OPTIMIZATION OF CRISPR/CAS9 GENE EDITING IN POTATO

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ABSTRACT

OPTIMIZATION OF CRISPR/CAS9 GENE EDITING IN POTATO

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Increased human population, climate change, and reduced water supply are demanding creative approaches in agricultural production. Now, a new method of gene editing may allow needed improvements possible.

This gene editing technology is called CRISPR / Cas9 technique which is less demanding than that of the other programmable editing technologies such as ZFNs and TALENs. The CRISPR / Cas9 model is developed based on the innate immune system observed in bacteria and archaea that directly degrades nucleic acids of bacteriophages. The safe and effective editing of potato genome especially against disease causing pathogens is of a great value in agriculture. The objective of this research is to explore the capacity of CRISPR / Cas9 technique for editing two potato genes; eukaryotic translation initiation factor 4E (eIF4E) and vacuolar invertase enzyme (VInv)). The eIF4E when mutated generates Potyvirus X defense, VInV prevents acrylamide synthesis, thus allowing long-lasting taste and safer food alternative against cancer. For both of the genes guide RNAs were engineered and were tested for effectiveness of generating mutations. In this research, gene editing is planned to be conducted in potato protoplasts. A high percentage of intact and healthy potato protoplasts were obtained by use of one-month-old plantlets grown in soil, long dark treatment (four days), and lower centrifugation speed during the isolation. Overall, it was possible to set up CRISPR / Cas9 tests in vitro and optimize in vivo studies with the use of better quality potato protoplasts that can be used for regeneration.

Keywords: Potato (S. tuberosum), gRNA, CRISPR/Cas9, eEIF4E, Vlnv

ÖZ

PATATESDE CRISPR/CAS9 GEN DÜZENLEME YÖNTEMİ UYGULAMASI

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Büyüyen nüfus, iklim değişikliği ve su temini gibi değişkenler nedeniyle, tarım sektöründe yeni gereksinimler son yıllarda artmıştır. Bu nedenle, daha az depolamada daha fazla yemek üretmek çok büyük bir sektör haline gelmiştir.

Bu genetik mühendisliği yönteminin önemli bir bileşimi var. Bazı deneyler, CRISPR / Cas9 tekniğinin ZFN'ler ve TALEN'ler gibi diğer programlanabilir nükleaz yöntemlerine ulaştığını göstermiştir. CRISPR / Cas9 modeli, kısa ribonükleik asit (RNA) kullanarak denizaşırı nükleik asitleri doğrudan parçalayan bakteri ve arkada gözlenen doğal bir bağışıklık sistemi üzerine kuruludur. Deoksiribonükleik asit (DNA) patates genomu güvenli işlemesi tarıma elverişli olabilir. Bu araştırmanın amacı, CRISPR / Cas9 tekniğini kullanan, DNA'sız genetiği değiştirilmiş bir organizma (GDO), patates üretme kapasitesini araştırmaktı. İki aralayıcı (20 bp; iki patates referans genini hedefleyebilir; ökaryotik translasyon başlatma faktörü 4E (eIF4E) ve vacuolar invertaz enzimi (VInv)), iki tek kılavuz RNA (sgRNA) taşıyan genleri oluşturmak için alt klonlandı. Transkriptler, sgRNA'lardan ve Cas9'dan in vitro transkripsiyon kullanılarak üretildi. Cas9 proteini ve sgRNA'ları, konfigürasyonu onaylamak için in vitro olarak karıştırıldı ve Cas9 proteininin, eEIF4E ve Vlnv PCR materyallerini seçme ve sindirme kapasitesini gösterdi. Cas9 transkriptleri ile kombinasyon halinde sgRNA'lar kullanarak az miktarda korunmuş protoplast ile düzenleme, bu önlemleri in vivo olarak gerçekleştirmeye çalışıldı, ancak bu başarısız oldu ve daha fazla optimizasyon gerektirdi.

Anahtar Kelimeler: Parates (S.tubersoum), gRNA, CRISPR/Cas9, eEIF4E, Vlnv

Dedicated to my past and future

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LIST OF ABBREVIATIONS

AmpR – antibiotic, amphicilin resistance

BP - base pair

BSA - Bovine Serum Albumin

CCD - change-coupled device

CRISPR/Cas9 - clustered regularly interspaced short palindromic repeat (CRISPR)- associated protein9 (cas9)

crRNA – CRISPR-derived RNA

CTAB - cetyltrimethyl ammonium bromide

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphates

DSB - double-strand break

EIF4E - Eukaryotic translation initiation factor 4E

GMOs – genetically modified organism

gRNA – guide RNA (also referred to as a single guide RNA "sgRNA")

HDR - homology directed repair

IPTG - Isopropyl β-D-1-thiogalactopyranoside

KanR - antibiotic, kanamaycin resistance

Kb - kilo base pair

LB - Luria-Betani

MCS - multiple cloning site

MEGA - Molecular Evolutionary Genetics Analysis

MES - 2-(N-morpholino)-ethanesulfonic acid

Mg2+ - magnesium

MLO - MILDEW-RESISTANCE LOCUS

MS – Murashige and Skoog

NHEJ - non-homologous end joining

ORI - origin of replication

PAM – protospacer adjacent motif

PCR – Polymerase Chain Reaction

PDS - phytoene desaturase

PEG - polyethylene glycerol

PET - pre-enzyme treatment

RGENs - RNA guided endonucleases

RNA - ribonucleic acid

SDS-PAGE - sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SIPIF4 - phytochrome interacting factor PIF4 (Solyc07g043580.2.1)

SSN - sequence-specific nuclease

TAE - tris-acetate-EDTA

TALENs - transcription activator-like effector nucleases

TBE - tris borate EDTA

TGS - tris-glycine-SDS

tracrRNA - trans-activating CRISPR RNA

Vlnv - vacuolar invertase

ZFNs - zinc finger nucleases

CHAPTER 1

INTRODUCTION

1.1 DNA free editing

Due to growing population, climate change, limited water resources, and other difficult circumstances create an urgent need for fresh agricultural developments for producing more products in less storage while preserving the ecosystem and obeying the local or international laws for the production and the consumption of genetically modified organisms (Kanchiswamy 2016). Genetically modified organisms (GMOs) have been accessible for more than two centuries and are thought to accomplish many of these objectives, but GMOs are not permitted owing to rigid legislation in many nations (Wunderlich and Gatto 2015). This has resulted in a rise in the search for renewed and more appropriate techniques that can both fulfill these obstacles for the future and meet the requirements established by the regulators.

1.1.1 Cell function

The cell includes several parts containing genetic information. Most of the genes are discovered in the nucleus that is the cell's command center. Deoxyribonucleic acid (DNA) is arranged as chromosomes in the nucleus. Chromosomes comprise genetic information in as a lengthy molecule of DNA with several proteins that create up a chromatin structure. An illustration of a plant cell is shown in Figure 1.1.

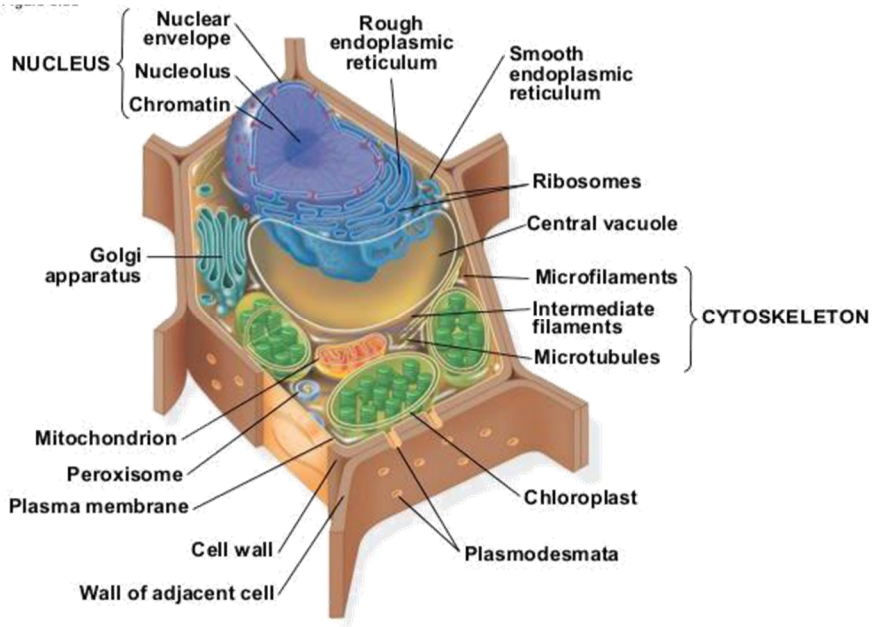

Figure 1.1. Eukaryotic Plant Cell (Reece et al. 2011)

Genetic mutations are crucial for studying cellular fuctions. Many significant biological mechanisms were explored and understood by analyzing natural mutants. However, random mutagenesis can contribute to many undesirable mutations and it is therefore restricted (Ma et al. 2016), but inverse genetics can be implemented to allow particular genes to be targeted in order to study their roles. Reverse genetics allows first the the mutant phenotype to be discovered. The point of departure for reverse genetics is to discover the set of proteins and end with the mutant phenotype (Griffiths et al. 2000).

1.1.2 Genome editing methods

Alternative technologies has been rapidly increasing to solve the challenges of spontaneous mutagenesis. Four techniques of genome editing are used widely: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic

repeat (CRISPR) -associated protein 9 (Cas9) (Yin et al. 2017). These methods use inverse genetics where in vitro or in vivo random mutagenesis is conducted.

Programmable nucleases such as ZFNs, TALENs and Ribonucleic Acid (RNA) guided endonucleases (RGENs) may promote genome processing, by enhancing the effectiveness of homologous recombination. These techniques both take time and there are several challenges in optimization (Ma et al. 2016).

ZFNs were the first installment of sequence-specific programmable nucleases (SSNs) that offered a major advance in the domain of genome processing. Double-stranded breaks (DSBs) could be caused by SSNs at chromosomal locations that could be restored either by the error-prone non-homologous end joining mechanism (NHEJ) or the homologous directed repair (HDR) mechanism (Symington and Gautier 2011). For instance, ZFNs have been used to edit plant genomes, but this method is restricted owing to problems in manipulating different species and the cost (Ramirez et al. 2008).

The other instrument for genome processing, TALENs, has been developed from knowledge gained studying Xanthomonas (Moscow and Bogdanove 2009). In essence, Xanthomonas bacteria secretes TAL effector enzymes through the stage III secretion system when interacting with a foreign entity such as viruses. To withstand the attack, the bacteria mutate genes of the pathogen (Malzahn et al. 2017). Although this technique has been much easier to use than ZFNs, complicated construction of tandem repeat domains of TAL proteins is required (Ma et al. 2016).

On the other hand, CRISPR-Cas9 technology has been under inquiry in many distinct fields in recent years. This technique has been shown to greatly enhance the capacity of many distinct species to alter genes (Kanchiswamy 2016).

1.2 CRISPR/Cas9

1.2.1 Natural immune system in bacteria

The CRISPR / Cas9 technique is focused in bacteria and archea on a innate immune system using brief RNA to guide overseas nucleic acid degradation. Since 1987, CRISPR has been recognized in the Escherichia coli population for the first time (Ishino et al. 1987). CRISPR chains are key elements of the immune system of bacteria. It consists of brief palindromic DNA clusters that have between each loop so-called spacers. These are distinctive spacers. The Cas genes are various genes associating with CRISPR and making Cas proteins (Jinek et al. 2012; Wiedenheft et al. 2012). These are both DNA-unwinding helicases and DNA-cutting nucleases.

There will be several reactions in this framework when a bacterial phage assaults the bacteria. Figure 1.2 (Lab 2012) illustrates the system for this method. The spacers will work as the genetic memory for prior bacteria assaults and either acknowledge or activate the bacterial phage. If the spacer is enabled, the overseas DNA will be copied to the CRISPR framework. This will provide a library of short CRISPR-derived RNA (crRNAs) containing the additional structure of the attacking nucleic acid (Jinek et al. 2012; Wiedenheft et al. 2012). Figure 1.3 (Reis et al. 2014) provides a more comprehensive overview of this method.

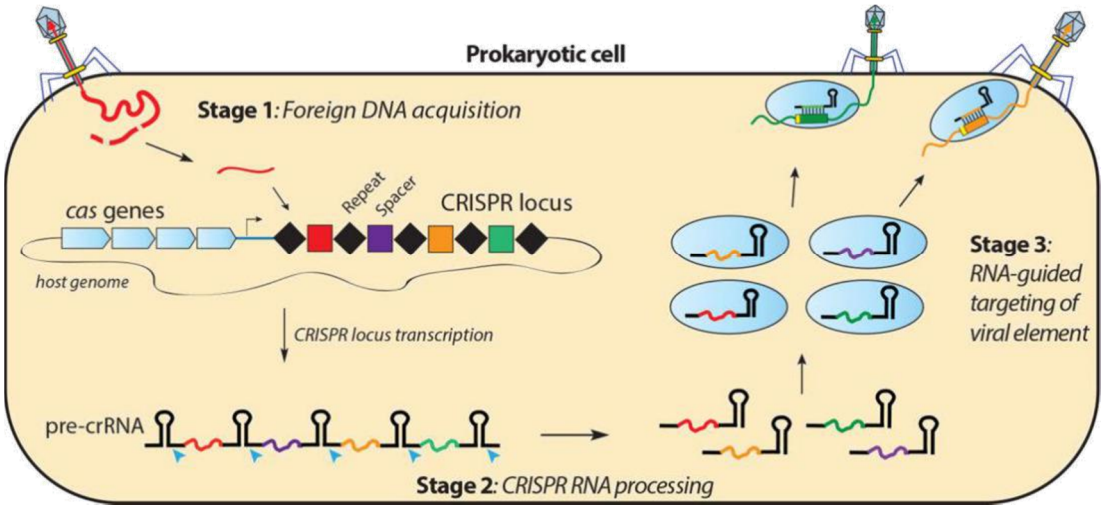

Figure 1.2. CRISPR mechanism in a prokaryotic cell (Lab 2012). The CRISPR mechanism reacts when a bacterium is assaulted by a bacterial phage. CRISPR consists of brief palindrome DNA repetitions with so-called separators between each repetition. These are special spacers. The Cas genes are various CRISPR related genes that produce Cas proteins. Such spacers act as the genetic memory of preceding bacterial assaults and acknowledge, or activate, the bacterial phage. That foreign DNA will be copied into the CRISPR when the spacer is enabled. This gives a collection of brief CRISPR-derived RNAs (crRNAs) which are supplementary to the attacking nucleic acid sequences. Figure 1.3 provides a more comprehensive overview of the system

Three CRISPR editing enzymes were found, the most researched being class II (Makarova et al. 2011). CRISPR category II was discovered in Streptococcus pyogenes (Jinek et al. 2012) and is believed to be dependent on only one enzyme (Cas9 (formerly Csn 1)) for overseas DNA gene silencing (Sapranauskas et al. 2011). It is believed that Type I and III are more complex and retain some prevalent characteristics as well. They have dedicated Cas endonucleases that digest pre-crRNA until it matures before each crRNA forms a big protein complex that can acknowledge and cleave nucleic acids in addition to crRNA (Jinek et al. 2012).

Another method shown in figure 1.3 (Reis et al. 2014) will happen in Type II structures.

