGCTCTTTTAGG
GTTTCTTACTAGTCTCAACTGTATTATCTCTCTGATTATAGGTTGTCTTTATTTAATTAACCTACTAGTAACATATTTTGAAGGAAA **ATGGAGAAATTA**
TAATCACTAAGGGGAGAATCAAGCTCAAGAGGTAATCTCTTTATGTTGGAATTCAGTTCTTGCACTCCAAATCTCTTATTTATGATGATCAAT
CTCAAGTAGTGGCAGACCCAATAGTAAAAAGACGAGGAGGCAGCAGTAATCTCTCATACATCATCAATTTCAATACCTTCGATTAATTC
GTAGTGGTCACTCAACATCAGACGGTGCATATCAATCAGGAAGTGAAATTTCTCTGCTGAAGTGTGGGCTTAAAAAGCCAA**GATTCCTGC**
CAATCCTCAGTCTCCAGTCTCTTTGGATGTCATATGCAATGAGCTGCAAAAGGTATAAACACATTITTTCT

The above sequences of the exon, gRNAs, RE sites and PAM are from the internet source. To confirm that the exact same sequences were present in the Kn1 gene of the Petunia under experiment and it does not contain any single nucleotide polymorphism (SNP), further steps were followed to amplify the targeted exon fragment by markers described in section 4.2.2 and sequence them (section 4.2.7).

4.2.4 PCR Reactions for DNA Fragment Amplification

The molecular markers described in section 4.2.2 were sent to [IDT Technologies](#) and after receiving the markers the dilution of the markers was done by adding deionized water according to the prescribed water volume by the

company. The dilution was done under sterile condition. The initial concentration of the stock solution of the markers was 100 μ M/1 μ l. The stock solution was then stored at -20°C. Following steps were followed to run the polymerase chain reaction (PCR) for all the primer pairs.

4.2.4.1 PCR optimization for MG1 and MG2 primer pairs

1st PCR: The further dilution of the markers MG1 and MG2 was done to make a working solution of 1 μ M/ μ l by adding 99 μ l deionized water and 1 μ l marker from the stock solution. PCR reaction was run to check if the genes were present in the petunia variety under experiment in the laboratory. PCR mix was made according to the standard volumes of the PCR given in the Table 4.10.

Table 4.10. PCR composition for the 1st PCR of MG1 and MG2 primer pairs

Chemical name	1x PCR mix volume
Taq buffer (5x)	5 μ l
dNTPs (10mM)	0.5 μ l
Forward primer (1 μ M)	1 μ l
Reverse primer (1 μ M)	1 μ l
H ₂ O (Deionized)	15.28 μ l
Taq polymerase (5U/ μ l)	0.25 μ l
DNA (10ng/ μ l)	2 μ l
Total	25μl

PCR tubes of 200 μ l volume were used. Standard PCR protocol was followed. The initial denaturation at 95 °C for 1 min, 30 cycles (denaturation at 95 °C for 2 min, annealing for a gradient of temperature at 57-62.5 °C for 1 min, extension at 72 °C for 2 min), and the final extension at 72 °C for 5 min. The primer MG1 was placed at 57.4 °C and MG2 was placed at 61.4 °C during PCR and the control was put at 59 °C. This PCR protocol was followed with the primer concentration was 1 μ M and the annealing temperature for MG1 was 57.4 °C while for MG2 it was 61.4 °C. The DNA concentration was 10ng/ μ l.

2nd PCR: To further optimize the PCR of the markers MG1 and MG2, the volumes of the PCR mix were a bit changed as given in Table 4.11:

Table 4.11. PCR composition for the 2nd PCR of MG1 and MG2 primer pairs

Chemical name	1x PCR mix volume
Taq buffer (5x)	5 μ l
dNTPs (10mM)	0.5 μ l
Forward primer (1 μ M)	1 μ l
Reverse primer (1 μ M)	1 μ l
H ₂ O (Deionized)	15.375~16.375 μ l
Taq polymerase (5U/ μ l)	0.125 μ l
DNA (10ng/ μ l)	1~2 μ l
Total	25μl

PCR mix was made in such a way to check different volumes of DNA, as one sample had 2 μ l DNA, 1 sample had 1 μ l of DNA and remaining samples had 1.5 μ l of DNA. The PCR was now set to an initial denaturation at 95 °C for 2 min, 30 cycles (denaturation at 95 °C for 40s, annealing at a gradient temperature at 55-62°C for 40s, extension at 72 °C for 1 min), and a final extension at 72 °C for 5 min. Three PCR tubes were placed at 61.5 °C, two were placed at 55 °C and the control was placed at 56 °C. The same PCR protocol for MG1 and MG2 primers was repeated to confirm if it was optimized and was providing reliable results each time. This PCR protocol was followed for two times. In the first repetition, the DNA concentration was 10ng/ μ l and different amounts of DNA were added to increase or decrease the concentration in the PCR mix. 2 μ l DNA was added to MG2A, 1.5 μ l DNA was added to MG2C, MG1A and MG1B while 1 μ l DNA was added to MG2B. The annealing temperature for MG2A, MG1A and MG2B was 62°C and for MG1B and MG2C it was 55°C. A second repetition was followed to amplify MG2 fragments with 1 μ l DNA quantity (10ng/ μ l concentration) and the annealing temperature was set to be 61.5 °C for MG1A and MG2A, 59.3 °C for MG1B and MG2B, 56.4 °C for MG1C and MG2C and 55 °C for MG1D and MG2D.

3rd PCR: The concentration of the markers was increased for this PCR. The markers of MG1 and MG2 were diluted to a concentration of 10 μ l/ μ M. With this new marker concentration, the PCR mix was prepared according to the composition provided in Table 4.12.

Table 4.12. PCR composition for the 3rd PCR of MG1 and MG2 primer pairs

Chemical name	1x PCR mix volume	7x PCR mix volume
Taq buffer (5x)	5 μ l	35 μ l
dNTPs (10mM)	0.5 μ l	3.5 μ l
Forward primer (10 μ M)	0.5 μ l	To be added individually
Reverse primer (10 μ M)	0.5 μ l	To be added individually
H ₂ O (Deionized)	17.875 μ l	125.125 μ l
Taq polymerase (5U/ μ l)	0.125 μ l	To be added individually
DNA (10ng/ μ l)	0.5 μ l	To be added individually
Total	25μl	

Hot start PCR technique was used to avoid any unexpected marker binding. For this reason, all the constituents were added to the PCR Eppendorf tubes. Tubes were put in the PCR machine following the same PCR protocol as followed in 2nd PCR of section 4.2.4.1 except that as soon as the PCR lid temperature reaches 100°C, the process was paused, taq polymerase was added to each tube and then the PCR process was resumed. The same PCR mix and protocol was followed to check if it continuously produces reliable results. 3 samples of each primer pair were run at different annealing temperatures. The annealing temperature for MG1A and MG2A was 61.5 °C, for MG1B and MG2B it was 57.7 °C and for MG1C and MG2C it was 55 °C. The same PCR was repeated to confirm the results.

4.2.4.2 PCR optimization for Kng11, Kng12 and Kng2 primer pairs

The primers of Kng11, Kng12 and Kng2 were first diluted to 100 μ M/ μ l concentration by adding deionized water in an amount recommended by the

company IDT Technologies. Then, a working concentration of 10 μ M/ μ l was made by adding 90 μ l deionized water and 10 μ l of primer solution from the master solution of 100 μ M/ μ l. These dilutions were carried out under sterile environment. To avoid unexpected mistakes, 3 repetitions of each primer pair were decided to run in PCR along with 1 control reaction. The annealing temperature for sample Kng11A, Kng12A and Kng2A was 60.6 °C, for sample Kng11B, Kng12B, Kng2B it was 57.7 °C, for sample Kng11C, Kng12C, Kng2C it was 55.5 °C. The composition of PCR mix for these 10 samples is given in Table 4.13.

Table 4.13. PCR composition for the PCR of Kng11, Kng12 and Kng2 primer pairs

Chemical name	1x PCR mix volume	10x PCR mix volume
Taq buffer (5x)	5 μ l	50 μ l
dNTPs (10mM)	0.5 μ l	5 μ l
Forward primer (10 μ M)	0.5 μ l	To be added individually
Reverse primer (10 μ M)	0.5 μ l	To be added individually
H ₂ O (Deionized)	17.875 μ l	178.75 μ l
Taq polymerase (5U/ μ l)	0.125 μ l	To be added individually
DNA (10ng/ μ l)	0.5 μ l	To be added individually
Total	25μl	

The hot start method and PCR protocol was followed as same as explained in 2nd PCR of section 4.2.4.1. This same PCR protocol for the primer pairs Kng11, Kng12 and Kng2 was followed to further confirm the reliability of the results. A mixture of these primer pairs was made to amplify a larger part of the targeted fragment for the sequencing. Table 4.14 shows the details of the mixture of the primer pairs. For further experimentation code “PC” (Primer combination) was given to each primer pair.

Table 4.14. Details of primer combinations of Kng11, Kng12 and Kng2

Primer pair		Code name to be used in experiment	Amplified fragment length
Forward primer of	Reverse primer of		
Kng2	Kng12	PC1	1721 bp
Kng2	Kng11	PC2	1313 bp
Kng11	Kng12	PC3	1322 bp
Kng11	Kng2	PC4	902 bp

Another PCR was run with the new primer combinations. Two repetitions of each PC were run at two different temperatures. PC1A, PC2A, PC3A, and PC4A were run at 60.6 and PC1B, PC2B, PC3B, and PC4B were run at 57.7. These temperatures were chosen according to the individual temperature of each primer and an average was taken. PCR composition was used as described in Table 4.13.

4.2.5 Gel Electrophoresis

The PCR products were run on the agarose gel. TAE buffer was prepared as explained in section 3.3. The TAE buffer was diluted to 1X concentration and pH was set to 8.2 by using NaOH. For PCR samples of 1st PCR of section 4.2.4.1, 1.5% agarose gel was prepared to run the PCR samples. 2µl of 6X blue/orange loading dye by Promega (section 3.3) and 1µl of GelRed (acts as a staining agent, attaches to the DNA and produces fluorescence under UV light) was added to each PCR sample for the fluorescence of DNA and to make it heavy to be submerged in the well of the gel. To prepare the ladder, 2µl of loading dye and 1µl of GelRed was added to 5µl of 100bp ladder provided by Promega at concentration of 0.13µg/µl (section 3.3). Figure 4.5 shows the expression of ladder on 2% gel. The gel was run at 135V for 20-50 minutes (depending upon the size of the expected DNA band) in the smaller gel electrophoresis container, and on 220V for 30-50 (depending upon the size of the expected DNA band) in the larger gel electrophoresis container. The gel was then placed on a UV light source and the results were observed.

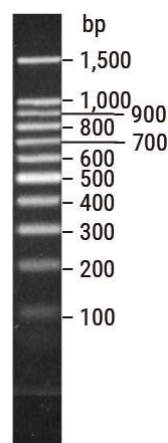


Figure 4.5. 100bp Ladder by Promega.

For all the remaining PCR reaction, 2% agarose gel was prepared while the concentration of TAE buffer, the constituents of the ladder and mixture of the PCR sample to be run on the gel, remained the same.

4.2.6 DNA excision from the gel

After successful PCR, the DNA bands were cut from the gel to isolate the amplified DNA segments to send them for sequencing. The DNA isolation kit was used by Promega company (section 3.3). The gel band was excised from the gel by using sterilized scalpels and was stored in a 1.5ml microcentrifuge tube. The weight of the gel was 70mg so 700 μ l Membrane Binding Solution was added (as per the instruction given in the manual to add 10 μ l membrane binding solution in 10mg of gel sample). To completely dissolve the gel the mixture was incubated at 50-65 °C.

The SV minicolumn (provided with the kit) was placed into the collection tube and the gel mixture was poured into it by using pipette. This mixture was incubated at room temperature for 1 minute and then was centrifuged at 16000 x g for 1 minute. The flow-through was discarded and the minicolumn was reinserted into the collection tube. Then, 700 μ l Membrane Wash Solution was added to the minicolumn and it was centrifuged for 1 minute at 16000 x g. The Flow-through was thrown away and the same step was repeated with 500 μ l Membrane Wash Solution and was centrifuged for 5 minutes at 16000 x g. The flow-through in the

collection tube was thrown away, the minicolumn was reinserted and was centrifuged again for 1 minute so that any remaining ethanol could evaporate. The minicolumn was then carefully placed into a new clean 1.5ml labelled tube and to elute the DNA 50µl Nuclease-free water was added to the minicolumn. After incubation for 1 minute at room temperature it was centrifuged at 16000 x g for 1 minute. The minicolumn was thrown away and DNA was stored at -20°C. The flow-through contained DNA and 1µl of this DNA + 2µl of loading dye + 1µl GelRed + 6µl nuclease free water was run on gel to confirm that DNA excision was successful, and the DNA had expected size of the bands.

4.2.7 Sequencing of the genomic fragments

The DNA samples were sent for sequencing to confirm the sequences of the amplified fragments. It was done to check if the sequences downloaded from solgenomics and the sequences of the plant under experiment are same or to observe the difference. The excised DNA fragments were sent for sequencing to the LGC company and to Eurofins. Each company offers various methods and option for sequencing ranging from simple to complex and from cheaper to expensive. Each sequencing protocol requires a specific concentration of the DNA so in some samples, the excised DNA was frozen at -20°C and was immediately put in lyophilizator for freeze-drying. The lyophilizator was from Analis company model number Alpha 1-2 LDplus. This DNA was later diluted to a required concentration by adding nuclease free water.

Sequencing from LGC company: The [instruction manual](#) was downloaded according to which the DNA concentration was maintained at 10ng/µl for samples between 200-500bp size, at 20ng/µl for samples between 500-1000bp and at 40ng/µl for samples between 1000-2000bp. The required primer concentration was 5µM/µl. So, DNA samples were prepared accordingly, and final volume of the sample was 15µl for sequencing. Different samples were sent to this company 3 times.

For the samples of Kng11, Kng12 and Kng2 primer combinations, 8 different samples were sent to this company. PC1 and PC3 were amplified to send

for sequencing. Each primer combination was sent separately with forward primer and reverse primer to allow sequencing from both ends of the fragments. For further optimization, both PC1 and PC3 samples were also freeze dried, so in total 8 samples were sent for sequencing for these two primer combinations. Details are as follows:

1. PC1 freeze-dried + forward primer of Kng2
2. PC1 freeze-dried + reverse primer of Kng12
3. PC1 without freeze drying + forward primer of Kng2
4. PC1 without freeze drying + reverse primer of Kng12
5. PC3 freeze-dried + forward primer of Kng11
6. PC3 freeze-dried + reverse primer of Kng12
7. PC3 without freeze drying + forward primer of Kng11
8. PC3 without freeze drying + reverse primer of Kng12

Sequencing from Eurofins: [Mix2Seq kit](#) was used from this company. The DNA samples were prepared accordingly. This kit required DNA concentration of 1ng/μl for sample between 150-300bp, DNA concentration of 5ng/μl for samples between 300-1000 bp and DNA concentration of 10ng/μl for samples over 1000bp. The required primer concentration was 10μM/μl. Final volume of the samples was required to be 17μl. The samples were prepared accordingly and were sent to the company for sequencing. To avoid freeze drying, more PCR mix was made to have more PCR product and was eluted in 35μl water instead of 50μl (section 4.2.6). The amplifications of Kng1, Kng11, PC1 and PC3 were sent for sequencing, each with forward and reverse primer separately. Total 6 samples were sent this time which are detailed below:

1. Amplification of Kng11 with its forward primer
2. Amplification of Kng11 with its reverse primer
3. Amplification of Kng12 with its forward primer
4. Amplification of Kng12 with its reverse primer
5. Amplification of PC1 with its forward primer
6. Amplification of PC3 with its forward primer

4.2.8 PCR cloning

To overcome the hurdle of sequencing of heterozygous gene, PCR cloning technique was used to separate the heterozygous fragments and to send those fragments separately for sequencing. [CloneJET PCR Cloning Kit](#) was used from ThermoFisher Scientific Company. The kit contained all the necessary enzymes and buffers for the cloning. *Agrobacterium tumefaciens* was used for the cloning was taken from the already growing culture from the lab. The Agrobacteria were first converted to competent cells according to the protocol described by (Debler, 2019; Wang, 2006). Already competent agrobacterium cells were available in the lab and were stored at -80 °C.

PCR product of Kng12 amplification were run on the gel and DNA was excised from the gel as explained in the section 4.2.6. The sticky-end cloning protocol was followed. As explained in the kit manual, the DNA blunting enzyme was first used to blunt the ends of the PCR products. The cloning vector and ligase enzyme was then added to the mixture so that the enzyme ligate the PCR products to the vector. The control experiment was run side by side and control PCR product provided within the kit was used for it. These ligation mixtures were then stored at -20°C. Heat shock method was decided to follow to transform the agrobacterium. The stored agrobacteria were taken from -80 °C and were put on ice (0 °C). 10µl of the ligation mixtures were added to each tube of the competent bacterial mixture and was shaken gently. This mixture was incubated at 0 °C for almost 15-20 minutes. A quick heat shock was given to the bacteria by placing them in water bath for 60-90 seconds at exactly 42 °C. 500µl SOB media (Sambrook et Russell, 2006) was added to each tube making the total volume 600µl. These tubes were closed tightly and were put on the shaker for almost 40-50 minutes at 37 °C. Petri plates of 30ml LB media were prepared with 100µg/ml ampicillin in it. 60µl of the bacterial mixture was spread on one LB plate. Remaining mixture was concentrated 10 time and remaining 10X concentrated bacterial mixture was spread on another petri plate. All the petri plates were labelled and put in the incubator at 37 °C for 16 hours. After 16 hours, the growth of the bacteria was observed.

12 single colonies of bacteria were taken into a new LB plate with ampicillin in it. Each colony was transferred to the new plate very carefully under sterile environment. The plate was incubated at 37 °C for 16 hours. After 16 hours, the growth of the bacteria was observed, and the plate was stored in the cold chamber. These colonies were used to isolate the plasmid and send it for sequencing.