1.2.2 CRISPR Type II System

Acquisition of overseas DNA at the CRISPR loci is the first phase of CRISPR-mediated protection (Wiedenheft et al. 2012). When a bacterium is assaulted by a virus, two kinds of short RNA will be generated. This will occur when CRISPR loci during crRNA biogenesis is translated and transferred into crRNA. CrRNA will comprise an attacking nucleic acid series. These two RNAs create a cluster of Cas9 proteins during the disturbance stage, a nuclease that breaks DNA. To accomplish site-specific DNA identification and cleavage, the interaction between Cas9, crRNA and a distinct tracrRNA, which is partly supplementary to crRNA, is required. The corresponding structure recognized as the RNA manual (gRNA), which is a crRNA structure and trans-activating CRISPR RNA (tracrRNA) consisting of a series of 20 nucleotides (spacer), will discover its destination within the host community (Jinek et al. 2012).

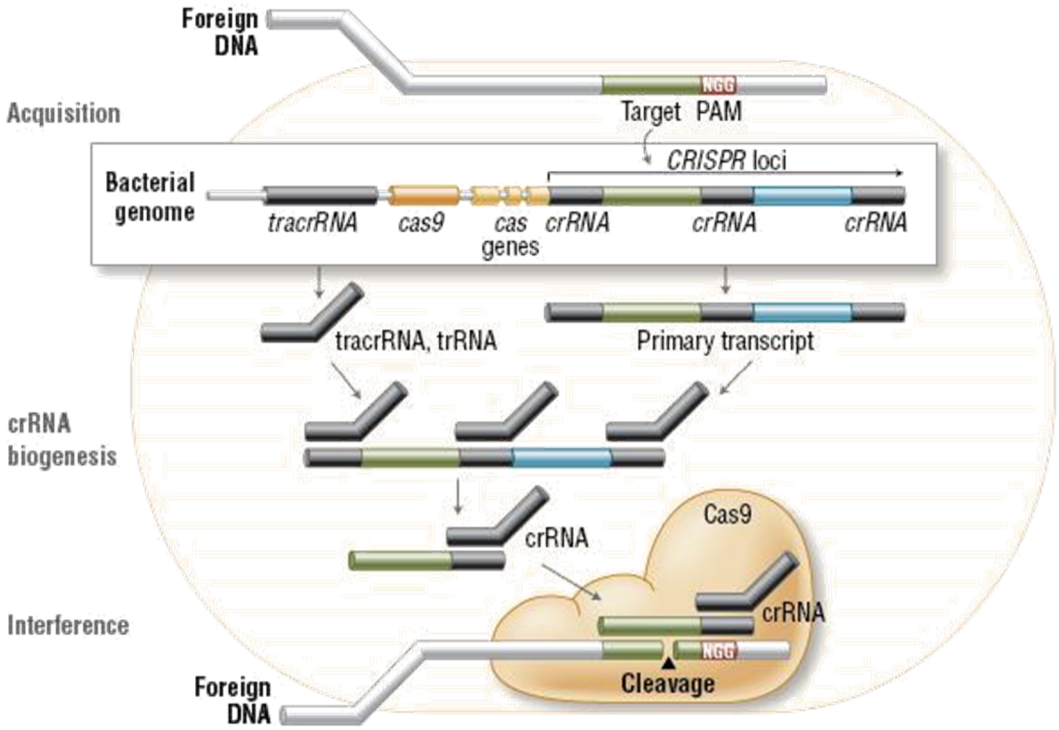

Figure 1.3. CRISPR prokaryotic defense mechanism : Once CRISPR loci is transcribed and obtained into CRISPR RNA (crRNA) throughout crRNA biogenesis, two distinct kinds of brief RNA will be generated if a bacterium is assaulted by a virus. CrRNA will comprise an attacking nucleic acid chain. After the interference stage, such two RNAs will create a structure with a protein called Cas9. The Cas9 will break the destination DNA when the corresponding structure recognized as a reference RNA (gRNA) finds its destination inside the viral genome. The virus will be disabled (Reis et al. 2014)

When the structure is inside the nuclease, Cas9 (Jinek et al. 2012) will lock on to an adjoining NGG protospacer pattern (PAM) as shown in Figure 1.3 (Reis et al. 2014). Cas9 has two effective locations, RucV and HNH, whereas one of the supplementary DNA fibers will be sliced by each of these locations (Ma et al. 2016).

The HNH domain will split the additional layer, while the non-complementary layer is split by the RuvC field (Jinek et al. 2012). DSB can be restored in two forms; either through the NHEJ or HDR process. NHEJ is susceptible to error and is the prevalent path. Usually this path contributes as tiny deletion, introduction or frameshift to distinct mutations. The virus may be disabled by these mutations. Figure 1.4 (Addgene 2017b) shows this system.

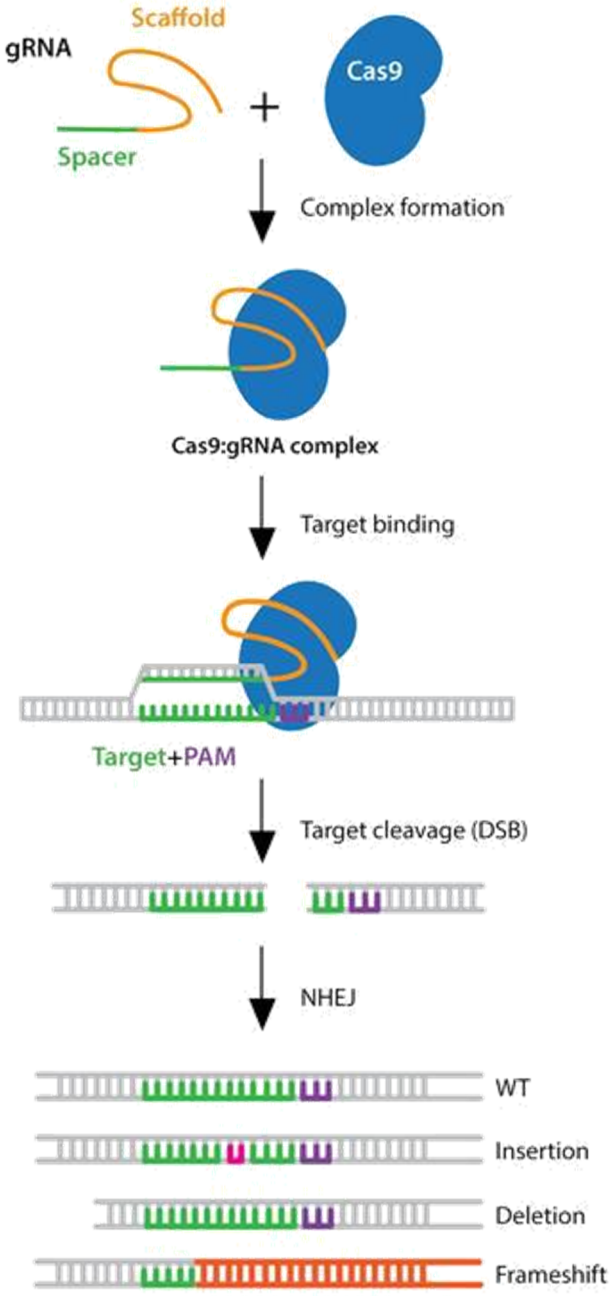

Figure 1.4. NHEJ pathway. A structure will be formed by the guide RNA (gRNA) as well as Cas9 that will hold on a brief adjacent protospacer motif (PAM). Cas9 has two effective locations, each with one supplementary DNA strand sliced. Generally the non-homologous end joining (NHEJ) mechanism will restore the double-strand

break. This path leads to various mutations like tiny removal, incorporation or frameshift changes (Addgene 2017b)

Studies have shown that, by altering the gRNA to suit the destination, this scheme can break any DNA sequence at a specific place (Jinek et al. 2012; Ma et al. 2016). You can do this by incorporating another bit of DNA with the required pattern. There are many distinct variables affecting the Cas9/sg RNA bonding specificity as both the gRNA-DNA stem combination and the PAM area with NGG structure instantly upstream of the destination area (Lin et al. 2014). The Cas9 protein domains are cleared to produce a DSB and then re-combined and changed to a different variant in the initial series (Jinek et al. 2012; Jinek et al. 2014; Nishimasu et al. 2014; Sternberg et al. 2014). This has been performed to edit genes in various species, including bacteria, yeast, crops, livestock, and even in groups of animal cells (Woo et al. 2015 ; Cho et al. 2013 ; Cong et al. 2013 ; DiCarlo et al. 2013 ; Friedland et al. 2013 ; Lemay et al. 2017 ; Malnoy et al. 2016).

In contrast to earlier techniques, the CRISPR / Cas9 mechanism can aim many genes at once (Pan et al. 2016), which has a huge benefit in both plant studies and complicated animal illnesses induced by multiple genes working together. In many areas such as fundamental studies, forestry and medication production, this technique can be used.

1.2.3 CRISPR/Cas9 experiments

There are distinct methods to start up an experiment based on the primary objective of CRISPR / Cas9. CRISPR / Cas9 can be used to generate a full and lasting reduction of gene activity or feature (knock-out), mutant gene action, or boost or reduce the destination gene expression. These various techniques of genetic manipulation involve distinct parts of CRISPR. One of the general goals in this research was to alter genes with gRNA(s) through an encoding scheme consisting of Cas9 enzyme (and/or Cas9 mRNA transcripts). For in vitro transcription, Cas9 mRNA and gRNA plasmids are used to produce mature Cas9 mRNA and gRNA supplied to host cells, which can also be used to produce transgenic crops (Addgene 2017b).

Different techniques of transmission can be implemented, such as remote protoplastic transfection carried out in this research. Several trials in grapevine, tobacco, apples, lettuce, rice, Arabidopsis thaliana and Petunia hybrida have used Cas9 protein with sgRNA to make a nuclease-gRNA combination. For effective transmission of Cas9 and gRNA, some of these also used separated protoplast (Malnoy et al. 2016; Woo et al. 2015; Subburaj et al. 2016). Cas9 mRNA and gRNA, however, have been implemented in wheat (Zhang et al. 2016), Cas9 DNA and RNA were both used with sgRNA, but the author has not yet implemented the understanding of this in potato crops.

The next stage is to pick a destination series and design the gRNA(s) once the distribution technique has been selected. *Figure 1.5* (Addgene 2017b) demonstrates a graph in this method of suggested measures. The cell line and genome sequence to be used in the experiment must be determined. It is suggested to order the region to be used in the experiment before developing gRNA(s) to reduce variables that may contribute to decreased cleavage owing to pattern differences (Addgene 2017b).

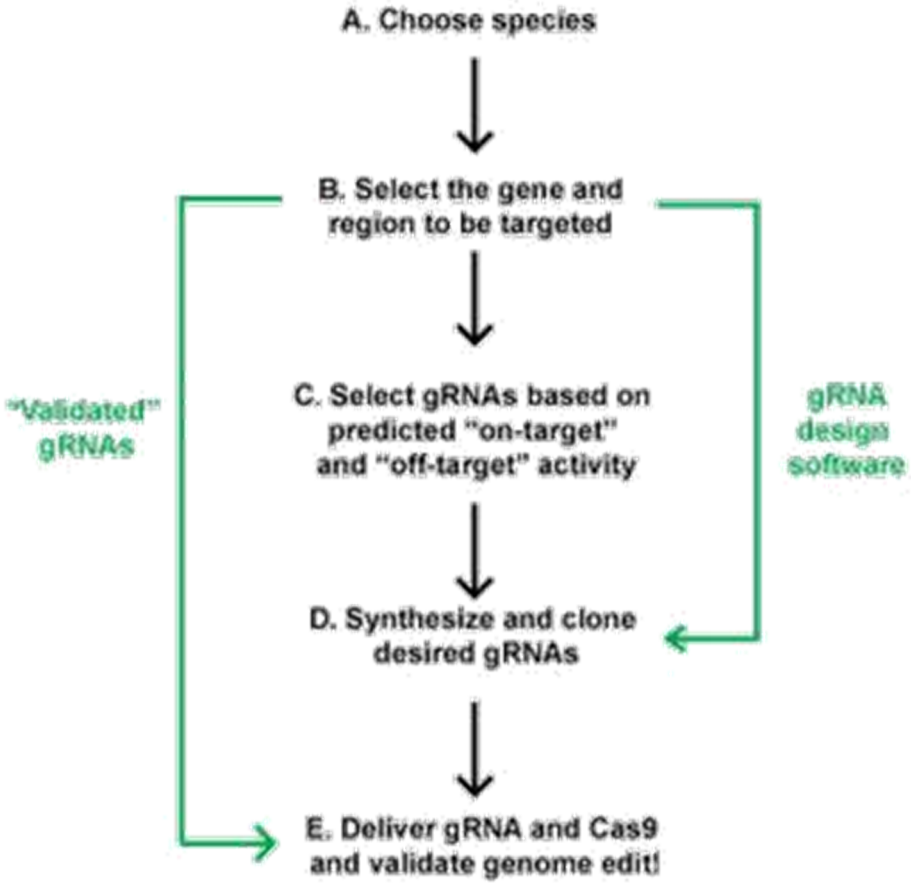

Figure 1.5. Selecting target sequence and designing gRNA(s) (Addgene 2017b)

Before selecting gRNA(s), the identification of the genome sequence to the target gene must be performed in order to manipulate genes using the CRISPR / Cas9 system. To select gRNA(s), it is necessary to target all PAM genes in the genetic region before choosing which location is most probable to occur in on-target conception. There are various variables that need to be conscious of when selecting gRNA(s). One of the most important variables is the matching of the gRNA pattern to the destination sequence. It is also essential that the destination location of gRNA

does not fit other genome locations, resulting in so-called "off-targets" (Xie et al. 2014).

There are various instruments for building gRNA(s) such as CRISPR-PLANT (Xie et al. 2014), a software instrument where CRISPR / Cas9 can be selected for destination locations. Off-targets are not inherently a critical issue in crop studies for future off-target genes that could be separated by intersection, contributing to only particular strains that can be used for further fundamental studies and crop reproduction (Zhang et al. 2016). Off-targets are a critical variable in other areas, such as clinical studies. The simplest route to prevent this is to carefully build the gRNA(s).

To build the desired gRNA(s) in the experiment, oligos must be designed and cloned into a vector. For instance, this can be accomplished by synthesizing, annealing and inserting blocking oligos into plasmids where gRNA is available by normal limitation requirements. Cas9 and gRNA(s) distribution will rely on the technique of consignment selected for the experiment. Optimizing the protocol for this purpose might be necessary, for example in the experiment to isolate protoplasts in the specific target specie (Addgene 2017b).

The final phase in the CRISPR / Cas9 test will be to test for effective dna alteration. For this intent, different techniques can be implemented. The outcome will be a distinct feasible genotype discovered in the corresponding "mutant" community of cells in many species. Some of the cells may be wild-type after the experiment is performed due to lack of expression of gRNA(s) and/or Cas9, or cells carrying gRNA(s) and Cas9 may reduce the destination cleavage. Cells altered can be either homozygous, where both alleles have been changed, or heterozygous, where only one of the alleles has been adjusted. Methods that can be used to check whether cells have been modified will rely on the experiment's objective. Methods that can be implemented include mismatch-cleavage assay for NHEJ fixed DSBs, PCR and limitation digest for HDR fixed DSBs, and for HDR or NHEJ, PCR amplification

and liquid electrophoresis, PCR amplification, sub-cloning, Sanger sequencing and PCR amplification and next-generation sequencing (Addgene 2017b).