5. RESULTS

The results were taken and recorded on regular basis. The results were analyzed to plan the direction of further experiment. In this section various results will be discussed related to various activities performed in section 4.

5.1 Results of Petunia Tissue Culture

Petunia normally produces very good results for the tissue culture and grows easily in the hormones provided MS media. However, the growth of petunia or any plant is also dependent on the genotype and based on it, a plant can be very easy or difficult to multiply *in-vitro*.

Results of Shoot regeneration: Shoot regeneration of petunia had been very successful. In the first experiment of shoot regeneration 13 plants were used to make 120 shoot cuttings to be planted in 20 jars (6 cuttings per jar). Out of these 120 cuttings almost 108 cuttings grew within a week giving 90% success. The plants grew normally, and no deformation was observed (Figure 5.1). This chimera might have developed due a mutation as petunia very easily develops mutations or another reason might be the response of the plant to light. This chimera also showed some branches which reverted to normal green color leaves with small whitish dots. However, only in one pot a chimera was observed.



Figure 5.1. Chimera of petunia due to mutation or physical stress.

The second shoot regeneration gave almost 92% success rate. In third shoot regeneration experiment 9 plants were used to make 120 shoot cuttings to be placed 6 cuttings in each jar and total of 20 jars were prepared and 0% results were obtained. For this experiment, plants started to grow in a very unexpected pattern and each branch started to subdivide into further branches, an irregular growth pattern called hyperhidrosis (Figure 5.2).



Figure 5.2. Hyperhidrosis - unusual growth pattern in Petunia.

It was concluded that there might have been contamination of growth hormones in the MS media or the plants might need more space to grow properly. So, another shoot regeneration experiment was performed, and 10 jars were made where 3 plants were placed in each jar. The plants showed the same growth pattern and did not recover to the normal growing habit.

Later it was revealed that this occurred due to lack of oxygen for the plant. The jars which were used this time did not allow for gaseous exchange and the plants showed this type of growth. To overcome this problem, the plants from the first shoot regeneration stock were used for another shoot regeneration. 6 plants were used to prepare 30 shoot cuttings. 3 cuttings were placed in one jar and bigger jars were used this time which had a little opening for allowing gaseous exchange. The plants started to grow normally and there was no irregular pattern observed.

Results of the Leaf Initiation of Petunia: The 1st initiation of petunia leaves did not give any results because the whole experiment got contamination (Figure 5.3). The contamination happened because of unsterilized paper being used during the cutting and transplanting the leaf section.



Figure 5.3. Contamination in 1st leaf regeneration experiment of petunia.

The same experiment was repeated and this time no contamination was found. After almost one-month results of callus formation was taken (Figure 5.4). However, no further growth was observed, and the experiment was discarded after almost two months. Callus growth was observed in 87% of leaves.



Figure 5.4. 1-month callus growth of petunia leaves under BA, TDZ, Z and 2iP hormones.

The results of the 2nd regeneration of petunia showed vigorous callus growth in CIM after one week. The callus producing leaves started to grow callus in the SIM but did not produce any shoots even after 2 months. The growth of the callus also seemed to be stopped (Figure 5.5). Almost 96% of leaves showed very good callus growth but no further growth was observed.

1-week growth in CIM

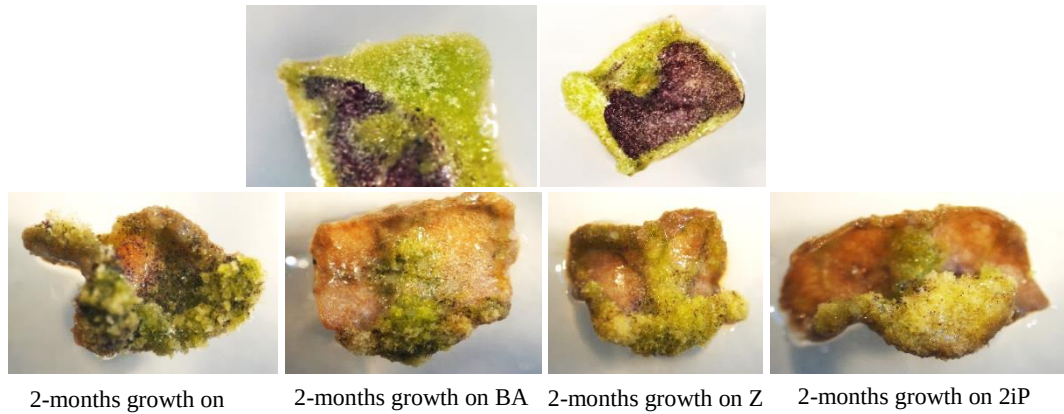


Figure 5.5. 1-week callus growth on CIM and 2-months callus growth on SIM.

In the 3rd regeneration experiment of petunia, all the leaves started to turn brown and after 3 weeks of initiation, no confident callus growth was observed. The callus kept growing and after 3 weeks of the experiment results were still not confident and a little callus growth was observed in some treatments (Figure 5.6). Overall only 5% of plants made little callus growth.

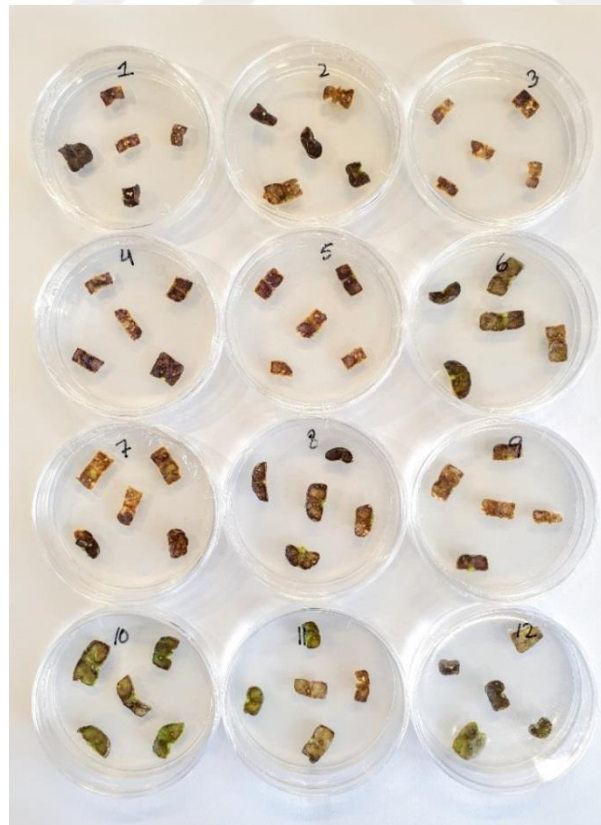


Figure 5.6. 3-week callus growth in in-silico designed cytokinin oxidase inhibitors

Results of the Agrobacterium Transformation for GFP Expression: The petri dishes were examined after one week of initiation, no visible callus growth was observed. After almost 4 weeks all the leaves turned pale brown (Figure 5.7) and the experiment was discarded because of no growth. 0% GFP expression was observed.

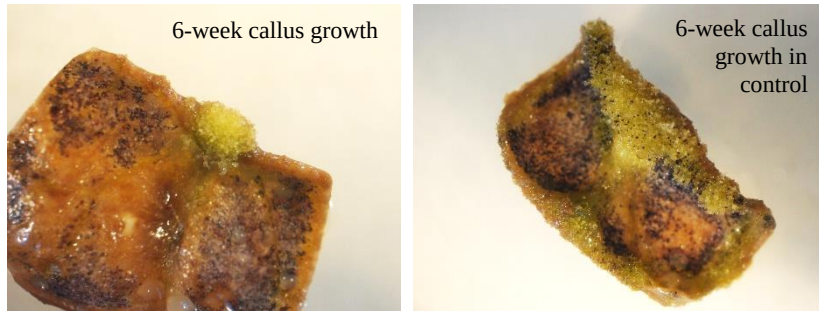


Figure 5.7. Browning of petunia leaves under Agrobacterium transformation for GFP expression.

Results of Leaf Infiltration for GFP expression: After two days of leaf infiltration, the leaf was observed for GFP expression. Excellent results were observed. Figure 5.8 shows a side by side comparison of the injected leaf under normal light and under UV light. A clear boundary was observed in the leaf where the expression took place while the remaining part of the leaf had no GFP expression.

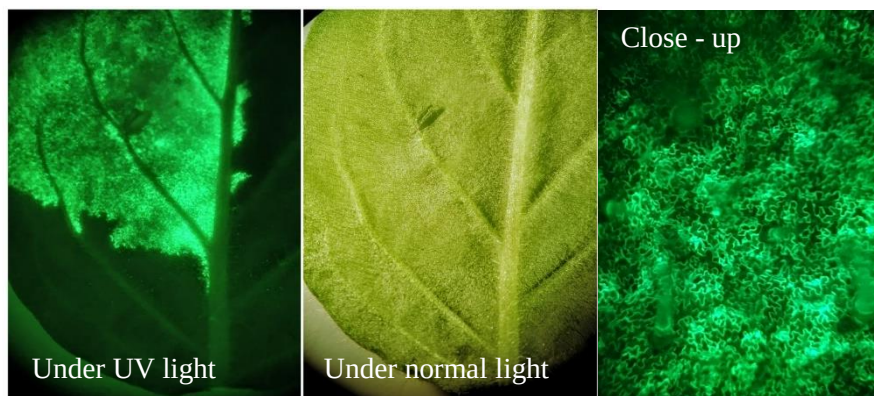


Figure 5.8. GFP Expression in petunia leaf.