1.2.4 Plasmids

Plasmids are tiny, distinct DNA molecules from chromosomal DNA that can reproduce separately. As tiny linear double stranded DNA, they are most prevalent in bacteria, but can sometimes be discovered in ancient and eukaryotic species. Although plasmids comprise only a tiny amount of genes, their preservation as antibiotic resistance has some useful characteristics (Reece et al. 2011).

Artificial plasmids are frequently used as vectors in molecular cloning where they will have a DNA plug from another origin. This will lead to a molecule of recombinant DNA. Then the plasmid is transferred to a bacterial cell where it produces and replicates a recombinant bacterium. When a given cell produces numerous clones, it is called gene cloning. Usually a vector comprises of various parts making it very beneficial. Vectors often have in prevalent that they represent a replication origin (Ori) where many versions of the vector in the cell can be produced, a multiple cloning site (MCS) with many restriction sites where DNA components can be attached and a template that can be distinguished. They are generally also tolerant to antibiotics (AmpR, KanR, ChlR), and therefore the selectable marker is often an antibiotic resistance element that will offer noticeable colonies where the recombinant plasmid is placed, which is showed as Figure 1.6 (Reece et al. 2011).

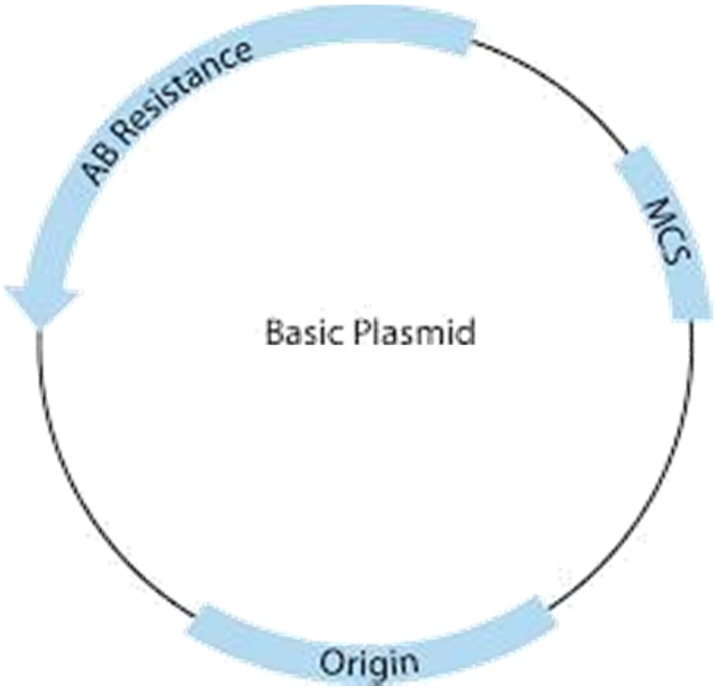

Figure 1.6. Basic plasmid regions

1.3 Potato (*Solanum tuberosum*)

1.3.1 History and description

Potato, *Solanum tuberosum*, refers to the Solanaceae group of nightshades and is generally acknowledged by its typically yellow phenotype (Abelanda et al. 2011) as shown in Figure 1.7 (Old Farmer's Almanac 2019).

Potato is rated the world's fifth largest food plant (Barrell et al. 2013). It has a strong dietary importance and produces per hectare a strong energy yield. Not only is potato important as a food crop, it is also one of the most important plants produced for starch manufacturing (Ellis et al. 1998).Tetrasome inheritance and elevated heterozygosity of cultivated varieties makes the development of potato cultivars with traditional cross-breeding complex, difficult (Muthoni et al. 2015). Thus,

reproduction techniques in which only one or a few characteristics can be brought into an aristocratic context are of great concern to potato. Genetic modification (GM) has lengthy been a commonly used technique in crop studies and reproduction through robust inclusion of DNA content (Barrell et al. 2013).

However, the commercialization of the GM plants produced has been limited by a lengthy and costly deregulation phase in Europe and elsewhere. New cultivation methods that do not introduce or maintain recombinant DNA on crop genome have been promising, and debate is being held about whether this leads to occurrences that should be controlled as GMOs (Jones 2015; Waltz 2016).

Figure 1.7. Potato, Solanum tuberosum, is a major crop globally and is renowned for its traditional yellow phenotype (Almanac 2019 by Old Farmer).

1.3.2 CRISPR/Cas9 editing in potato

In plant studies and reproduction, gene silencing is commonly used to study gene features and to create fresh cultivars. For this objective, site-directed mutagenesis (SDM) has recently been implemented. The SDM methods are intended to be target-specific in comparison with chemical and physical mutagenesis, which is accidental with numerous mutations brought throughoutthe genome (Quetier 2016). SDM constructs can be stably integrated into the genome through transformation or expressed transiently to create mutations in vivo. The primary SDM methods presently in use are zinc finger nuclease (ZFN), TAL effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated enzyme 9 (Cas9) (Schiml and Puchta 2016).

CRISPR-Cas9 development has recently gained a great deal of interest because it is a more user-friendly and cost-efficient method for generating target-specific constructs relative to ZFN and TALEN. In 2013, the CRISPR-Cas9 method in higher plants was first recorded as effective (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). The technique is focused on a brief single-guide RNA (sgRNA), with a 20 bp guide structure complementing a destination region, a promoter, and a sgRNA scaffold that can cause mutations in a destination region of selection in conjunction with a Cas9 nuclease (Jinek et al. 2012). The subsequent double strand break (DSB) is restored through the cell's own attachment system, either by non-homologous end joining (NHEJ) or by homologous recombination (HR) (Britt 1999). NHEJ is susceptible to mistake and often causes to random inserts or deletions (indels) that can lead cell feature to be knocked out.

Potato, offers an optimal chance for genome editing in a plant species to develop methology and reagents. As one of the first genetically engineered crops, maize is extremely tissue-friendly and can be regenerated with elevated effectiveness from branch and root cells (Banerjee et al., 2006; Chakravarty et al., 2007). A amount of

potato cultivars have created agrobacterium-mediated conversion, but other techniques of distribution have also been effective (Barampuram and Zhang, 2011).

Similar to other crop organisms that have effectively used CRISPR / Cas9, potato cell conversion is effective and the DNA structure of double-haploid DM and diploid RH is accessible, making potato an optimal choice for this DNA processing mechanism (Potato Genome Sequencing Consortium 2011). The potato genome launch has increased potato studies and offered essential instruments for crop operational genomics (PGSC, 2011). The database of the potato genome enables consumers to scan for genes of concern in the recent genome assembly and identify sequences that combine identific The capacity to scan for possibly unnecessary dna or gene fragments of the potato genome offers vital information for developing reagents for dna processing (Curtin et al., 2012; Voytas and Gao, 2014; Liu et al., 2013). Here, we used the CRISPR / Cas9 mechanism to effectively generate gene knockouts in potato, providing an outstanding basis for potential gene activity research.ation or resemblance with a particular outcome (Hirsch et al., 2014; Sharma et al., 2013).

1.3.3 eEIF4E and Vlnv genes

1.3.3.1 eEIF4E

In clonally propagated plants such as potatoes, viruses are particularly difficult because they have a high probability of being transmitted vertically between generations. For seed potato manufacturers, potato virus Y (PVY) has long been a massive challenge, and fresh strains of the virus have emerged in Europe and North America in latest years that cause devastating tuber necrosis. These strains have a negative effect on the quality of seed tubers and commercial potatoes, triggering trade restrictions. Seed certification programs have been moderately successful in decreasing PVY incidence below financial thresholds, but this is getting harder with

the prevalence of strains that cause mild foliar symptoms and escape detection during visual field inspections. Because of these variables, PVY has become North American seed potato industry's most significant problem (Gray et al., 2010). A latest study discovered more than one-third of North American seed lots had detectable PVY amounts (Gray et al., 2010), and the amount of seed lot rejections is increasing. In Europe, where the tuber necrotic strain has moved the normal strain, many cultivars are no longer cultivated due to their extreme susceptibility to tuber necrosis (Rolland et al., 2008). You can see this infected potato plant in figure 1.8 (Bruce Watt, University of Maine, Bugwood.org, 2010)

One of the most effective methods for managing viral diseases in crop species is host plant strength (Khetarpal et al., 1998). Resistance strains that are efficient against all recognized PVY varieties have been recognized in potatoes and several plant cultivars with severe resistant have been published through cultivation programs, particularly in Europe (Plaisted et al., 2001; Solomon-Blackburn and Barker, 2001). Though, introgression of resistant genes into a tetraploid potato frequently occurs at the cost of other beneficial culinary and horticultural features. Due to sector choice for current plants, the acceptance of durable cultivars, particularly in North America, has been restricted (Douches et al., 1996). This is demonstrated by the reality that, in the United States and Canada (USDA 2008),' Russet Burbank,' a cultivar published over 100 years earlier, tends to grow more commonly than any other. Therefore, genetic engineering is an appealing option to standard plant reproduction because it can lead to the growth of opposition to disease while preserving all other desirable features. Numerous study organizations have used a range of international gene constructs to develop genetically engineered PVY-resistant potatoes. The Monsanto affiliate NatureMark has marketed a durable chain showing the PVY cap protein mutation coupled with the Bt insecticidal protein. In the mid 1990s and mid 2000s, this crop,' Newleaf Y' was extensively introduced (Borlaug, 2000; Duncan et al., 2002). Although, sector worries about the presence of overseas genes in potato caused the item to be withdrawn and transgenic potatoes to be banned from the

sector (Kaniewski and Thomas, 2004). Developing durable potatoes using an intragenic approach wherein the plant is converted using genes from within its own genome can tackle both the issues of preserving the horticultural characteristic and accepting the industry.

Innumerable plants have recognized and implemented recessive potyvirus resistant genes for many centuries, and these genes have lately been described at the molecular stage (studied in Truniger and Aranda, 2009). In host protein translation along with viral infection, the extremely preserved eukaryotic translation initiation factor 4E (eIF4E) performs an important part. The protein eIF4E connects to the 5′ tag of mRNA and helps the carrier ribosomal cluster to be recruited (Gingras et al. 1999). Several crop parasites, e.g. potyviruses, with single-stranded RNA genomes also interfere with eIF4E, often covalently linked at the 5′ terminus of the viral dna via a pathogen-linked protein (VPg) (Leonard et al., 2000; Schaad et al., 2000). This communication is essential for effective virus infection and is believed to promote virus genome transcription, replication, and/or cell-cell mobility (Gao et al., 2004; Kang et al., 2005; Robaglia and Caranta, 2006).

Mutations at the eIF4E locus have advanced in so many plant species that interfere with VPg, leading to virus-resistant beings (Cavatorta et al., 2008). The pepper and tomato loci pvr1 and pot-1 respectively offer additional virus strength to many types of potyvirus and have been shown to encode orthological eIF4E versions (Ruffel et al., 2002, 2005; Kang et al., 2005). In relation to pepper and tomato, barley (Stein et al., 2005), lettuce (Nicaise et al., 2007), melon (Nieto et al., 2006) and pea have been recognized as recessive strength alleles at the eIF4E node (Gao et al., 2004). Naturally current durable variants of eIF4E vary just by one to five modifications in amino code from the vulnerable structure of the protein. Predicated on the solidified framework of murine and wheat eIF4E, several of these polymorphisms are grouped in the protein region expected to connect to VPg (Marcotrigiano et al., 1997; Gao et al., 2004; Kang et al., 2005; Ruffel et al., 2005; Monzingo et al., 2007; Nicaise et al., 2007).

Intimate knowledge of how modifications in the eIF4E protein can lead to efficient virus tolerance while not interrupting the main role of target mRNA translation provides fresh instruments for virus command in many economically significant species of plants. In a growing population, marker-assisted selection of eIF4E strength alleles may be used to quickly pick people who are purportedly susceptible (Yeam et al., 2005). Moreover, given the existence of the sensitive endogenous potato gene, transgenesis of an eIF4E deficiency allele from pepper has been shown to confer wide-spectrum potyvirus strength in vegetables (Kang et al., 2007). In plants that haven't really obviously evolved eIF4E-mediated resistant, such as potatoes, this technique of transmitting virus strength between plant species can tackle virus disease issues.

Figure 1.8. Potyvirus Y disease infected potato (Bruce Watt, University of Maine, Bugwood.org, 2010)

1.3.3.2 Vlnv

Frequently collected potato tubers (Solanum tuberosum L.) are stored at temperatures ranging from 4 to 8 °C to stretch the end-harvest shelf life by decreasing sprouting and decomposition from bacterial and fungal diseases. Cold housing also provides personal customers and the potato sector with an uninterrupted distribution chain of fresh tubers. Cold housing, though, is followed by concentration of sugar reduction (mainly sugar and fructose) in potato tubers via incremental starch deterioration, a condition known as cold-induced sweetening (CIS) (Dale and Bradshaw 2003). This is indeed a recurrent and expensive issue for the potato industry because CIS has a negative impact on the quality of crisps, frites and other goods that entail processing at temperatures above 120 °C. Each year and, approximately 30 million kg of potatoes are handled into organic consumer products such as French rolls and chips (Keijbets 2008), according to one survey. The high reduction sugars respond in cold-sweetened potatoes through the Maillard response with free radical α-amino acids, creating sour smelling through-products that blacken French rolls to brown (Stadler et al. 2002). In addition, cooked potatoes may comprise high amounts of acrylamide ($CH_2CHCONH_2$, CAS Registry No. 79-06-1), neurotoxin, and potential human carcinogen (Friedman 2003; Parzefall 2008). At the biochemical and molecular concentrations, CIS' biological process has been widely defined. Scientists recognized CIS as a complicated genetic trait connected to various quantitative trait loci (QTLs) containing various proteins through genetic linkage mapping (Nägele et al. 2010). During CIS, numerous biochemical procedures, along with starch production and mobilization, and also some sucrose formation due to glucose and fructose hydrolysis, are recorded as critical measures in increasing cold-stored tubers ' reduction in sugar quantity (Li 2008). The mixed operations of certain proteins, such as UDP-Glucose pyrophosphorylase, sucrose phosphate synthase, sucrose phosphate phosphatase, β-amylase, and acid invertase, are causing these molecular occurrences (McKenzie et al. 2005). The sucrose generated by these enzymatic reactions is ultimately transferred to the vacuole,

where glucose and fructose are even further hydrolyzed (Blenkinsop et al. 2004). The concentration of sugar reduction within the vacuole is associated with the operation of the vacuolar invertase enzyme (VInv), which performs a critical part in hexogenic processes by controlling the starch-sucrose proportion (McKenzie et al. 2005).

The Maillard reaction as well contributes to the creation of acrylamide, which is the prevalent viable amino acid in potato tubers by decreasing sugars and asparagine. Acrylamide is a potentially carcinogenic and neurotoxic polymer (Friedman, 2003). Recently, the Food Standards Agency (FSA) published a study on ingredients with elevated capacity for acrylamide production, focusing on activities to decrease this compound's nutritional consumption (FSA, 2015). Silencing of the Vlnv gene in tubers leads to a decline in the capacity for acrylamide formation as it reduces the accessibility of sugar decrease (Ye et al, 2010). This indicated complete gene decrease or transient denial, see Figure 1.9.

Minimizing the availability of toxins such as acrylamide in food materials is becoming a concern for potato manufacturers as well as several reproduction programs are aimed at generating "cold chippers" crop cultivars that produce reduced sugar concentrations during short processing (Xiong et al. 2002; Hamernik et al. 2009). Multiple native Solanum species, including Solanum raphanifolium (2n=2x=24), demonstrated the ability to decrease the CIS event and were the finest hot chippers (McCann et al. 2010). Throughout freeze containment, farmers have produced restricted progress towards creating native germplasm-derived cultivars that demonstrate decreased rates of concentration of sugars (Hamernik et al. 2009). Nevertheless, with continuously reduced CIS, there seems to be no environmentally common cultivar to date. Thereby, for either the global potato sector, CIS continues one of the most significant and continuous issues.

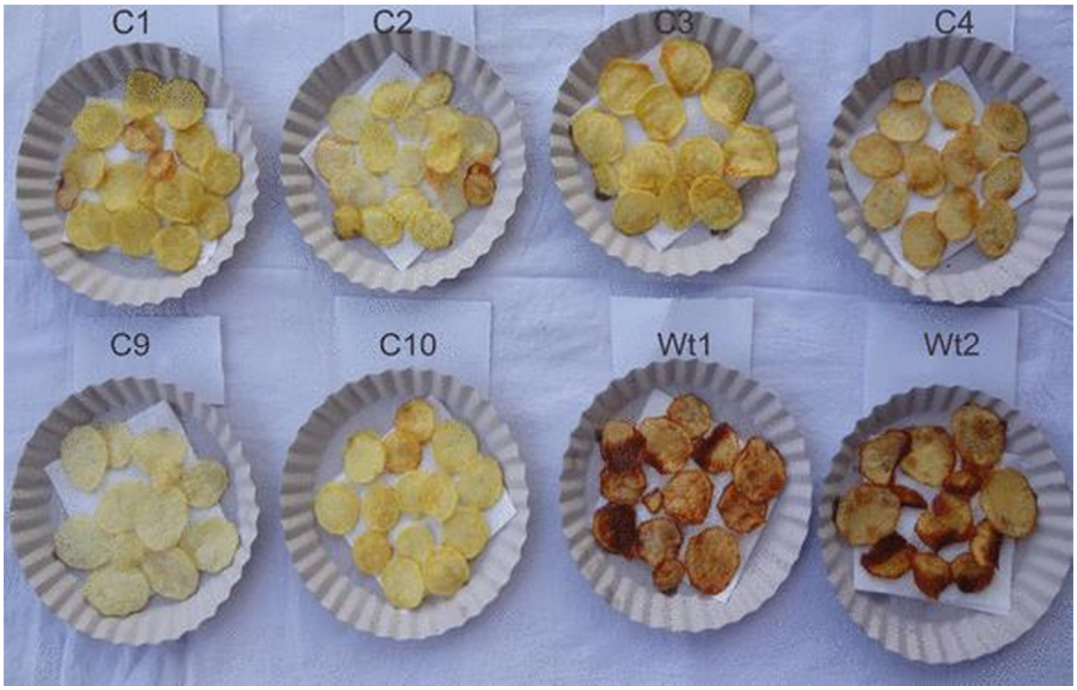

Figure 1.9. Acrylamide synthesis in potatoes cooked due to color change. An image of the potatoes C1-C2-C3-C4-C9-C10, in which the V1nv gene is inactive, as well as the Wt1 and Wt2 potatoes in which the V1nv gene is active (Hameed et al. 2018).

1.4 Tissue culture

1.4.1 Growth of plants in vitro

Tissue culture in a fluid, semi-solid or strong development environment is the in vitro development of tissue, cells or the entire plant under regulated dietary and climatic circumstances. It would be frequently like to make plant clones (Murashige and Skoog 1962; Thorpe 2007). Tissue culture allows seasonal and weather-independent crops to develop. It really is a significant instrument for methods to

biotechnology such as genetic engineering that rely on an effective scheme of regeneration of plants in vitro (Hussain et al. 2012).

1.4.2 Important Crop Improvement Tool

Tissue culture has enabled a big amount of secondary plant products to be produced and is regarded to be the most effective instrument for improving crops. And can be used for several reasons such as plant cultivation, DNA transformation, secondary metabolite manufacturing, disease abolition and the development of various species with regard to salt tolerance, moisture and thermal stress (Hussain et al. 2012). Figure 1.10 demonstrates a graph for various studies in tissue culture.

Another of the latest elements of plant cell and tissue culture is the chance of genetic transformation that allows genes with desirable qualities to be transferred to target crops and transgenic plants to develop. The tissue culture method uses the totipotentiality of plant cells in order to regenerate an existing plant, implying that even a single cell can convey the complete genome through cell division, and also some cells can change their own environment, growth and development (Hussain et al. 2012).

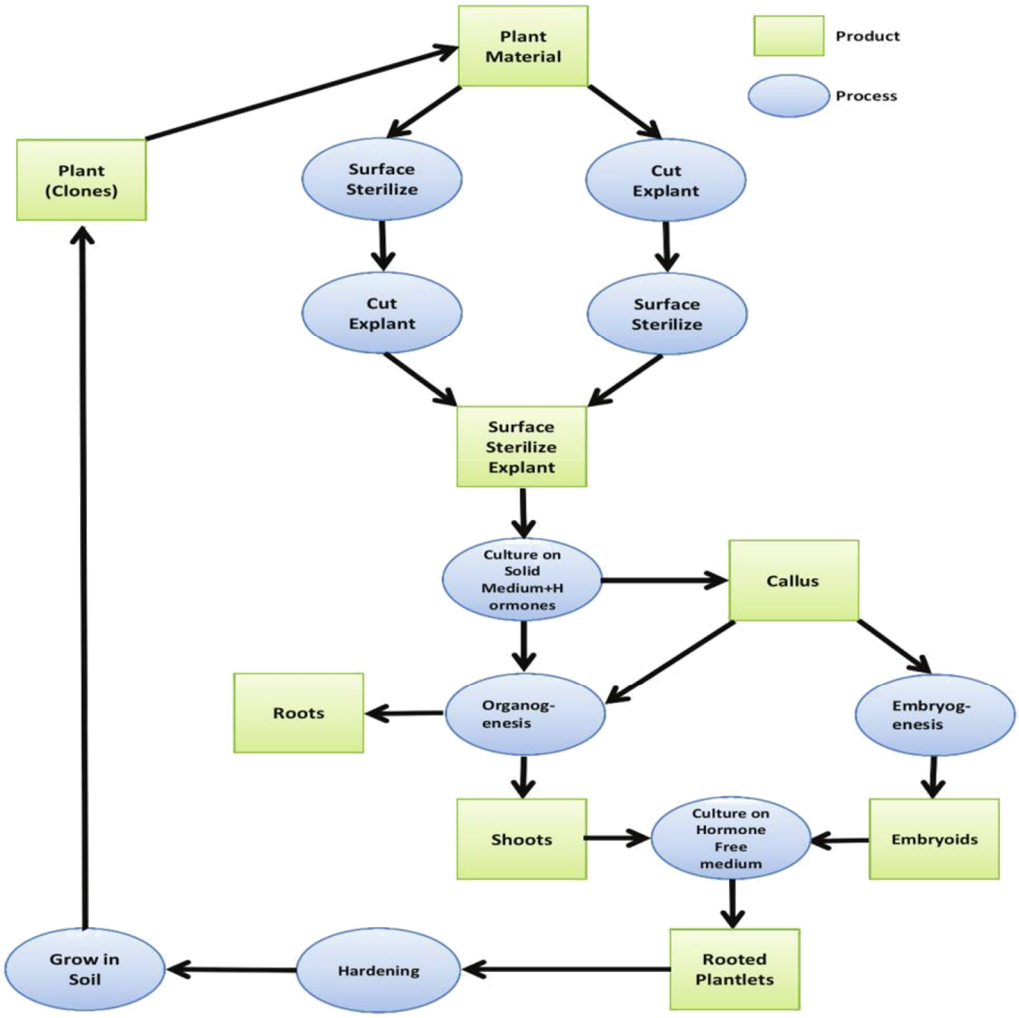

Figure 1.10. Schematic image for separate studies in tissue culture (Hussain et al. 2012).

1.4.3 Optimization of tissue culture

As of 1902, when the German physiologist Gootleib Haberlandt first attempted to cultivate intact bacteria from flowers in the saline bath of knop with sucrose, bone cultivation has been greatly improved (Hussain et al. 2012). Several chemicals and circumstances need to be achieved in order to optimize cell culture.

Broadly speaking, the medium must therefore comprise substances such as macronutrients, micronutrients, vitamins, amino acids or carbonsource(s) of nitrogen, growth regulators, solidifying inhibitors and sometimes undefined nutritional substances. Medium pH, temperature, gaseous and liquid properties are also essential in the setting. The environmental pH is generally 5.4-5.8 (Saad and Elshahed 2012; Hussain et al. 2012). Murashige and Skoog medium (MS) is by far the most widely used within vitro cultivation method for far too many crop organisms (Hussain et al. 2012).

1.5 Objectivities of present study

Whenever a procedure for DNA-free editing has been used as a potato plant in crops, this can clear up the opportunity of producing potato plants in many nations according to rigid legislation when it goes to customarily GMOs that have new leftover different DNA from shipping methods (Andersson et al., 2018).

DNA-free alteration with CRISPR / Cas9 has not yet been carried out in the significant plant potato to the author's comprehension. In this study, it was required to use this fresh technology for fundamental and applied research reasons to edit particular genes. This was recorded effectively using a mixture of the sequence of Cas9 protein and gRNAs and their subsequent conversion into plant protoplasts (Woo et al. 2015). To accomplish this goal, this study's primary concerns were:

- Trying to plant and cultivation of potato seeds and tissue culture.
- The tissue cultivation system for the regeneration of crops from detached potato protoplasts is implemented.
- Guide RNA genetically modifying, which may aim one or even more genes. Cloned gRNA and Cas9 in vitro transcriptions.

- CRISPR / Cas9 in vivo genetic manipulation of Cas9 protein (and / or transcribed gRNAs) by the polyethylene glycerol (PEG) transfections of selected protoplasts with the markers Vlnv and eEIF4E.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Kits

Table 2.1. provides an outline of the various devices used in this research.

Kits	Function	Supplier
Plasmid Mini Kit (100)	Plasmid Isolation	QIAGEN
GeneJET Plasmid Miniprep Kit	Plasmid Isolation	Fermentas
MinElute Gel Extraction Kit	DNA Gathering after PAGE	QIAGEN
Taq DNA Polymerase	Routine PCR applications	New England Biolabs
Q5 Hot Start High Fidelity Taq DNA polymerase	Colony PCR applications	New England Biolabs
HiScribe™ T7 Quick High Yield RNA Synthesis	sgRNA transcription from plasmid in vitro	New England Biolabs
RNAs MegaclearTM kit Purification	cleanup of transcription reaction	Thermo Fisher Scientific
HiScribeTM T7 ARCA mRNA	Cas9 mRNA in vitro transcription	New England Biolabs
Cas9 Nuclease	Site specific cleavage of DNA with Cas9	OriGENE

2.2 Methods

Experiments were carried out in three parts: potato plants, gRNA and Cas9 and protoplastic separation and transfection.

Sterilized and cultivated potato plants both on soil and on tissue cultivation. There has also been the spread of potato crops. In the gRNA / Cas9 portion, numerous experimentation were handled with in vitro transcription and in vitro digestion using Cas9 nucleases, including the nealing of primers, restriction ligation, transformation, sequencing, digestion of plasmids, of gRNA and Cas9.

Before T7 Endonuclease I test, the final experiment was done with the transfer of the gRNA and Cas9 in vivo with the transfer of detached potato protoplasts.

2.3 Potato

During this research, PepsiCo potato plants were used. The fruits of PepsiCo that were Lady Olympia and Lady Amarilla were delivered by Ali Mehmetoğlu. The International Potato Genome Sequencing Consortium (PGSC) released and published the potato genome sequence in 2011. The potato genome inserted is the one of the paired monoploid S. Phureja DM1-3 (DM; AEWC00000000) tuberosum group; the genome assembly is 726 MB, 86 percent of which is attached to the genetic map and includes 39,031 genes annotated. Additionally, a second clone, published BAC and entire genome shotgun sections. S. tuberosum group Tuberosum RH89-039-16 (RH; ERP000627).

2.3.1 Sterilization of potato seeds

Two techniques have been conducted to sterilize Potato plants. The first technique was ethanol while Ca-hypochlorite was the other technique. All of this describes both techniques.

2.3.1.1 Sterilization with calcium hypochlorite

25 mL. one third (w / v) of Ca-hypochlorite was blended with one piece of Tween and continued to rest. One mL. of (supernatant) product was drawn in 9 mL. of 96 percent ethanol. Seeds were put in tubes of Eppendorf (five seeds in each tube) and one mL. of ethanol / hypochlorite solution was mixed, rubbed and allowed to sit for a total of five minutes (longer duration seed can be killed). Supernatant and one mL were taken. Ethanol was added to 95 percent. Ethanol was then separated and performed twice the cleaning measures. Seeds were kept in a sterile cover to stay overnight, or cleaned with 2 mL. then water autoclaved and the same day sown. Each seed has been put with MS medium in an autoclaved box.

2.3.1.2 Sterilization with ethanol

In a 1.5 mL eppendorf bottle, one mL 70% ethanol and 0.01% triton were attached to seeds and incubated on a shaker for 15 minutes. The solution was withdrawn in a sterile hood and added and incubated one mL of pure ethanol on a shaker for 10

minutes. The ethanol was lifted, adding one mL of pure ethanol and inverting the tube a couple of occasions before the ethanol was withdrawn again. The Eppendorf tube was kept with the cover accessible for washing in a sterile cabinet. Seeds were cultivated or deposited at four degrees centigrade after drying.

2.3.2 Sowing of potato seeds

Various techniques have been used to sow potato seeds and propagate potato crops.

2.3.2.1 Potato seeds on MS-agar

For plant regeneration, the Murashige & Skoog (MS) method is the most appropriate and widely used fundamental tissue development method. Here we define the parts used in tissue culture of the MS medium, inventory preparing and transport. As designated stock solutions, nutrient salts and vitamins are ready. Stocks at 4 ºC are deposited. To prepare 1L of a medium, the desired amount of concentrated stocks is mixed.

Petri dishes were mixed 30 mL of MS agara (see table 2.2) with agar with either 3% sucrose and kept to dry for about 30 minutes. Seeds were planted with toothpick and placed in daylight for 16 hours and dark room for 25 °C for eight hours.

Table 2.2. Table shows content for MS medium.