5.2 CRISPR-Cas9 Protocol

The CRISPR-Cas9 protocol was followed after careful literature study. Several experiments were running side by side to obtain results in the short time. The results of these experiments are explained below:

5.2.1 Identification of the target gene

All the genes which are mentioned in section 4.2.1. were studied thoroughly. The first focus of the project was to make serrated petunia leaves. The miR164A, CUC2 and AS1 genes were involved in controlling leaf margin serrations in Arabidopsis. The miR164A was a microRNA and in literature it was found that it was very difficult and time-taking to knock-out such coding parts of genome. So, miR164A was not considered a valuable option to work on. Next was to consider CUC2 but the BLAST result of this gene showed that it was not found in petunia. The BLAST results of AS1 gene sequence also showed the that it was not present in Petunia. The literature study of WUSCHEL and PHAN genes also suggested that these genes were not confident to be worked upon. Several other articles were also found to provide evidence about these genes controlling leaf serrations or leaf shape in general. Knotted-1 homeobox gene was found to be confident as the suppression of this gene in different plants showed different results so based on such evidences, this gene was targeted to see the effects of suppression of this gene in Petunia. The BLAST results of this gene showed that it was found in Petunia (Figure 5.9), the sequence is available at NCBI (code: [GQ409544.1](#)) and the complete sequence of this gene was downloaded from [Solgenomics JBrowse](#).

Petunia x hybrida KNOTTED1-like protein mRNA, complete cdsSequence ID: [GQ409544.1](#) Length: 1074 Number of Matches: 3Range 1: 619 to 868 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
335 bits(181)	3e-91	227/250(91%)	0/250(0%)	Plus/Plus
Query 62227172	AAGTGTGAGGGTGTGGATCATCAGAAGAGGATCAAGACAACAGTGGTGGAGAACTGAA	62227231		
Sbjct 619	AAGTGTGAAGGTGTGGATCATCAGAAGAGGACCAAGACAACAGTGGTGGAGAACTGAA	678		
Query 62227232	CTTCCTGAAATAGATCCAAGAGCAGAAGATAGAGAACTTAAGAATCACTTGTGAGAAAA	62227291		
Sbjct 679	CTACCAGAGATTGATCCACGAGCAGAAGACAGAGAACTGAAGAACCATTGTTGAAAAAA	738		
Query 62227292	TATAGTGGCTATTTAAGTAGCCTCAAAACAAGAACTTTCAAAGAAGAAGAAAAAGGGGAAA	62227351		
Sbjct 739	TACAGTGGCTACTTAAGTAGTCTCAAAACAAGAACTTTCAAAGAAGAAGAAAAAGGGGAAA	798		
Query 62227352	TTGCCCAAAGATGCTAGGCAGAAATTGATCACTTGGTGGGAGTTGCACTACAAGTGGCCA	62227411		
Sbjct 799	TTGCCCAAAGATGCTAGGCAGAAATTGCTCAGTTGGTGGGAGTTGCACTACAATGGCCA	858		
Query 62227412	TACCTTCGG	62227421		
Sbjct 859	TACCATCGG	868		

Figure 5.9. NCBI BLAST results of homology of Kn1 gene in petunia.

5.2.2 Designing Molecular Markers

At first, two molecular markers MG1 and MG2 were designed to check whether this sequence is present in the plant under experiment in the lab. These primer pairs were designed at NCBI primer designing tool and the purpose was to amplify the first exon of Kn1 gene. The primer pairs showed positive results which are showed later in this section. Three more molecular markers Kng11, Kng12 and Kng2 were designed later. The purpose of these markers was to amplify a region of the kn1 gene's exon1, which adds an extra at 200bp (at least) before and after the exon. These primers were important to get the complete sequence of the exon before and after the experiment as the sanger sequencing starts gives lesser results than the amplified DNA fragments. So, these extra 200bp were amplified as a control to be able to get complete sequence of the exon1 itself. The details of all these primer pairs and visual demonstration has been given in section 4.2.2.

5.2.3 Results of PCRs, Gel Electrophoresis and Gene Sequencing

Since the primers were newly designed so it took some repetitions of the PCR to finally optimize it and to get good results. Several factors of the PCR were altered to optimize it and literature was studied on how to optimize PCR.

Following figures and discussions explain the PCR optimization and planning for MG1, MG2, Kng11, Kng12 and Kng2 genes. The reference for the bands (fragment size) of the DNA ladder is given in section 4.2.5.

5.2.3.1 Results of MG1 and MG2 primer pairs

PCR and gel electrophoresis results:

One PCR was followed for the **1st PCR** (section 4.2.4.1). Figure 5.10 shows the electrophoresis results of the first reaction where 1 μ M primer concentration was used. It was observed that neither the bands are clear nor the DNA ladder. An off-target amplification can also be observed as the expected size of the band was 319bp and the one in the figure is larger than 1kbp. It was concluded that there could be a few reasons, either the gel was not good, the annealing temperature was not good or the DNA concentration, which was used, was not enough. Because a few articles suggested that 1 μ M primer concentration works fine. So, at this time, the primer concentration remained the same for the next reaction.

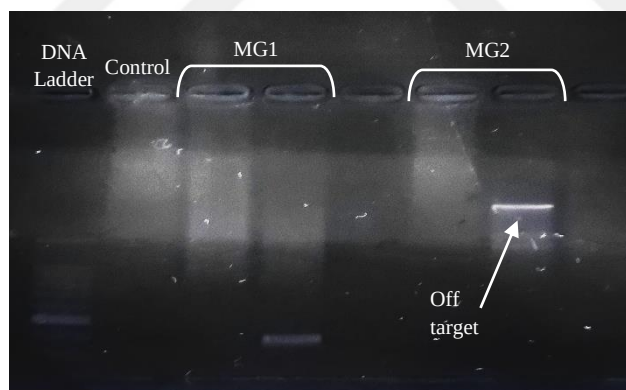


Figure 5.10. 1st PCR protocol with 1 μ M primer concentration.

Several repetitions were followed for **2nd PCR** (section 4.2.4.1). Figure 5.11 shows the results where different DNA concentrations were used. It shows good results for MG2 but for MG1 results were unexpected, as the difference in the fragment lengths of MG2 and MG1 was only 13bp. The bands in the gel should be almost at the same level while it can be observed that the band of MG1A was somewhere around 350bp and MG1B did not appear.

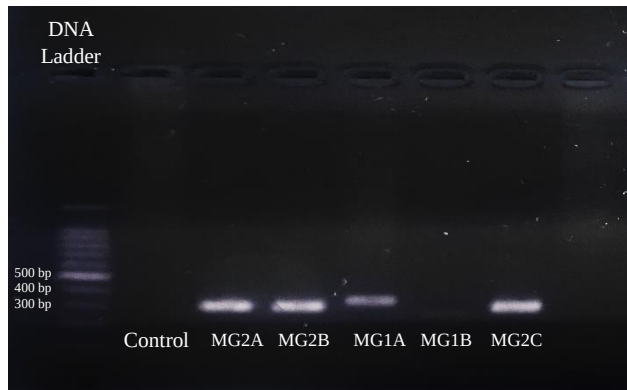


Figure 5.11. 2nd PCR protocol with different concentrations of DNA.

Based on previous results, as MG2 showed better results, a second repetition was followed where annealing temperature was varying. Figure 5.12 shows the results of this PCR and the MG1A (61.5 °C) and MG1B (59.3°C) did not give any result. MG1C (56.4 °C), MG1D (55 °C), MG2A (61.5 °C), MG2B (59.3 °C), MG2C (56.4 °C) had varying results, and MG4D (55 °C) gave one result. It was concluded that either there was off-target amplification or there had been a mistake while carrying the experiment. The same experiment was repeated, and almost similar results were observed. So, literature was studied to further optimize the experiment.

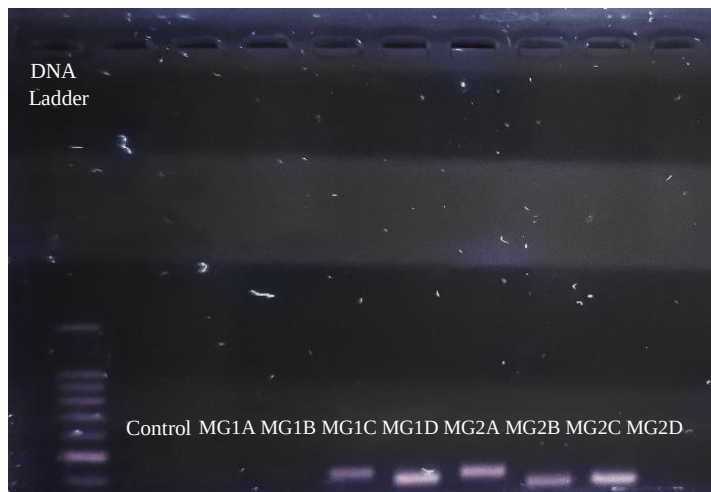


Figure 5.12. 2nd PCR protocol with varying annealing temperatures.