Stock solutions	**mg/L with exceptions**	**MS medium (500 mL)**	**Stock (20X) for 500 mL**
MS Major Salts			
NH_4NO_3	1650		16.5 g
KNO_3	1900		19.0 g
$CaCl_2.2H_2O$	440		4.4 g
$MgSO_4.7H_2O$	370		3.7 g
KH_2PO_4	170		1.7 g
MS Minor Salts			
H_3BO_3	6.2		620 mg*
$MnSO_4.4H_2O$	22.3		2230 mg*
$ZnSO_4.4H_2O$	8.6		860 mg*
Kl	0.83		83 mg*
$Na_2MoO_4.2H_2O$	0.25		25 mg*
$CoCl_2.6H_2O$	0.025		2.5 mg*
$CuSO_4.5H_2O$	0.025		2.5 mg*
MS Vitamins			
Thiamine-HCL	0.1		10 mg
Niacin	0.5		50 mg
Glycine	2.0		200 mg
Pyridoxine-HCl	0.5		50 mg
Other chemicals			
Sucrose	30 g.	15 g	
Agar	8 g.	4 g	
Fe/EDTA Solution			**12.5 mL**
Na2•EDTA	373		
$FeSO_4.7H_2O$	279		
Note: pH was adjusted to 5.8 and autoclaved			

* : Amount Required for 500 mL. Stock (200X)

2.3.2.2 Propagation of potato

The propagation of potato seeds was accomplished by removing a small portion of the stem from Germini crops already cultivated. They were put in MS + 0.8 percent agar-agar and 3 percent sucrose boxes and placed at 25 °C in 16 hours daylight and 8 hours dark room.

2.3.2.3 Potato plants on soil

Some of the potato crops were transmitted with fungus after seeding potato seeds on MS + agar (three percent sucrose). After 14 days, these plants were transferred to the soil for later surface sterilization.

Soil was 3/1 proportionally combined with vermiculite and put in plant containers. Water was put one to two hours in the container until the soil was moist from top to lower part. Potato plants have been placed in each plant box and placed in daylight for 16 hours and dark room for 25 °C for eight hours.

2.3.3 Seed germination

From the protocol described in "Totipotency of potato protoplast" (Shepard 1977), seed germination was performed according to the totipotency chart of potato protoplast (Figure 2.1). (Shepard, J., and Totten, Roger E. (1977). Potato Protoplasts of Mesophyll Cell: Isolation, Proliferation and Regeneration of Plants. Physiology of

plants, 60(2), 313-316. Retrieved from http:/www.jstor.org/stable/4264979) Medium MS + agar (3% sucrose) potato crops, 3-12 days after germination to 50 mL TM-1 medium boxes (Table 2.3). Roots were sliced and moved to the TM-1 medium with a part of the hypocotyl. Planted potato boxes were then positioned for two to six weeks in 16 hours of daylight and eight hours of dark at 25 °C until protoplast isolation was done.

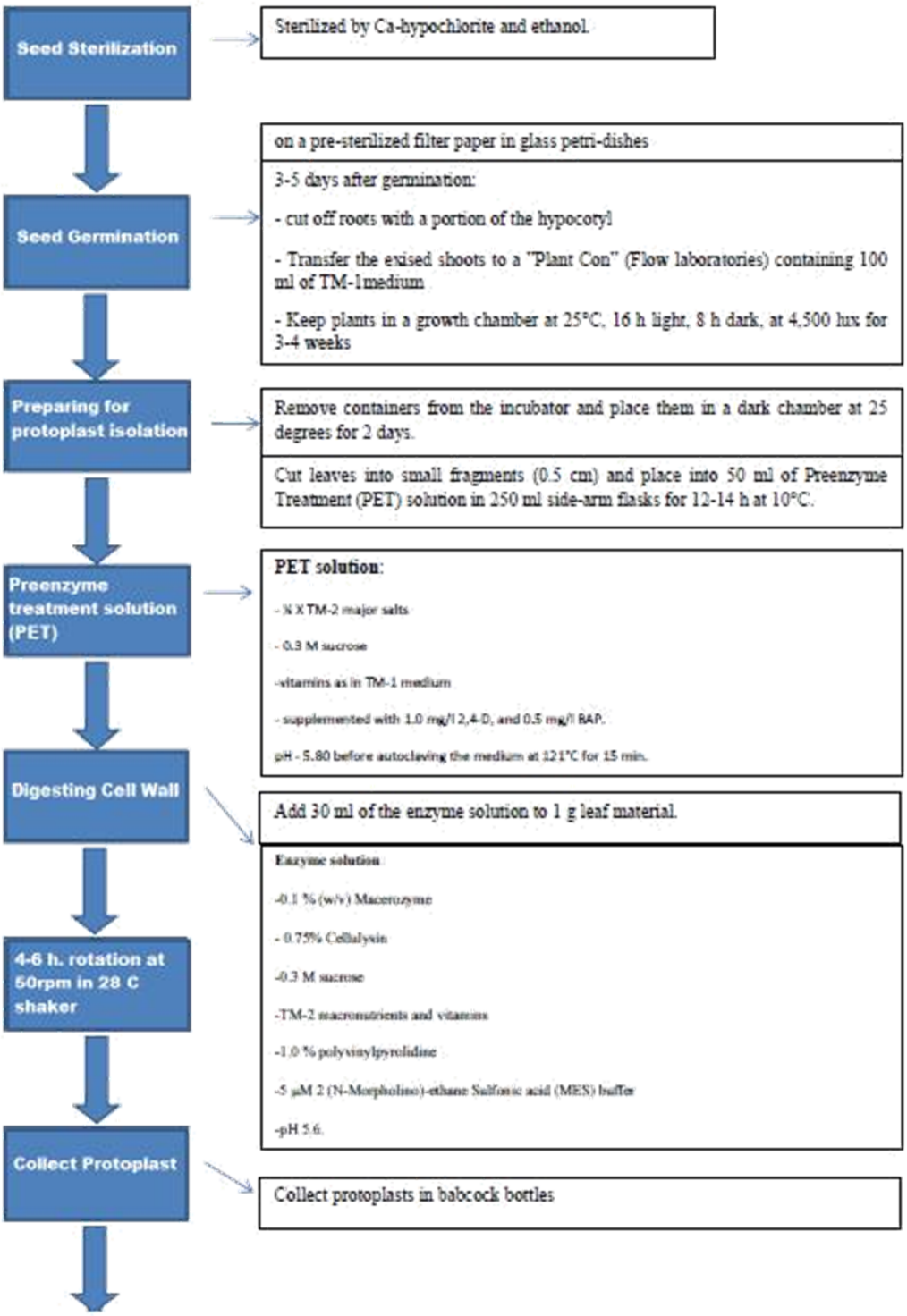

Figure 2.1. Potato protoplast totipotency. Shows the entire protocol that was scheduled to follow during this research (Shepard, J., and Totten, Roger E. (1977).

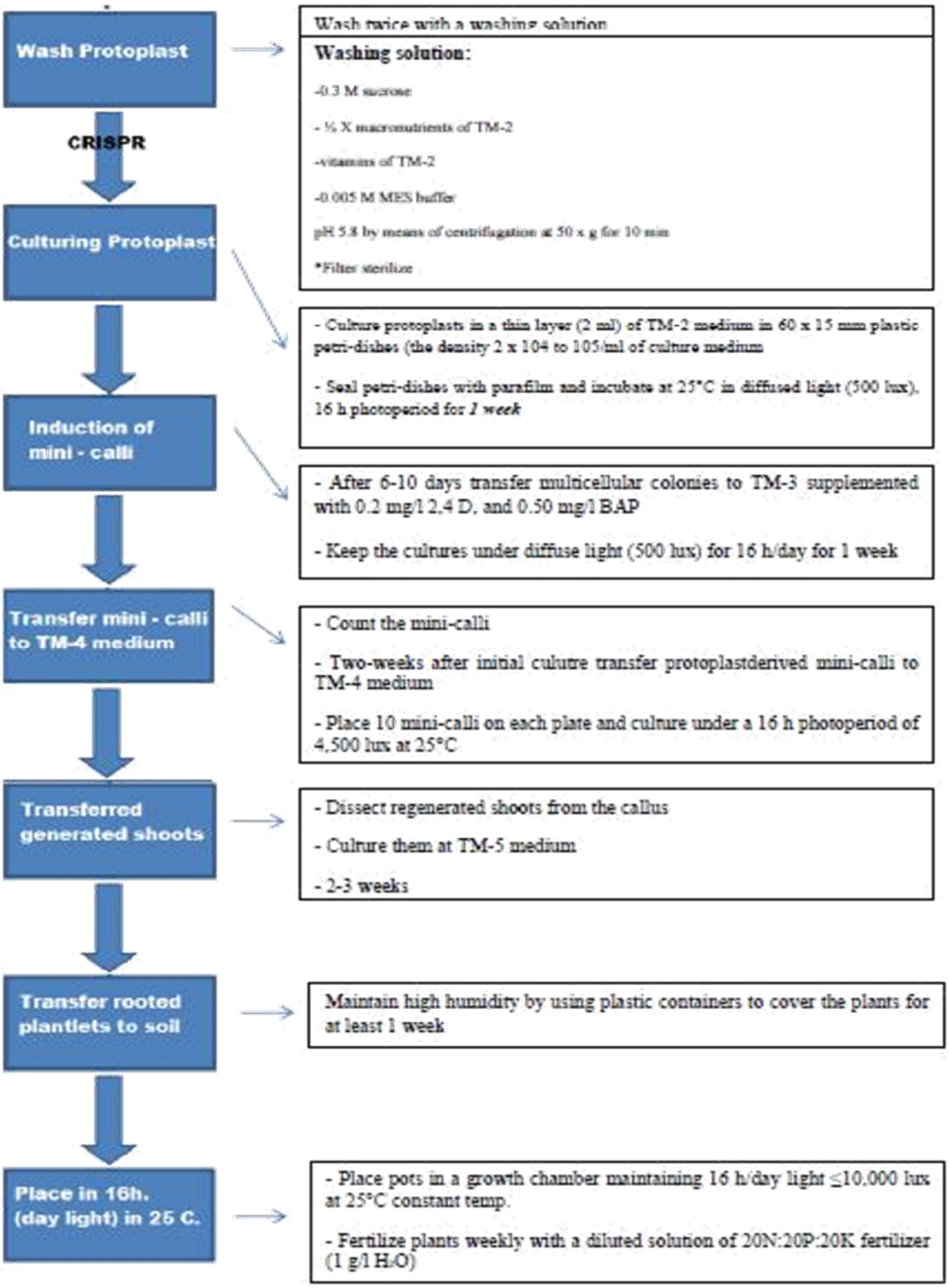

Figure 2.1 (cont'ed). Potato protoplast totipotency. Shows the entire protocol that was scheduled to follow during this research (Shepard, J., and Totten, Roger E. (1977).

Table 2.3. TM-1 solution. g/ L except when notated. Solutions were made according to the original protocol.

TM-1			
Components	1 L (g/L)	Components	1 L (g/L)
Macronutrients		**Vitamins**	
CaCl2• 2 H2O	0.15	Nicotinic acid	0.0025
KNO3	2.53	Thiamine HCl	0.01
NH5NO3	0.32	Pyridoxine HCl	0.001
NH4H2PO4	0.23	Folic acid	500 µL*
(NH4)2SO4	0.134	Biotin	50 µL*
MgSO4•7H2O	0.25	D-Ca-Pantothenate	500 µL*
Micronutrients		Choline chloride	100 µL*
KI	380 µL*	Glycine	500 µL*
H3BO3	0.0062	Cacein hycrolysate	0.05
MnSO4• 4H2O**	0.0223	L-Cysteine	1000 µL
ZnSO4 • 7H2O	0.0086	Malic acid	0.01
Na2MoO4• 2H2O	250 µL*	Ascorbic acid	500 µL*
CuSO4 • 5H2O	25 µL*	Myo-inositol	0.1
CoCl2• 6H2O	250 µL*	Riboflavin	250 µL*
FeSO4• 7H2O	0.0139	**Others**	
Na2• EDTA	0.0185	Sucrose	30
		Agar	6
pH	5.8		

* = stock solutions. 100 mg/100 mL stock solution were prepared for KI, $CuSO_4$ x $5H_2O$, Na_2MoO_4 x $2H_2O$ and $CoCl_2$ x $6H_2O$. 10 mg/10 ml stock solution were made for the rest.

**0.00845 g MnSO4 x H2O was used instead.

2.4 gRNA, Cas9

2.4.1 Making gRNAs and Primers

Spacers and other details for sgRNA 1, 2, 3 and 4 were discovered and recognized as shown in Table 2.4 from the use of software techniques.

Queries were for, eEIF4E is PGSC0003DMG400013421 in chromosome three from 831,240 to 836,333 and Vlnv is PGSC0003DMG400013856 in chromosome three from 39,255,053 to 39,259,538.

To find flanking primers for amplification of the Polymerase Chain Reaction (PCR) around the target of CRISPR, sgRNA was aligned with the potato genome in Plant Ensemble where the target location was found. The primers were designed using this. Steps are provided in the section for building the gRNAs and primers.

Table 2.4. List of some web-based tools to design CRISPR/Cas systems.

Tool	Features	Web Addresses
CRISPR Design	sgRNA designing, prediction of off-site targeting against a limited number of reference genomes including Arabidopsis thaliana	http://crispr.mit.edu
Cas-OFFinder	Multi-featured, prediction of potential off-site targeting against many reference genomes, sgRNA designing, PAM type option for different CRISPR/Cas variants	http://www.rgenome.net/cas-offinder/
CCTop	sgRNA target selection, off-target prediction	http://crispr.cos.uni-heidelberg.de/
CHOPCHOP	Multi-featured, Target sites for CRISPR/TALENs systems, Off-site targeting against many reference genomes	http://chopchop.cbu.uib.no
CRISPOR	Design, evaluate and clone guide sequences for the	http://crispor.tefor.net/

	CRISPR/Cas9 system and prediction of off-site targeting	
E-CRISP	Multi-featured, Designing of CRISPR constructs, Prediction of off-site targeting against a limited number of genomes	http://www.e-crisp.org/E-CRISP/reannotate_crispr.html
CROP-IT	CRISPR/Cas9 target sites in the input sequence, prediction of off-site targeting	http://www.adlilab.org/CROP-IT/homepage.html
CRISPR-P	sgRNA designing in plants, prediction of off-site targeting	http://cbi.hzau.edu.cn/crispr/
CRISPRseek	Target-specific sgRNA design tool, Prediction of off-site targeting	http://bioconductor.org/packages/release/bioc/html/CRISPRseek.html
CRISPR-PLANT	CRISPR/Cas9-sgRNAs designing tool limited to plants	http://genome.arizona.edu/crispr/
SYNTHEGO-tool	CRISPR/Cas9-sgRNAs designing tool limited to plants	https://design.synthego.com/#/

2.4.2 Annealing of reverse and forward primers

Annealing of reverse and forward primer was performed to get complementary oligo nucleotides. Two protocols were used for this purpose with some modifications (Liu et al. 2013, Li et al. 2013). Vlnv1Fwd and Vlnv1Rev primers were used to make gRNA 1, Vlnv2Fwd and Vlnv2Rev primers were used to make gRNA 2, EIF4E1Fwd and EIF4E1Rev primers were used to make gRNA 3 and EIF4E2Fwd

and EIF4E2Rev primers were used to make gRNA 4. An over primer sequences, restriction enzyme and other information is given in table 2.5.

Table 2.5. Primers used to make gRNA 1 (E1), gRNA 2 (E2), gRNA3 (V1) and gRNA4 (V2). Primers EIF4E1Fwd and EIF4E1Rev were used to make gRNA 1, EIF4E2Fwd and EIF4E2Rev were used to make gRNA 2, Vlnv1Fwd and Vlnv1Rev were used to make gRNA 3, Vlnv2Fwd and Vlnv2Rev were used to make gRNA 4. Sequence, restriction enzyme, subcloning vector for primers are given.