By studying further literature, for the **3rd PCR** (section 4.2.4.1) new working solution of primer pairs was made with 10 μ M concentration. Figure 5.13 shows the PCR results of this new primer concentration while the remaining protocol remained same. All the primer pairs at all the annealing temperatures showed very good and confident results. These results were according to expectations as the bands were aligned correctly at around 300bp fragment length. So, it was confirmed that the sequence of exon1 was present in the plant being experimented in the lab. The same experiment was repeated and Figure 5.14 shows that the same results were obtained which confirms that the PCR had finally been optimized and expected band size was obtained each time.

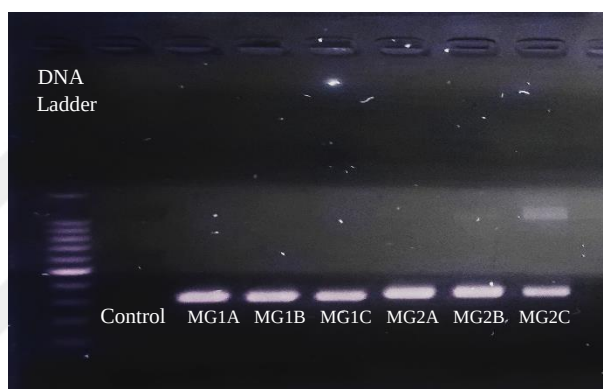


Figure 5.13. 3rd PCR protocol with 10 μ M primer concentration.

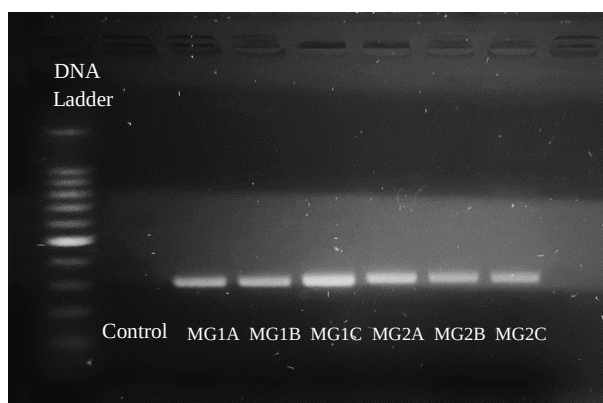


Figure 5.14. 3rd PCR protocol confirmation of the PCR optimization.

Sequencing results:

As explained in section 4.2.7 that the samples were prepared according to the manual of the sequencing companies. From the previous PCR reaction, it was decided that DNA amplification of MG2 primer pair will be sent for sequencing to LGC company. The DNA was isolated from the gel (Figure 5.13) according to the gel excision protocol explained in section 4.2.6. The DNA concentration was checked on nanodrop and it was less than required so the DNA was freeze-dried, and then enough water was added to make required concentration. It was observed that DNA had become heavier or self-ligation might have happened. As the DNA fragment after freeze drying became much bigger. To resolve this problem, DNA was incubated at 94 °C for 5 minutes and was cooled down at room temperature. This DNA was run on the gel again and Figure 5.15 shows the side by side comparison of the DNA fragment after excision from the gel, after freeze drying and after incubation.

After receiving the sequence from the company. The downloading sequence from solgenomics and the sequences of the Kn1 of the petunia under experiment in the lab were aligned together to see the homology. The sequence which was received from the company was not according to expectation and was not complete 319bp. Figure 5.16 shows the visual representation of the homology and many SNPs were observed. The start and end of the exon could not be sequenced and the gRNA1 was present at the ending part of the exon. Many SNPs were observed (highlighted in red). One SNP was also observed in gRNA2 and this one SNO could cause off-targets. So, to be more confident about not having off-targets, the specificity of gRNA was necessary. It was observed in the homology that as the sequence proceeds it had more and more SNPs. Following this trend of more SNPs, it was thought that the gRNA1 might have even more SNPs than gRNA2.

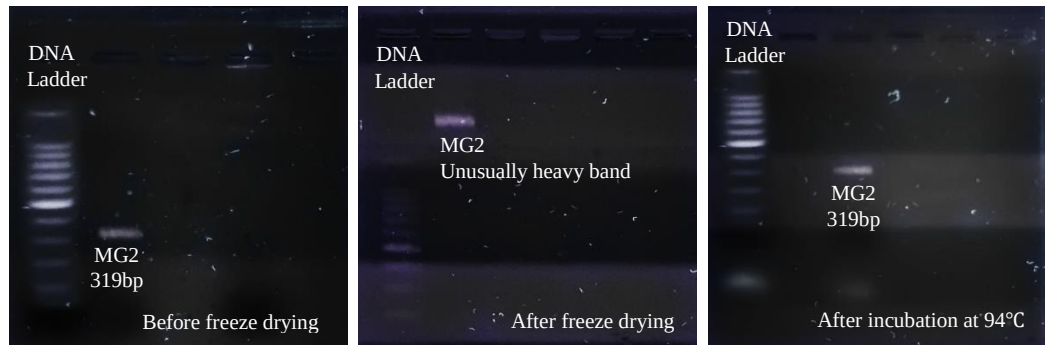


Figure 5.15. Gel electrophoresis of before and after freeze drying and after incubation.

Sequenced Kn1	-----CAAAGAGGTAACCTCTTTTATGGTGGAAATTCAGTTCTTGACCT	45
Sequence from Jbrowse	GGGGAGAACTCAGCTCAAAGAGGTAACCTCTTTTATGGTGGAAATTCAGTTCTTGACCT	60

Sequenced Kn1	CCAAATCTTCTATTTATGATGATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACA	105
Sequence from Jbrowse	CCAAATCTTCTATTTATGATGATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACA	120

Sequenced Kn1	GAAGGAGGCAGCAGCAGTAATACTTCTCATCATCATCATACTTCAATACCCTTCGATA	165
Sequence from Jbrowse	GAAGGAGGCAGCAGCAGTAATACTTCTCATCATCATCATACTTCAATACCCTTCGATA	180

Sequenced Kn1	ATTCGTAGTGGTCATCATCAATCAGACGGTTCATAATCATCATCACGAAAGAGTGAAAA	225
Sequence from Jbrowse	ATTCGTAGTGGTCATCATCAATCAGACGGTTCATAATCATCATCACGAAAGTGAAAA	240

Sequenced Kn1	TCCTTCTGGTGAAAGTGGTGGGC-----	248
Sequence from Jbrowse	TC-TTCTGGTGAAAGTGGTGGGCTTAAAGCCAGAGATTCTGCCCCATCCTCAGTGTTCAG	299
	* ***** *	
Sequenced Kn1	-----	248
Sequence from Jbrowse	TCTTTTGGATGCTTACATGG	319

Key: Non-sequenced; SNPs; Heterozygosity; gRNA1; gRNA2; PAM; Restriction site

Figure 5.16. Homology of the sequenced Kn1 fragment with the downloaded sequences from solgenomics Jbrowse.

The sequence of the gRNA1 was not clear so samples were sent to the company again for sequencing. This time small sequences were received of only a few nucleotides. Upon discussion with the representative of the company, it was found that there could be several reasons for not getting good sequencing, either the DNA is too concentrated, DNA is less concentrated, primer is self-ligating, primer has off-target in the sequence, primer concentration is not good, or there can be some other problem.

The chromatogram of the sequence was analyzed to see the nucleotide's peaks and to understand the problem. Figure 5.17 shows the screenshot of the chromatogram. Another SNP was observed in the chromatogram which was due to heterozygous nucleotide bp. This heterozygous bp was present in the restriction site of the gRNA2 which meant that gRNA2 could have 2 SNPS and 4 possible sequences. These SNPs could greatly decrease the specificity of the gRNA2. So, it was important to get the sequence of gRNA1 and analyze if gRNA1's sequence is without SNPs and if that can be a confident gRNA sequence with high specificity.

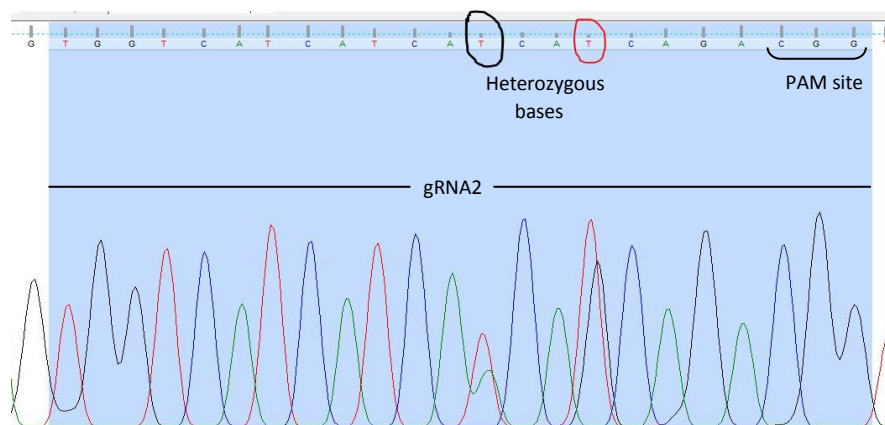


Figure 5.17. Chromatogram of sequence of Exon1 showing heterozygosity.