Primers	Sequences	Restriction Enzyme	Subcloning Vector
EIF4E1Fwd	AGTAGTGATTATGATACGGCGTCGTATTTGGTTTTAGAGC	BsaHI	pUC119-gRNA
EIF4E1Rev	GCTCTAAAACCAAATACGACGCCGTATCATAATCACTACT		
EIF4E2Fwd	AGTAGTGATTAGTCGTTAGTGTCCGGTCTAGTTTTAGAGC	BsaWI	pUC119-gRNA
EIF4E2Rev	GCTCTAAAACTAGACCGGACACTAACGACTAATCACTACT		
Vlnv1Fwd	AGTAGTGATTTTTAAGGGACTTCCGGTGGCGTTTTAGAGC	BsaWI	pUC119-gRNA
Vlnv1Rev	GCTCTAAAACGCCACCGGAAGTCCCTTAAAAATCACTACT		
Vlnv2Fwd	AGTAGTGATTTGAAGTGCTGCATGCGGTTCGTTTTAGAGC	SphI	pUC119-gRNA
Vlnv2Rev	GCTCTAAAACGAACCGCATGCAGCACTTCAAATCACTACT		

2.4.2.1 Protocol for annealing of reverse and forward primer to make a gRNA strand

These primers are like connections between the two components that you want to put together to make an overlapping PCR construct. By using web-based instruments (e.g., Primer3, Primer Design, or Primer-Blast), you will purchase two primers that complement each other. By using the online tool (Oligo Calculator), each of these primers will have a 60 ° C Tm with one portion and the same Tm with the other portion. The "end primers" will have no additions and are probable to have only locations with restrictions. Oligos have been diluted to reach 100 µM (Figure 2.2).

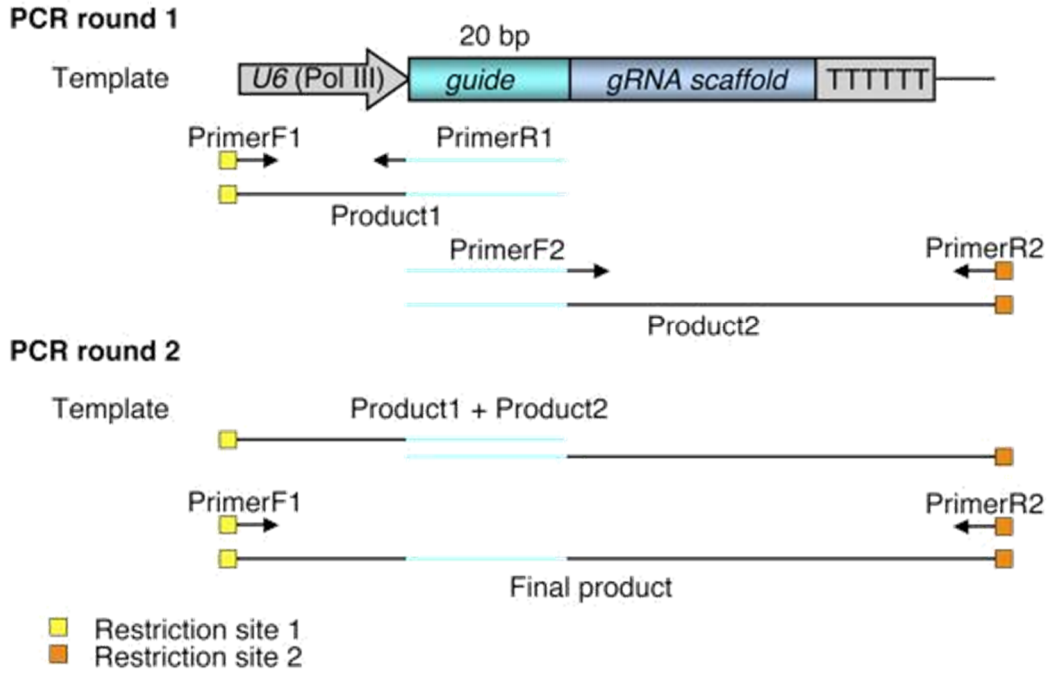

Figure 2.2. Fast assembly chart of a new gRNA build by overlapping PCR. Across PCR primers, PrimerR1 and PrimerF2, the custom guide sequence is implemented into a new gRNA in the first round of PCR. (Li et al, 2013)

2.4.2.2 Polymerase chain reaction (PCR)

PCR is a DNA strand amplification method. Materials such as DNA-primer, DNA polymerase, nucleotides, deoxynucleotides (dNTP), buffer , magnesium (Mg2+) and DNA template were required to perform PCR (Li et al, 2013). We used DNA template as pUC119_gRNA (Figure2.3) plasmid to get our modified gRNAs after PCR process. Table 2.6 blended reaction parts before double-stranded DNA was heated to 95 C for 5-10 minutes to denature the cells and enable the polymerase. Usually the temperature is fixed to 60 C or smaller after this phase. It will attach

stronger for 55 C, but less particular will be the beginning result. The single strands can respond at this temperature and connect to brief DNA segments (primers) with around 20 bases complementing the enhanced pattern. Then the temperature will be raised to about 68 C, which is ideal for replicating DNA with polymerase. DNA polymerase only produces DNA in the direction of 5'-3.' Until enough samples of DNA are produced, this method will be reiterated. As shown in table 2.6, PCR was conducted.

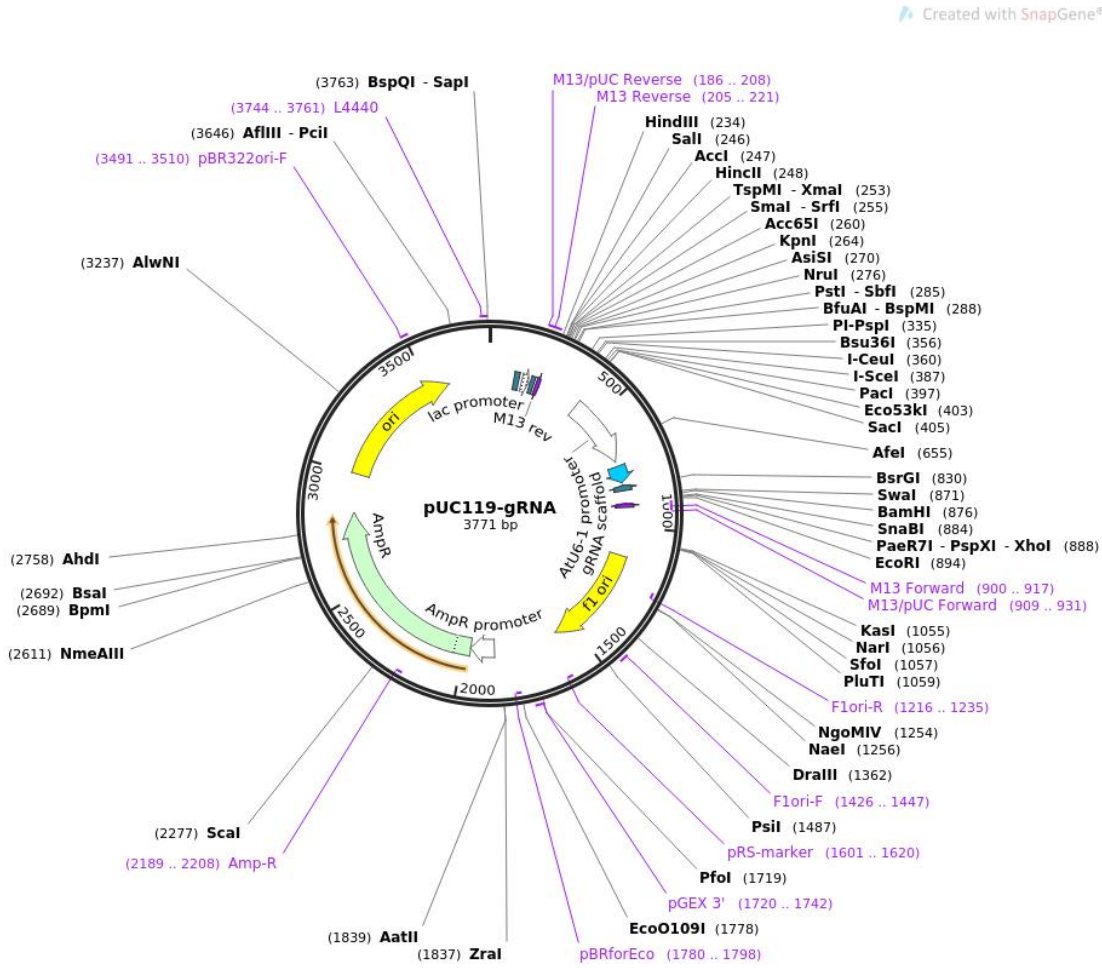

Figure 2.3. pUC119-gRNA DNA template vector. Plasmid used for producing gRNA casettes by overlapping PCR system. pUC119-gRNA was a gift from Jen Sheen (Addgene plasmid # 52255; http://n2t.net/addgene:52255; RRID:Addgene_52255)

Table 2.6. Overlapping PCR conditions and components.

Component	25 µL Reaction	
10X Standard Taq Reaction Buffer	2.5 µL	
10 mM dNTPs	0.5 µL	
10 µM Forward Primer	0.5 µL	
10 µM Reverse Primer	0.5 µL	
Template DNA	1000 ng	
Taq DNA Polymerase	0.125 µL	
Nuclease-free water	Upto 25 µL	
Temperature	**Duraiton**	
95°C	5 minutes	
95°C	30 seconds	35 cycles
60 °C	30 seconds	
68 °C	40 seconds	
68 °C	5 minutes	
4°C	hold	

2.4.3 Restriction-ligation

In order to link the gRNA templates into the plasmid, restriction and ligation reactions were performed after PCR. Figure 2.4 displays that pFGC-pcoCas9 plasmid activity was a Jen Sheen donation (Addgene plasmid #52256). Based on table 2.7 and table 2.9, solutions for restriction and ligation responses have been established. The EcoRI and XmaI restriction enzymes are used, as shown in table 2.8. The content was well blended and incubated at 37 ºC for three hours, then was denatured at 65 ºC for 30 minutes. After the first reaction (double cut of the expression plasmid), the ligation method began and the material was well combined and incubated at 4 Cº for overnight (16 hours), then was denatured at 65 ºC for 20 minutes.

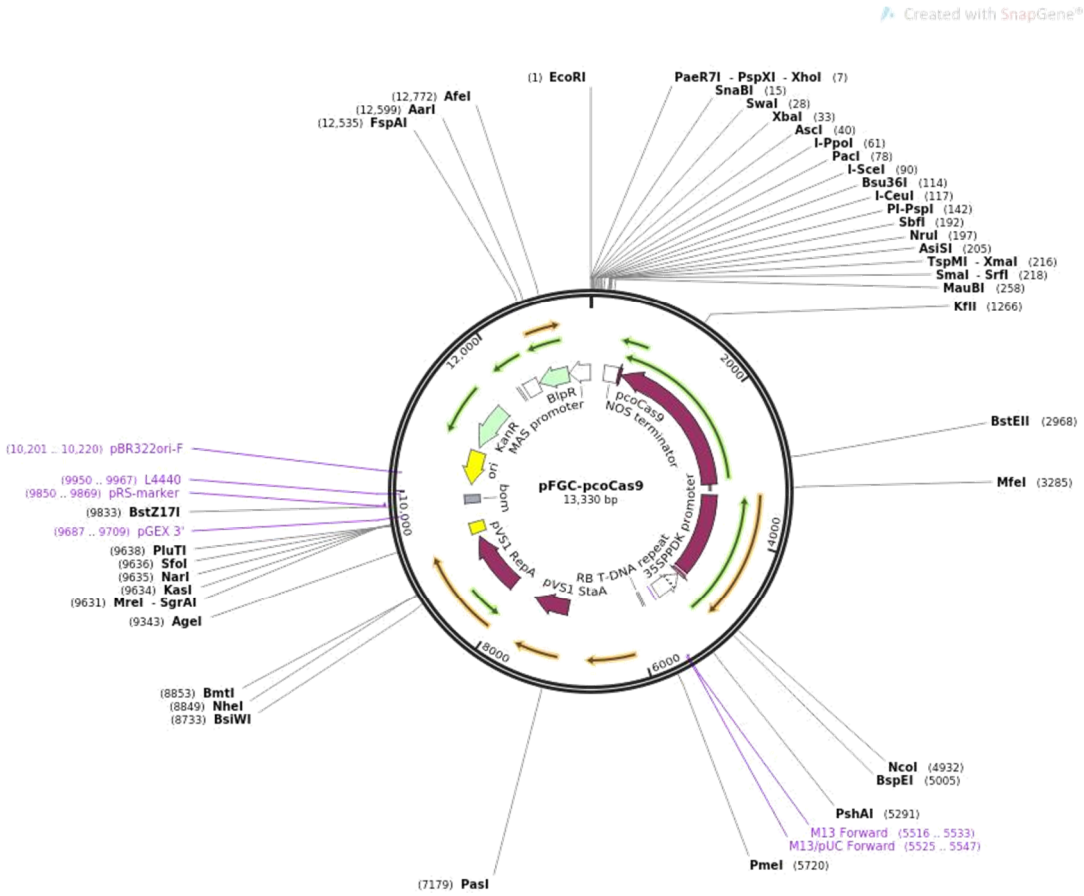

Figure 2.4. pFGC-pcoCas9 used as expression vector. gRNA casettes were ligated into this plasmid for CrispR-Cas9 mechanism. pFGC-pcoCas9 was a gift from Jen Sheen (Addgene plasmid # 52256 ; http://n2t.net/addgene:52256 ; RRID:Addgene_52256).

Table 2.7. Double digestion solution components and system mechanics

EcoRI	**1 µL**
XmaI	1 µL
CutSmart® Buffer	5 µL
DNA	1 µg
Nuclease Free Water	Upto 25 µL
Incubation Time	3 hours
Denaturation Time	30 minutes
Incubation Temperature	37 °C
Denaturation Temperature	65 °C

Table 2.8. EcoRI and XmaI restriction enzymes used in double restriction solution. Restriction sites is given as well as buffers and sources.

Restriction enzyme	Restriction site	Buffer	Source
EcoRI	5′...G▼AATTC...3′ 3′...CTTAA▲G...5′	CutSmart®	*Escherichia coli*
XmaI	5′...C▼CCGGG...3′ 3′...GGGCC▲C...5′	CutSmart®	*Xanthomonas malvacearum*

Table 2.9. T4 DNA Ligation solution components and system mechanics

T4 DNA Ligase Buffer (10X)	2 µL
Vector DNA	0.020 pmol
Insert DNA	0.060 pmol
T4 DNA Ligase	1 µL
Nuclease Free Water	Upto 20 µL
Incubation Time	4 °C
Denaturation Time	65 °C
Incubation Temperature	16 hours
Denaturation Temperature	20 minutes

2.4.4 Transformation

The transformation of competent cells into *E.coli* Top10 was carried out. The aim of creating competent cells, like a plasmid, is the capacity of cells to pick up safe, extracellular genetic material. *E.coli's* Top10 bacterial strain is a helpful source for multiple reasons as conversion of various vectors and manufacturing of single-stranded DNA from M13 or phagemid vectors (Invitrogen 2019). Top10 sample from *E.coli* from Invitrogen was used in this experiment. In this experiment, transformation was carried out several occasions for both gRNAs and Cas9.