5.2.3.2 Results of the Kng11, Kng12 and Kng2 primer pairs

PCR and gel electrophoresis results

The new primer pairs were designed to obtain larger sequence of the Kn1 gene. As it is explained in the previous section that sequencing that 319bp exon was not providing sequence of the expected length. It was decided to prepare new primers which may amplify a larger fragment of the Kn1 gene and will be used to send for the sequencing. Figure 5.18 shows the results of the 1st test of the PCR. All the primer pairs were successfully optimized, and all the fragments sizes were according to expectations. The same PCR protocol was followed to confirm the results and exactly same results were obtained. At this point PCR protocol was successfully optimized and it was also discovered that annealing temperature does not always disturb the reaction as in this case the primer pairs worked on each given temperature.

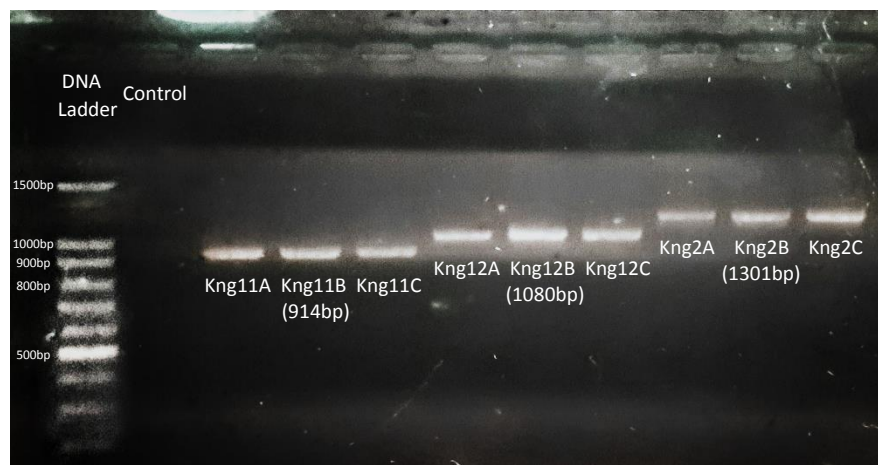


Figure 5.18. PCR amplification of Kng11, Kng12 and Kng2 primer pairs.

Primer combinations were made to amplify even a larger segment of the Kn1 gene. As the samples were to be sent to Eurofins company, it was decided to send a larger segment to have all the sequencing at once. All the possible combinations were tried to avoid the possibility of primer-dimer formation or primer-incompatibility. The PCR results of the primer combinations were also very good. Figure 5.19 shows the results of each primer combination, and all the amplifications were according to expectations and all the primer combinations amplified the targeted part of the gene at both annealing temperatures. So, once again, it was observed that annealing temperature is not a key factor for success of a PCR. As all the primer combinations were according to expectations, further experimentation was decided based on these results.

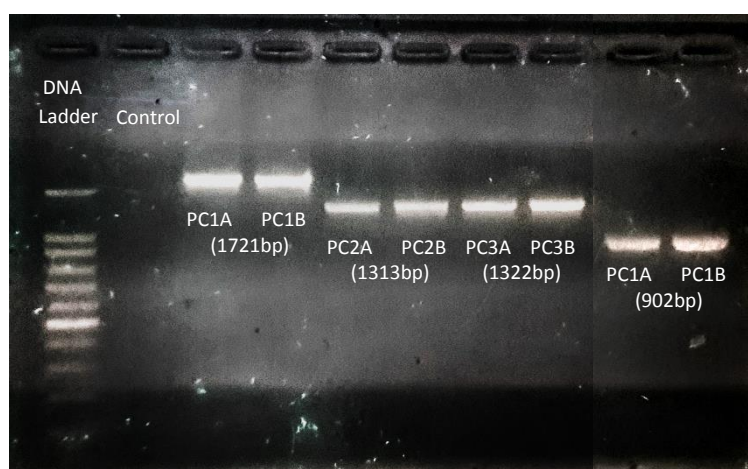


Figure 5.19. PCR amplification of the primer combinations of Kng11, Kng12 and Kng2.

PC1 and PC3 were chosen to be amplified and sent for sequencing to LGC sequencing company. 100µl PCR mix was made for each primer combination. PCR protocol was run as previous, amplifications were observed on the gel after electrophoresis, DNA was excised from the gel (section 4.2.6). The excised DNA was then run on the gel to confirm the excision (Figure 5.20). Details of the samples sent for sequencing are in section 4.2.7.

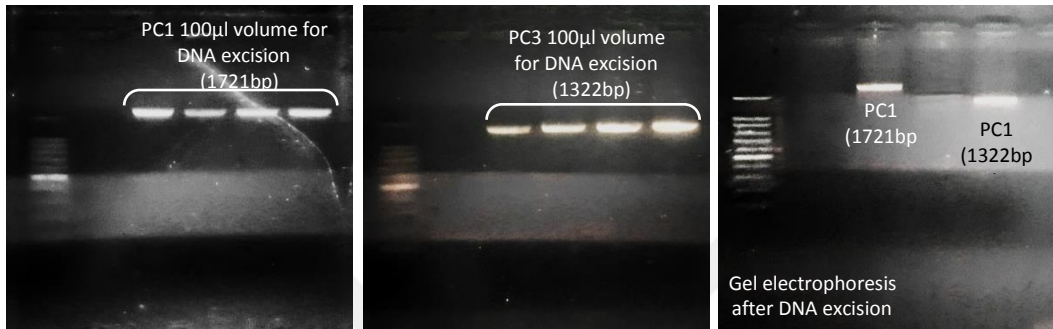


Figure 5.20. Amplification of PC1 and PC3 and gel electrophoresis after DNA excision.

Sequencing results:

The results of this sequencing were also not good. The comparison of the freeze-dried samples with the samples which were not freeze dried could not justify the reason of not getting a good sequencing. The company representative answered that the DNA concentration might not be good. Out of all the sent sample only few samples were amplified for sequencing and very short sequences were obtained as shown in Table 5.1.

Table 5.1. Sequences received from the Eurofins company

Sample	Sequence
Kng11 with reverse primer	GGCACACCCAATAGTGAAAACAGAAAGGAGGCAGCAGCAGTAATACTTCTCATCATC ATCATACATTTCAATACCCCTTCGATAATTTCGTAGTGGTCATCATCAACAGCAGACGG TTCATAATCATCATCACGAAAGTGAAAATCTTCTGGTGAAGTGTGGCTA
Kng12 with forward primer	CTCTGATGATATTTTCATGCCTCATCTTTAATTAAGATACAAAACTTCTTCACTTCAA CTTCTCCTTATCTAGTACACATCCAATTCATTACTTTTACCAATAAAAAAAGAGATCT CCAAGTGTGAATTAACGTGTGCTACTACTAC
Kng12 with reverse primer	TACGAATTTTATTGCAATATCATTGAAAATAAATACTACCAATA
KnG12F	CTCTGATGATATTTTCATGCCTCATCTTTAATTAAGATACAAAACTTCTTCACTTCAA CTTCTCCTTATCTAGTACACATCCAATTCATTACTTTTACCAATAAAAAAAGAGATCT CCAAGTGTGAATTAACGTGTGCTACTACTTC
PC3 with forward primer	CTGTATATTTTTTCTAAAAAATCAATACATGCAATTATATTGTCACTATAAGTA TTTAAGTAATTGATGATCGAGATAAATATGCTAACATGTTTTTACATGTTAAACCAT GTTTATAATGTGAAACGCTTTTATATATTTTGTAGTACTTAATCCCATTTGGTATAT AGGTAAAAATAATAACAATCTGTTGTCATATGTTAAACTATACTAACGTGTAAT

After all these sequences results, the final homology was checked. The chromatograms of each sequence were analyzed, and it was hypothesized that the targeted gene Kn1 in the plant under experiment is probably heterozygous and that is why there are so many SNPs in the sequence. This heterozygosity might be the potential reason that the sequencing was not successful and after a few nucleotides the chromatogram always shows multiple peaks and no further single nucleotide peak is observed Figure 5.21.

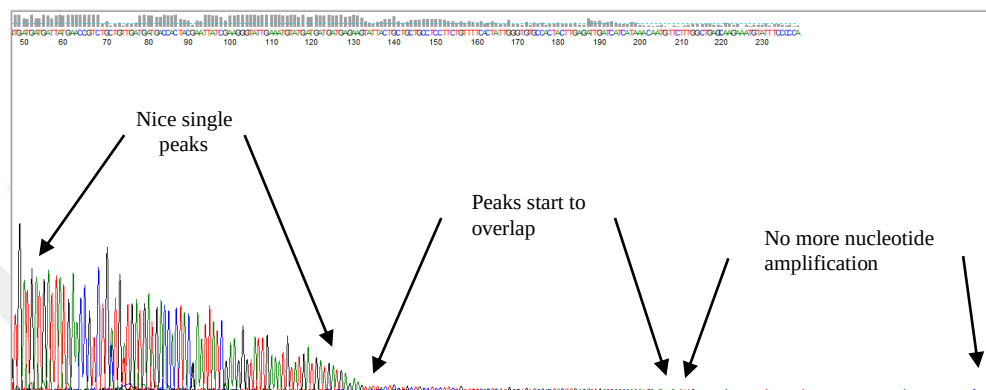


Figure 5.21. Chromatogram of a heterozygous genomic sequence.