2.4.4.1 Competent cells

Competent cells have been kept on ice for 20 minutes, all the item has been mixed with competent bacteria (10 µl for each tube into competent cells) and left on ice for 30 minutes at 42 C for 45 seconds, before heat shocking and then put away into ice for five minutes. Added, stirred and incubated LB broth (see section 2.10) 500 µl for 2 hours and 20 minutes (at least 75 minutes) for 37 C. At 37 C overnight, 500 µl of the item was then plated on agar sheets (see table 2.10).

Table 2.10. LB broth medium with kanamycin/ampicillin. For transformation of gRNA LB broth with ampicillin was serviced, and for transformation of Cas9 LB broth with kanamycin was serviced.

gRNA		**Cas9**	
Content	**Amount**	**Content**	**Amount**
LB Broth	5 mL	LB Broth	5 mL
Ampicilin	100 mg/mL	Kanamycin	100 mg/mL

2.4.4.2 Colony PCR

Colonies studied with colony PCR were using Q5 Hot Start High Fidelity Taq DNA polymerase (New England Biolabs) to determine if the converted bacteria were receiving gRNA in the vector. The reaction was accompanied by the spacer forward primers and the reverse primers (Sentegen, Turkey) shown in Table 2. 5. Table 2.11 provides a solution for the colony PCR.

Table 2.11. Colony PCR conditions and components.

Components	**25 µL Reaction**	
5X Q5 Reaction Buffer	5 µL	
5X Q5 High GC Enhancer	5 µL	
10 mM dNTPs	0.5 µL	
10 µM Forward Primer	1.25 µL	
10 µM Reverse Primer	1.25 µL	
Template DNA	1000 **ng**	
Q5 Hot Start High-Fidelity DNA Polymerase	0.25 µL	
Nuclease-Free Water	Upto 25 µL	
Temperature	**Duraiton**	
98°C	2 minutes	
98°C 62 °C 72 °C	10 seconds 30 seconds 30 seconds	35 cycles
72 °C	2 minutes	
4°C	hold	

2.4.5 Loading of colony PCR product onto 2 % agarose gel

The product was loaded onto 2% agarose gel after colony PCR. Agarose gel electrophoresis is a technique that distinguishes the sizes of the DNA macromolecules. The DNA molecules, positively loaded, will be moved against the anode. In a greater velocity than the larger molecules, small molecules will migrate against the anode. The lengths of the molecules can be determined by contrasting a norm with a ladder. A dye (Ethidiumbromide) is introduced to create the marks noticeable in a UV light. The DNA reacts with this colour. Samples are combined with the loading buffer and Ethidiumbromide. A loading buffer offers noticeable coloring that shows how soon the molecules have gone, and includes a elevated proportion of glycerol that raises the specimen size to fall into the well (Addgene 2017a).

For the production of two-percent agarose gel, a microwave buffer of two grams + 100 ml of onex Tris-acetate-EDTA (TAE) was chopped and fully dissolved prior to pouring into a leveraged jelly frame. Liquid had been abandoned until finalized for about 30 minutes. It was moved to an electrophoresis container packed with a buffer of 1x TAE after the gel was removed. Master mix was then created with the buffer loading. Two µL of load buffer were used for each test. Before the specimens 10 µL were supplied with the master mix in the remainder of the wells, the first well was filled with DNA ladder.

For 35 minutes, gel electrophoresis was executed at 90 V. Using UV transilluminator after gel electrophoresis spots were shown.

2.4.5.1 Overnight cultures

To obtain a large quantity of plasmid, cultures were produced overnight. One colony from favorable colonies was transmitted in a sterile hood to five mL LB broth produced with kanamycin or amphicilin. For 16 hours, cultures were incubated at 37 Cº.

2.4.6 Isolation of plasmids

Two industrial mini-prep sets were used to isolate plasmids:

1- Miniprep kit from QIAprep Spin Miniprep Kit from QIAGen

2- Miniprep kit from GeneJET Plasmid Miniprep Kit from ThermoFisherScientific

A detailed explanation of the QIAprep Spin Miniprep package from QIAGen.

Harvesting and lysing bacteria

The culture of the Eppendorf bacteria was poured out and centrifuged for one minute in full speed. Supernatant has been thrown away. It's been reiterated then. The pellet was supplemented with 250 µL resuspension solution and vortexed until dissolved. Added, reversed softly and removed 250 µl lysis solution for 2 minutes.

Prepare cleared lysate

In addition to treatment, 350 µL of neutralization were combined ten eight to occasions by reversal. Centrifuged at maximum velocity for 10 minutes.

Prepare binding column

Column prepataion solution of 500 µl was introduced to the sample tube binding column and centrifuged at maximum velocity for four minutes. Then the flow-through was dismissed.

Bind plasmid DNA to column

Cleared lysate was transmitted for one minute to the binding column and centrifuged. Flow-through for a spinjet to dry was removed and centrifuged for a minute.

Elute purified plasmid DNA

Column was moved to a fresh sample tube and 60 µL of nuclease free water was introduced and centrifuged for one minute and then frozen at-20 C. Samples from the freezer were then evaluated with nanodrop and permitted to operate on agarose gel for gel extraction.

2.4.7 Sequencing

Sequencing with 35SPPDK primers to Sentegen Biolabs, Turkey was carried out using the isolated plasmid with gRNA casettes. For the whole sequence, see Appendices Figure 3.

2.4.8 Digestion of Plasmids

Restriction enzymes are needed for plasmid digestion. Restriction enzymes are bacterial endonucleases that acknowledge and break DNA that is unknown to the bacteria.

They benefit from innate enzymes that split the DNA at particular locations.

Restriction enzymes, for instance are widely used in cloning. In this experiment, constraint enzyme BsaHI (New England Biolabs), BsaWI (New England Biolabs) have been used for gRNA and Cas9 has been used for SphI (New England Biolabs), see chart 2.12.

Digestion was conducted of effective plasmids that contain gRNAs using BsaHI, BswI and SphI. Samples were taken in accordance with Table 2.14 (Table 2.13 displays the intensity and calculation) and then stood for one hour at 37 C for incubation. Also permitted to operate undigested samples with the specimens + MM. Digested samples were subsequently permitted to operate on electrophoresis of one percent agarose gel. Several occasions plasmid digestion has been conducted. With both gRNAs and Cas9, it was conducted the second time. Figure 2.4 shows a table of pFGC-pcoCas9 (Shen et al. 2013), Jen Sheen donation (Addgene plasmid #52256). Two parallels were used for five μg in each test. For BsaHI and SphI 50 units of restriction enzyme and a little less for BswI were used. Table 2.15 demonstrates all components for ligation control of Cas9 plasmid with gRNA casettes in digestion procedure. After ligation we used XbaI and NruI for negative result. If ligation works XbaI and NruI couldn't cut the ligated plasmids, but if self ligation occur in pFGC-pcoCas9 plasmid, XbaI and NruI digestion gives two bands in agarsose gel result. Samples were incubated at 37 C overnight.

Table 2.12. BsaHI, BsaWI and SphI restriction enzymes used in gRNA restriction solutions. Restriction sites is given as well as buffers and sources.

Restriction enzyme	Restriction site	Buffer	Source
BsaHI	5′...GR▼CGYC...3′ 3′...CYGC▲RG...5′	CutSmart®	*Bacillus stearothermophilus CPW11*
BsaWI	5′...W▼CCGGW...3′ 3′...WGGCC▲W...5′	CutSmart®	*Bacillus stearothermophilus W1718*
SphI	5′...GCATG▼C...3′ 3′...C▲GTACG...5′	CutSmart®	*Streptomyces phaeochromogenes*

Table 2.13. Samples for digestion and concentration.

Samples	Concentration	Volume for 1 µg
E1	208.6 ng/µl	4.8 µl
E2	149.4 ng/µl	6.7 µl
V1	247.2 ng/µl	4.0 µl
V2	229.1 ng/µl	4.4 µl

Table 2.14. Digestion of the samples, amount and content for gRNAs.

Content	E1	E2	V1	V2
DNA	4.8 µl	6.7 µl	4.0 µl	4.4 µl
BsaHI	1 µl			
BsaWI		1 µl	1 µl	
SphI				1 µl
CutSmart®	5 µl	5 µl	5 µl	5 µl
Nuclease Free Water	14.2 µl	12.3 µl	15 µl	14.6 µl
Total	25.0 µl	25.0 µl	25.0 µl	25.0 µl

Table 2.15. Components for digestion of plasmids with samples from ligated Cas9/gRNA.

Components	Volume
Ligated Cas9/gRNA plasmid	1 µg
XbaI	1 µl
NruI	1 µl
CutSmart®	5 µl
Nuclease Free Water	Upto 25 µl
Total	25 µl

2.4.9 Glycerol stocks

Glycerol was stored in samples E1, E2, V1, V2, gRNA and Cas9. 500 µl of glycerol + 900 µl of bacteria. They were then placed in -80 ° C.

2.4.10 Gel extraction

The bands were sliced and evaluated in UV light. The QIAquick Gel Extraction Kit (Cat No./ID: 28704) from QIAgen was used in accordance with the protocol. 900 µL of solubilizing fluid has been used. Samples were evaluated using nanodrop after the gel extraction unit experiment was conducted.

2.4.11 gRNA In Vitro Transcription

gRNAs from plasmid HiScribe™ T7 Quick High Yield RNA Synthesis Kit from New England Biolabs (E2050S) were used for in vitro transcription. gRNA tubes were launched and placed in heat block for 45-75 minutes for 60 oC to get greater concentration in nanodrop tubes. As water evaporates, this will lead in greater density.

Before being tested with nanodrop again, tubes were reversed, centrifuged and placed on board. The synthesis protocol for RNA was then established. One µg was used for each test. RNA synthesis method was laid up as shown in table 2.16, response for short transcripts. An additional control with water (18 µl) was performed. Nanodrop were used for samples were then evaluated.

Table 2.16. RNA synthesis reaction with components.

Components	Volume
NTP Buffer Mix	10 µl
Template DNA	1 µg
T7 RNA Polymerase Mix	2 µl
Nuclease Free Water	Upto 30 µl
Total Reactipn Volume	30 µl

2.4.12 Cleaning RNAs

For only the cleaning of RNAs MegaclearTM kit Purification was used from Thermo Fisher Scientific for large-scale transcription reaction kit. User centrifuge technique has been selected (alternative 1 for elution). Nanodrop samples were evaluated after stage 6 and then congelated at -20 C

2.4.13 Cas9 In Vitro Transcription

To make Cas9 mRNA, New England Biolabs used a product from "RNA enzymes and gene assessment HiScribeTM ARCA mRNA kit with tailing. Using one µg DNA model. As shown in table 2.17, reaction was laid up and experiment was conducted with some limitations according to the protocol. Steps 3 and 4 were omitted and substituted by "RNA tidy and concentratorTM-25" set from Zymo Research (R1017) cleaning.

The remainder of the protocol from phase 5 was carried after cleaning measures. Samples were evaluated by nanodrop after this.

Table 2.17. Reaction components for in vitro transcription of Cas9 DNA template

Reaction components	Volume
Nuclease Free Water	3.4 µL
2X ARCA/NTP Mix	10 µL
Template DNA	4.6 µL
T7 RNA polymerase mix	2 µL
Total Volume	20 µL

2.4.14 Isolation of plant DNA

Protocol based isolation of plant DNA was conducted (Springer 2010). The plant processing buffer for Cetyltrimethyl ammonium bromide (CTAB) was provided and sterilized as per Table 2.18. Use of 10 mL of CTAB + 100 mL of B-mercaptoethanol.

Use and centrifugation of 500 μL of protoplasts at 700 rpm for one minute before discarding the supernatant. A PepsiCo leaf has been used as a control. In Eppendorf tubes (two tubes per sample with 1.5 mL in) plant tissue was gathered from tiny Petri dishes. Samples were centrifuged for a minute at 500 rpm. Supernatant has been transferred to another tube in Eppendorf to ensure that not all protoplasts have continued. Reversed and mixed 700 μL of CTAB fluid (CTAB crop extraction buffer) and incubated at 65 C for 30 minutes (mixed after 15 minutes). After this phase, the two Eppendorf tubes were transmitted for each sample to one tube. Mixed chloroform: isoamyl alchol 400 μL of 24:1 and blended with vortexing until there were no "stages" in the envelope. Centrifuged for 5 minutes at 10 000 g.

The aqueous phase (bottom phase) was transferred to a fresh Eppendorf tube. Added 300 μL isopropanol, mixed and centrifuged for 5 minutes at 10 000 g. Liquid has been spilled out. Added 500 μL 70% ethanol, combined with vortex and centrifuged for two minutes at 10 000 g. Then liquid was spilled out. Pellet was air-dried and one mM Tris (pH 8) resuspended at 100 μL. Nanodrop was used to measure samples and prepare them for PCR.

Before PCR was conducted, samples (100 μg) were permitted to operate on agarose gel.

Primers diluted to 100 μmol / L. Primers were centrifuged for the first time before adding water.

Vorting combined samples. Table 2.19 shows the primers used. Samples were then permitted to operate two percent agarose on gel electrophoresis. AppliChem loading buffer DNA II has been used. Samples could operate for 40 minutes at 90 V. Following this, samples were permitted to operate on 2% agarose gel for extraction at 80 V for 35 minutes. Macherey-Nagel sliced bands and set: "DNA, RNA and protein purification. DNA extraction from agarose gels" was used.

Added 400 µL of NT1 solution for every 100 mg agarose gel > 2 percent.

Protocol exceptions:

-Centrifuged for one minute rather than 30 seconds (phase two and three).

-Twice Elution stage (last stage in box before nanodrop)

-Step 5: Eluted DNA to 20 µL ddH2O (not buffer NE as in procedure)

Table 2.18. CTAB Solution

Reagent	Amount	Final concentration
Tris (1M pH 7.5)	100 mL	100 mM
NaCl (5M)	140 mL	700 mM
EDTA (0.5 M)	20 mL	10 mM
CTAB	10 g.	%1
ddH_2O	Upto 990 mL	
β- mercaptoethanol		%1

Table 2.19. Primers from genes EIF4E and Vlnv, sequences, PCR amplicon sizes and sgRNAs.

Primers	Sequence (5´----3´)	PCR	sgRNA
EIF4E1Fwd	AGTAGTGATTATGATACGGCGTCGTATTTGGTTTTAGAGC	649 bp	ATGATACGGCGTCGTATTTG
EIF4E1Rev	GCTCTAAAACCAAATACGACGCCGTATCATAATCACTACT		NGG
EIF4E2Fwd	AGTAGTGATTAGTCGTTAGTGTCCGGTCTAGTTTTAGAGC	649 bp	AGTCGTTAGTGTCCGGTCTA
EIF4E2Rev	GCTCTAAAACTAGACCGGACACTAACGACTAATCACTACT		NGG
Vlnv1Fwd	AGTAGTGATTTTTAAGGGACTTCCGGTGGCGTTTTAGAGC	649 bp	TTTAAGGGACTTCCGGTGGC
Vlnv1Rev	GCTCTAAAACGCCACCGGAAGTCCCTTAAAAATCACTACT		NGG
Vlnv2Fwd	AGTAGTGATTTGAAGTGCTGCATGCGGTTCGTTTTAGAGC	649 bp	TGAAGTGCTGCATGCGGTTC
Vlnv2Rev	GCTCTAAAACGAACCGCATGCAGCACTTCAAATCACTACT		NGG

As shown in Table 2.20, PCR reaction was laid up.