Homology of the Sequence:

According the above explained repetitions of sequencing the gene, following homology could be confirmed. As it can be observed that multiple parts of the targeted gene had been sequenced but complete sequencing could not be achieved due to the presence of heterozygous bases and SNPs. As a next step, PCR cloning technique was followed to get the sequence of each allele separately. PCR cloning technique is discussed in the next section.

Homologous (exactly same); heterozygous bases; SNPs; Targeted Exon; *gRNAs*

TGTATGAATAAAAGCTAAGATAAAATATGGAAGAAAGGAAAGTAGAATGAAAAAGTAACAAAATAGAAG
AGAAGTAGAAAGCAACAACAACAAAAGTAACATATAAAGGAATATGAATACTTCACCACATACAAAGGAGA
ATATGCCTGCCTATCTACTACTCTTCTATCCAAATCTTCGGCCTCCATCTTCTCTTAAATAGGAACATAATATA
TTTTTTTCTATAAAAAATCAATACATGCAATTATATTTGTCACATAAGTATTTAAGTAATTGATGATCGAG
ATAAATATGCTAACATGTTTTACATGTTAAACCATGTTTATAATGTGAAACGCTTTTATATATTTTGTAGT
ACTTAATCCCATTTGGTATATAGGTAAAAATAATAACAATCTGTTGCATATGTAAAACTATACTAACGTGT
AATTAAATAATTTTGGCTGGACATATTAATTATTTTCAATAATATTAACCTTAAAAGTCAAGAAATTGTTTGATT
TAGCCAAAGAAAAATATGGAAGGAAGAAGTCCCAAGAAAAAGAAAAAGAGGAGCTTAATGCCCCCGTG
AAAGCAATGGTAAAAAGCAAGGGGAAAAAAGTTGGAGGAGAAAAAAGAGAGAGAAGAAGTTC
CCTTTAACATTGAGTAGGATCATAGGGGAAATAGTAGTAAACCATAAATTTAATATATTTAATTTCCAATACA
AATATTTATTTATTCTCAACGAAGAAGCTTGTACCCACGGAGCCGCTACCAGCAGTGGCCACAAGCTT
CTCTTTCCCAATATGTTTCTTGTCAAAGCCACCATCTTGTAGATCTCTACCCTTTTATCCTCTAATGATAAT
TTCATGCCCTCATCTTTAATTAAGATACAAAACTTCTCACTTCAACTTCTCCTTATCTAGTACACATGCCAATT
CATTACTTTTACCAATAAAAAAAGAGATCTCCAAGTGTGAATTAACTTTTGCTACTTCTGTTCTAGTTACTA
GTTTATCCTATATTGTTGGGATAGTTGAAGCATAATAAGTCTTGGCTTTCTTAGGGTTTCTTACTAGTTCAAC
TGTTTATTATCTTCTTGATTTAGGGTGTCTTTATTTTAATTACTACTAGTAACATATTTTGGAGAAAAATG
GAGAATTATAATCAACTAGGGGAGAACTCAGCTCAAAGAGGTAAGTTCTT
TTATGGTGGAAATTCAGTTCTTGCACCTCCAAATTCTTCTATTTATGATG
ATCAATCTCAAGTAGTGGCACACCCAATAGTGAAAACAGAAGGAGGCAG
CAGCAGTAATACTTCTCATCATCATACATTTCAATACCCTTCGATAAT
TCGTAGTGGTCAATCAACATCAGACGGTTCATAATCATCATCACGAAAGT
GAAAAATTC(C)TTCTGGTGAAGTTGTGGCTTAAAAGCCAAAGATTCTTGCCC
ATCCTCAGTGTTCAGTCTTTTGATGCTTACATGGACTGTCAAAAGGTATA
AACACTTTTTTCTCAGATGTATATATAACAACATACTCAGAATACGGAGTGGTTCCTTTATTTATTTTGTG
TCCTTTTAACATACCATAAGGAATTAATTGACTTGGAAAAAATTATTCTTCGTCTCCGTACTTTCTTCAG
ATATTATTAAGATTTTATTGCAATATCATTGAAAAATACTACCAATAAAATACTCATGTAAAGTGTACTTAAT
TATCTATATGGAATAAAGAAAGTCGAACATGTTAGACGAAGTTAAATTATGTGTAAATATATTATTACAG
TTGTGAGTATATATTTTTCATAGGTGGGAGCACCGCTGAAGTGATGGCGAGGCTATCCACTTTACGGCAAG
AGTTTGAGATGAGGCAGCGAGCTTCATTGACCGGAAAAGATGTTGCCAAGGACCCAGAACTTGATCAATTCA
TGGTCAGTTTATTTTAACTTTTAAATCTCGTAATATATTTAAAT

5.2.4 Results of PCR cloning

PCR cloning was successfully done by following the protocol given in section 4.2.8. Figure 5.22 shows the growth of the bacteria in the control plates and in ligation vector plate (ligation of the PCR product of Kng12 primer pair with the pJet blunt vector provided in the kit of PCR cloning). Two plates were incubated for each ligation to observe the efficiency of the competent agrobacterium. 1X ligation plates show that there were 10 times less bacteria compared to the 10X plates. This 10 times difference in the concentration of bacteria was performed to have good results in case bacteria were not very efficient and at least some colonies could be found in 10X plates. However, all the plates show that the bacteria were very efficient, and many colonies were formed.

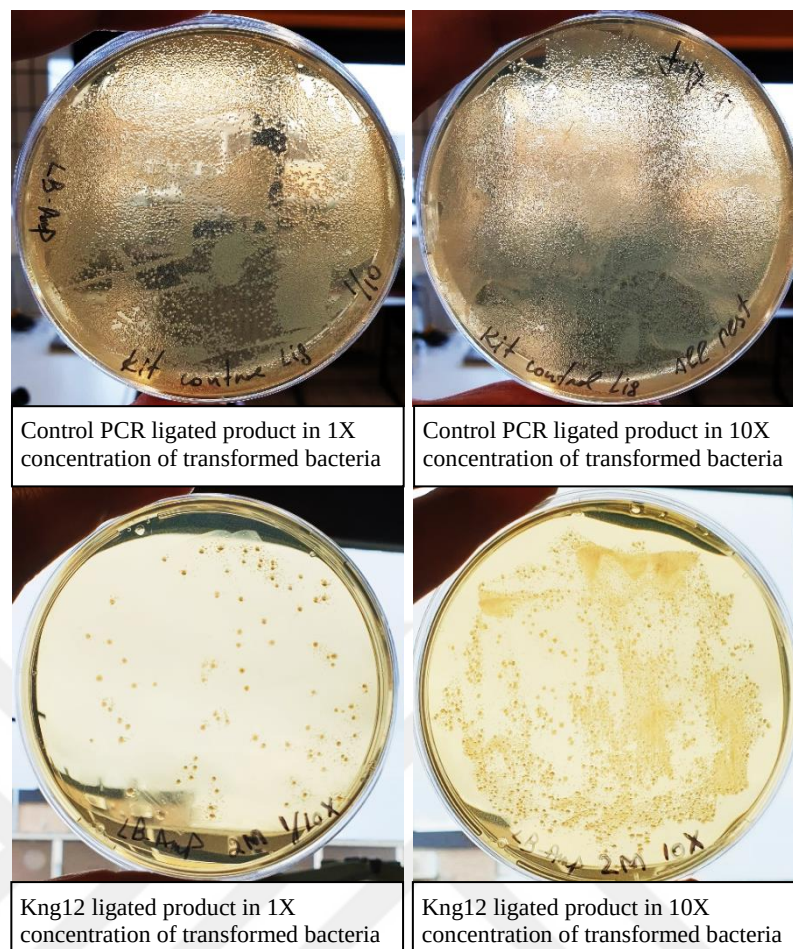


Figure 5.22. Growth of transformed *Agrobacterium* on LB media plates.

It was interesting to know that the pJet cloning vector has resistance gene to ampicillin and also has a suicidal gene in it which gets silenced if the ligation reaction is successful. If, somehow, the ligation is not successful, the suicidal gene kills bacteria after transformation. This was indeed a very intelligent technique to only let grow the transformed bacteria which means that all the colonies which are visible in the Figure 5.21 are transformed and there is not even a single colony which grows without the ligated gene. Further, ampicillin in the LB media kills any other contaminant and does not allow non-resistant bacteria to grow. So, it acts as a two-way control system. The suicidal gene kills all the non-transformed bacteria while the ampicillin kills any other contaminant. Thus, leaving the colony only with the transformed bacteria. However, there could be some other possibilities i.e. frameshift at the insertion point of the vector can still switch-off

the suicidal gene and bacteria will grow on LB plate but there will be no ligated gene.

12 single cell bacterial colonies were grown on another LB plate as shown in Figure 5.23 All the single cell colonies grew very well and were used for plasmid isolation and sequencing.

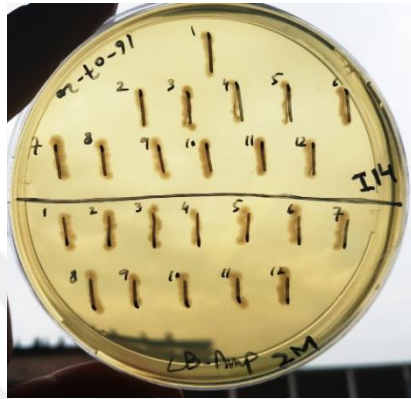


Figure 5.23. Growth of single cell colonies on LB plate.

6. DISCUSSION

Tissue culture of Petunia: Just like any other plant species, tissue culture of petunia can be very simple or highly complicated. It was observed that the success in tissue culture is highly dependent on the genotype of the plant. Different genotypes show different response to tissue culture growth (Compton et Koch, 2000). It was observed that petunia mutates very easily even while reproducing by asexual means. These mutations may occur while the plant is stressed during the time of regeneration (Evans and Sharp, 1983). The petunia plant being used in the lab was already heterozygous and had been regenerated several times. While its preferable to use an inbred line of the plant for such kind of experiments of CRISPR gene manipulations, the heterozygosity of the genome might exhibit off-target mutations resulting in various responses (Zhang et al., 2014). The results of the petunia being used in the lab were no different. The shoot cuttings from the same parent plant, while grown in MS media showed different growth pattern. Some shoots grew longer with small leaves while some made bigger thick leaves. These alterations could also have happened due to the difference in the micro-climate of each growing pot. However, within the same pot, different growth patterns were observed as well (Evans and Bravo, 1986). The production of chimera of petunia was also happened either phenotypically or there was a mutation (Marcotrigiano, 2001), as the parent plant from where chimera shoot cutting was taken, showed no signs of chimera. The production of hyperhidrosis also showed the phenotypic changes in petunia growth which was mainly happened due to oxygen stress (Csete, 2005). This provides an evidence that stress can change the phenotype of the plant quite easily but overall results of shoot regeneration from stem cuttings provided positive results.

During the leaf initiation of petunia, difference in responses was observed. While in literature growing petunia from leaves is quite simple (Trigiano and Gray, 1999), performing the same experiments in lab provided not so confident results and no visual growth of shoots was observed. The genotype of the plant, incompatible growing media, mutations in the plant, or unfavorable growing condition (Michalczyk and Michalczyk, 2000) could be a reason of unsuccessful growth of petunia leaf initiation. However, the callus formation was rather

successful in almost each experiment. The leaves almost always turned to purple color which was due to the stress. This provides an evidence that leaf initiation should always be optimized for specific genotypic samples of the plants, and each different genotype of the same species might need a different growing media or growth environment (Compton and Koch, 2000).

Literature study provides evidence of the success in *Agrobacterium* transformation as well as controlled protocol for successful transformation (Fraley et al., 1986; Koetle et al., 2015). However, the transformation during petunia leaf co-culture was not successful. In this experiment the leaves also did not turn purple, rather the leaves stayed green for almost two weeks and even callus growth was not observed. This could be because of the *agrobacterium* were not virulent, the plant did not accept the GFP plasmid, unfavorable growth conditions were provided or the defense system of plant resisted the transformation (Lin et al., 1995; Opabode, 2006). Leaf infiltration method of *agrobacterium* transformation provided to be highly efficient and quick method to incorporate the gene of interest in the plant cells. This provided the possibility that CRISPR-Cas9 construct will also work efficiently by following this method and later by sequencing, the gene-knockout can be observed.

PCR amplifications and gel electrophoresis: Optimization of PCR showed varying results which provides an evidence that several factors can affect the success of a PCR amplification. These factors might be coming from the primer pair itself, non-specificity of the primer pair, off-target amplifications, poor PCR protocol, unfavorable annealing temperature, DNA quality and concentration, the composition and concentration of agarose gel or there might be a problem in the loading or fluorescence dye. (Nations, 2000). However, in the experiments, it was observed that primer pairs work on a range of annealing temperature and it does not have to be very specific. Primer concentration and primer specificity on the other hand play, a specific role in PCR success. The PCR results may also go wrong due to human error. It was also observed that sometimes the PCR results are good, but the gel electrophoresis may happen (Bustin et al., 2020). In the experiments and repetition of the experiments conducted in the lab, the PCR protocol, was finally optimized and it was observed

that if some basic principles are followed, PCR almost always provided good results. Another observation was made that extensive care should be taken while designing the primers, as it is the first step and if anything goes wrong during the designing of the primers, no results would be obtained.

Sequencing of the Kn1 amplified fragments: The sequencing of the genomic fragments has become much easier and cheaper as compared to past. The method is simpler, convenient, accurate and almost anyone with basic knowledge can follow (van Dijk et al., 2018). Both companies, LGC genomics and Eurofins use Next generation sequencing (NGS) (Kulski, 2016). For the sequencing of the amplifications of MG1 and MG2 primer pairs, several samples were sent to the company, but not so confident results were obtained. It was thought there might be error in the DNA concentration, the primer specificity or primer-dimer formation (Wang and Song, 2017). Another possible reason of not getting good sequencing was also thought to be the freeze-drying of DNA as it was making the amplified fragment heavier. However, no confident literature was found on this possibility. Another possibility was thought to be that the company might not be efficient in sequencing and it was decided to try with another company.

The samples of amplifications of Kng11, Kng12 and Kng2 primer pairs and the samples of amplifications of PC1 and PC3 primer combinations which were sent to a new company, also did not provide reliable results. After another try, the chromatograms of each fragment were analyzed and compared. It was found that the gene was showing many SNPs. Further comparison of the chromatograms revealed that most of the SNPs were indeed heterozygous in the sequence of one amplified fragment compared to another amplification of the same fragment. This provided an evidence that the gene was indeed heterozygous at many points and so the sequencing was always being interrupted due to such heterozygous bases (Fujimoto et al., 2014).

To resolve these problem, two possible solutions were considered. First solution was to design new primer downstream and upstream of the gene until where the sequence has been received. The problem in this was to design new primer every time, not each sequence in the gene was good for making primers

and more time consuming and costly. As a second solution, PCR cloning was decided to clone each allele separately and then get the sequence of each clone.

PCR Cloning of amplification of Kng12: The amplification of Kng12 primer pair was decided to clone by PCR cloning as this fragment was covering enough length of the gene upstream and downstream of the gRNAs. The results of PCR cloning were promising, and single colonies were observed on the LB plates. These single colonies contained the individual copy of each allele so now it was easier to get the sequence of each allele after plasmid isolation (Jansen and Ledley, 1990).



7. CONCLUSION

It was concluded that for conducting such type of experiment, the choice of the parent plant is one of the most important factors. It is easier to conduct such experiment on an inbred line as there would not be heterozygosity and there will be less chance of gRNA off-targeting as it was observed in our study. The tissue culture of petunia must be established according to the genotype as the plant might show different response than expected. Petunia is subjected to mutations very easily and it also alters the response of plant towards different types of growth medias and growing environment. The primer designing and PCR protocol for this experiment was rather successful and showed confident results. It must be taken care of that the primer designing has certain rules and limitations which must be followed to get accurate results. The PCR protocol on the other, is always decided based in the primer characteristics and the purpose of the PCR. Gel electrophoresis is a comparatively simpler technique but human error or little deviation in composition might degrade the expected results. In our study, after a few optimizations, very good PCR results were obtained. If quick results of CRISPR-Cas9 are required, leaf infiltration method provides very efficient results. But care must be taken, that in this case, gRNAs must have restriction site and no off-targets. Leaf infiltration method makes the designing of gRNAs even more specific. In our study, it was very important to completely sequence the Kn-1 gene to design highly specific gRNAs due the limitation of RE sites. Lastly, for the sequencing of the heterozygous genes, it is easier to separate each allele by PCR cloning method and to sequence the alleles separately. This experiment clearly shows the scientific method of planning, experimenting, failure, planning, optimizing, hypothesizing, and acquiring results was followed. The whole experiment was greatly affected due to COVID-19 and many activities were slowed down. Further research must be conducted to optimize the growth of petunia under different climate situations as well as to resolve the issue of acquiring sequence of a heterozygous gene to further construct the CRISPR-Cas9 experiment.

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16 / 09 /2020

A handwritten signature in black ink, appearing to read 'Muhammad Moazzam' with a stylized flourish at the end.

Muhammad MOAZZAM

CURRICULUM VITAE

Muhammad Moazzam was born in Sheikhpura, Pakistan on 22nd April 1996. He went to Govt. High School Sheikhpura and Punjab College Sheikhpura for his secondary and intermediate education, respectively. In 2018 he graduated from University of the Punjab, Pakistan with degree in B.Sc. (Hons.) Agriculture. From 2018-20 he studied plant breeding under Erasmus Mundus Master Program in Plant Breeding (emPLANT) from Institut Polytechnique UniLaSalle, France and Ege University, Turkey as a joint master degree program.