Table 2.20. Q5 PCR conditions and components

Component	**50 µL Reaction**	
5X Q5 Reaction Buffer	10 µL	
5X Q5 High GC Enhancer	5 µL	
10 mM dNTPs	1 µL	
10 µM Forward Primer	1.25 µL	
10 µM Reverse Primer	1.25 µL	
Template DNA	1000 **ng**	
Q5 Hot Start High-Fidelity DNA Polymerase	0.25 µL	
Nuclease-Free Water	Upto 50 µL	
Temperature	**Duraiton**	
98°C	2 minutes	
98°C 62 °C 72 °C	10 seconds 30 seconds 30 seconds	35 cycles
72 °C	2 minutes	
4°C	hold	

2.4.15 Cas9 mRNA Gel (one % agarose gel)

These were permitted to operate on formaldehyde denaturing agarose gel to verify the value of Cas9 mRNA transcripts. Table 2.21 demonstrates the Cas9 mRNA gel buffer and gel. It was used to create the protocol of fluid and buffers (Kataya 2011). National Diagnostics used the "Preparation of Denaturing Agarose Gels" test preparing procedure.

Until dissolved, agarose and water were heated in the microwave and cooled down to about 60 C. Warmed buffer 10x MOPS and formaldehyde to 55 C and finally introduced GelRedTM. Gel was kept for an hour to lay down. Sample buffer No.1 was ready for 15 minutes using samples and boiled to 55 C. Sample buffer No.1 + 2.5 µLl (two µg specimen) of Cas9 was taken out 10 µL and cooked 55 C in thermal box for 15 minutes. Added specimen buffer No.2 2.5 µL after boiling. Gel electrophoresis was then permitted to operate at 90 V for 50 minutes.

Table 2.21. Buffers and gel for Cas9 mRNA gel

Buffers/Gel	Components	Amount
10 x MOPS gel running buffer (0.5 L)	0.4 M MOPS (pH 7.0) pH was adjusted with NaOH	41.85 g
	0.1 M sodium acetate	4.10 g
	10 mM EDTA	10 mL
Formaldehyde denaturing agarose gel (50 ml 1 % agarose)	Agarose	0.5 g
	Nuclease free water	36 mL
	10 x MOPS buffer	5 mL
	Formaldehyde (37 %)	9 mL
	GelRedTM	5 mL
Sample buffer NO.1 (1 ml)	Dimetylformamide*(65 %)	650 µl
	Formalmin(22 % from 37 % formaldehyde)	220 µl
	10 x MOPS (13 %)	130 µl
Sample buffer NO. 2 (1 ml)	Glycerol (50 %)	500 µl
	EDTA (1 mM EDTA 0.001M EDTA)	2 µl (from 0.5M EDTA)
	Bromophenol blue	3 µl (0.003g)
	Xylene cyanol	3 µl (0.003g)
	ddH2O	492 µl

Note. * = dimetylformamide was used rather than formamide

2.4.16 gRNA gel (denaturing PAGE/Urea Gel)

They were permitted to operate on denaturing PAGE / Urea fluid to verify the value of gRNA transcripts. According to gRNA gel was established (Summer et al. 2009). gRNA gel buffers are mentioned in table 2.22.

Gel was pre-run on two gels with onex TBE buffer. If any leakage occurred, it was verified.

Combs were deleted afterwards. Wells have been rinsed with a working buffer before applying the gel to prevent UREA. Samples were drawn from the ice straight.

3 µL specimen and 2 µL ddH2O + 5 µL load balance were added. This raised the thermal block at 80 C for a couple of minutes and introduced two µL of GelRedTM to the specimens. This was done in a hood.

Table 2.22. Buffers for gRNA gel (PAGE-Urea Gel)

Buffers	Components	Amount
10 x Tris Borate EDTA (TBE) buffer (0.5 L)	Tris base (0.9 M)	54.52 g
	Boric Acid (0.9 M)	27.2 g
	EDTA (20 mM from 0.5 M pH 8.0)	20 mL
Loading buffer 1 mL	Dimetylformamide* (90 %)	900 µL
	EDTA (0.5 %)	5 µl (from 0.5 M)
	Xylene cyanol (0.1 %)	1 µL (0.001 g)
	Bromphenol blue (0.1 %)	1 µL (0.001 g)
	Glycerol (9.3 %)	93 µL

Note. * = Dimetylformamide was used rather than formamide

2.4.17 In Vitro Digestion of DNA with Cas9

DNA digestion with Cas9 was conducted in vitro in accordance with kit "In vitro digestion of DNA with Cas9 nuclease and sgRNA, S. Pyogenes (Cat. No. TP790148) "OriGene. The Streptococcus pyogenes Cas9 nuclease was used in this unit for the digestion of in vitro double-stranded DNA with Cas9 and gRNA. Table 2.23 and Table 2.24 shows the reaction set-up and test method.

Table 2.23. Reaction compositon of Cas9 and gRNA to in vitro digestion of DNA

	Cas9	**gRNA**	**DNA**
Relationship	10	10	1
Concentration	1 µM	1 µM	20 nM
Volume	1 µL	1 µL	4 µL
Final Concentration	30 nM	30 nM	3 nM

Cas9, gRNAs (E1 & E2 & V1 and V2) and DNA (protoplasts) solutions were developed:

• Cas9 final density one µM

• gRNA final density one µM

• DNA (protoplasts) final concentration 0.02 µM

Samples were carefully combined and centrifuged at 37 C for an hour before incubation. After that, heat at 65°C for 10 min to deactivate Cas9 nuclease.

A fragment assessment with gel electrophoresis (2% agarose solution) was then performed.

Table 2.24. In vitro digestion reaction of DNA with Cas9 nuclease

Components	**Volume**
Nuclease Free Water	21 µL
10 x Cas9 nuclease reaction buffer	3 µL
1 µM sgRNA	1 µL (~30 nM final)
1 µM Cas9 Nuclease, S. pyogenes	1 µL (~30 nM final)
20 nM substrate DNA	4 µl (~3 nM final)
Pre-incubate for 10 minutes at 25 ºC (To make complex)	
Total	30 µl

2.5 Isolation of protoplasts and transfection

The previous alternatives were developed for protoplastic separation and transformation mentioned in Table 2.25 and Table 2.26. Some alternatives mentioned in Appendices in Table 1 and 2 have also been used for protoplastic separation.

Table 2.25. Solutions used for protoplast isolation

Solutions	Components	Amount	Sterilization
Enzyme Solution (50 mL)	Cellulase (%1.0)	0.5 g	
	Maceroenzyme (%0.5)	0.25 g	
	Mannitol (0.45 M)	4.10 g	
	2-(N-morpholino)-ethanesulfonic acid (MES) (20 mM pH 5.7)	2 mL (from 500mM Solution)	
	CaCl2 (1 M)	500 µL	
	Bovine Serum Albumin (BSA)	0.05 g	
PEG Solution (10 mL)	Polyethylene Glycerol (PEG) 4000	4 g	Filter Sterilized
	CaCl2 (1 M)	1 mL	
	Mannitol (0.5 M)	4 mL	
	Distiled Water	Upto 10 mL	
W5 Solution (500 ml) pH adjusted to 5.7 with KOH	2 mM MES (pH 5.7)	0.1952 g	Filter sterilized and stored at 4 °C
	154 mM NaCl	4.499 g	
	125 mM CaCl2	6.9363 g	
	5 mM KCl	0.1864 g	
MaMg (50 ml) pH adjusted to 5.7 with KOH	Mannitol (0.5 M)	4.555 g	Filter sterilized and stored at 4 °C
	MgCl2 x 6H2O (15 mM)	0.152 g	
	MES (0.1%)	0.100 g	
B5 (50 ml)	1 x B5	500 µL of all stock solutions	

Table 2.25 (cont'ed). Solutions used for protoplast isolation

		*	
	Glucose (0.45 M)	4.00 g	
	2,4-D (1 mg/L)	50 µL from 1 mg/ml stock solution	
	BAP (0.15 mg/L)	7.5 µL from 1 mg/ml stock solution	

Note. * = Stock solutions are listed in table 2.26

Table 2.26. Stock solutions in medium used to make B5 for transformation of protoplasts

	Concentration in medium (1x)		**Stock (100x)**	**Volume (ml) of stock to make medium (1x) 1L**
Macro Elements	**mM**	**mg/L**	**g/100 mL**	
CaCl2 x 2H2O	1.02	150	1.5	10 mL
KNO3	24.73	2500	25	10 mL
MgSO4 x 7H2O	1.01	250	2.5	10 mL
NaH2PO4 x 2H2O	1.09	170	1.7	10 mL
(NH4)2SO4	1.01	134	1.34	10 mL
FeNaEDTA	0.100	36.7	0.367 (10mM)	10 mL
Micro Elements	**µM**	**mg/L**	**g/L**	
CoCl2 x 6H2O	0.11	0.025	0.0025	10 mL
CuSO4 x 5H2O	0.10	0.025	0.0025	10 mL
H3BO4	48.2	3.00	0.30	10 mL
KI	4.52	0.75	0.075	10 mL
MnSO4 x 1H2O	59.16	10.00	1.0	10 mL
Na2MoO4 x 2H2O	1.03	0.25	0.025	10 mL
ZnSO4 x 7H2O	6.96	2.00	0.20	10 mL

2.5.2 Isolation of protoplasts

2.5.2.1 Surface sterilization of leaves

Two alternatives were developed to conduct flower leaf ground sterilization: 0.5% calcium hypochlorite and 70% alcohol 50 ml. Leaves were sliced and carried in a total of 60 seconds to Falcon tubes with 70% ethanol. Invert the tubes. Leaves were then transported in a Petri dish to a 0.5% solution of calcium hypochlorite and dipped for 20 minutes. Then leaves were transported with sterilized water to a Petri dish, separated, dipped into fresh sterilized water and performed again. Leaves were prepared to be moved to a remedy for preenzyme treatment (PET) after this sterilization. PET methods and components can be found in Appendices 4.

2.5.2.2 Preparation for protoplast isolation

Aluminum foil was put around propagated Germini crops and then returned for three days to the same place. Leaves were cut into small fragments and 30 mL of PET solution was transferred to large Petri dishes and placed in a sterile hood (15 ° C) overnight. PET solution in sterile cover was removed with pipette. For the digestion of cell wall enzyme solution 30 mL was inside and placed overnight in a shaker at 40 rpm for 28 ° C with parafilm over.

2.5.2.3 Collection of protoplasts

Protoplastic collection was conducted with changes in accordance with the procedure (Woo et al. 2015). After overnight treatment, plates with leaves in enzyme solution were removed.

Enzyme fluid was removed by cutting pipette edges and softly handled protoplasts with funnel and filter, pre-sterilizedwith alcohol (%70 Ethanol). In 15 mL Falcon tubes, protoplasts were gathered. At 600 rpm, protoplasts were centrifuged at 10-11 C for one min.

Supernatant has been separated and in each Falcon bottle five mL of W5 solution has been inserted.

Centrifuged for one min at 12 C at 600 rpm before removing supernatant. In each Falcon tube, 2.5 mL washing fluid was added and 100 µL was taken from this to two Eppendorf tubes and by using a hemocytometer, calculated protoplasts.

2.5.2.4 Microscopy

For the calculation and examination of protoplasts, two distinct microscopies were used during this research: light, and epi-fluorescence microscopy. Light microscopy is a well-known biological instrument used to detect tiny items that are not noticeable to the naked eye using light. A hemocytometer was used in this microscopy to calculate protoplasts.

In laboratories that work with life science, epi-fluorescence microscopies are usual to use. This microscopy was used for protoplastic examination. Usually the

microscope comprises of these vital parts: a light source, an incoming light filter, a dichroic beam splitter or mirror, an emitted light filter, and a CCD (Webb and Brown 2013; Scientific 2019) lens.

Both the illuminated and the emitted beam will pass through the same objective lens in epi-fluorescence microscopy. By focusing on the sample through the objective lens beam of the excitation wavelength. The incoming beam will restrict the light's range so that only the frequencies used to excite the specimen will pass. The dichroic beam splitter or mirror reflects the wave of excitation and only returns directly to the detector the heat produced from the sample. Filter for reflected light transmits the reflected light's wavelengths from the sample and blocks all the beam that has gone through the excitation filter. Fluorescence imaging utilizes a CCD camera linked to a computer screen to detect the emitted light (Webb and Brown 2013; Scientific 2019).

2.5.3 Transfection

Transfection was conducted after protoplastic isolation. W5 was separated and MaMg resuspended protoplasts. Petri dishes have been introduced DNA (summary in table 2.27) and MaMg resuspended protoplasts have also been introduced. Then pipetting combined in this well.

Before putting on the cover, 500 µL PEG was introduced to drop wise and stayed for 30 minutes. W5 was then wisely and very gently introduced to the Petri dish drop. First 0.5 mL, then 1 mL, 2 mL, and 3 mL with 6.5 mL in sum. Solution was then transmitted to a Falcon tube and at 14 C for one minute centrifuged for 500 rpm. Resuspended solution in three mL B5 and transferred in dark at 25 C to small Petri dishes and incubated them during the overnight. Table 2.27 provides an overview of the material of protoplast transfection Petri dishes.

Table 2.27. Protoplast transfections

Experiment	Content	Amount
1	Cas9 mRNA	42 µL
	gRNA	33 µL
	Protoplasts	300 µL*
2	Protoplasts	300 µL*

* : 250.000 protoplasts per 300 µL in Magnesium solution

Following transfection, protocol-based separation was conducted (Springer 2010).

Exceptions from the procedure were that 300 µL CTAB (CTAB crop extraction buffer) product was introduced and blended by reversal and incubated at 65 C for 30 minutes (mixed after 15 minutes by reversal). Nanodrop was used to measure samples and prepare them for PCR. Every sample was ready at 100 ng. PCR was established in accordance with Table 2.28.

Then, PCR products were permitted to operate two percent agarose on gel electrophoresis. There were two gels produced; one to cut and one to check. Samples were permitted to operate at 80 V for 50 minutes. Bands were sliced and evaluated in UV light. Extraction of the gel was then carried out.

Table 2.28. PCR setup of after transfection procedure

Reagent	Cas9 mRNA and gRNA
Template DNA	100 ng
Nuclease Free Water	Upto 50 µL
Forward Primer	1 µL
Reverse Primer	1 µL
10x Dream Taq Buffer	5 µL
dNTP Mix	5 µL
Dream Taq Poly	0.25 µL
Total	50 µL

2.5.3.1 DNA extraction from agarose gels

With some circumstances, protocol from Macherey-Nagel's Nucleospin Gel and PCR Clean-up was performed for DNA extraction. Instead of 30 seconds, centrifugation was used for one minute. Centrifuged for two minutes instead of one minute to dry silica membrane and incubated columns at 50 ºC for three minutes. Instead of buffer, NE, nuclease free water was used for elution of DNA. Samples were then frozen overnight at -20 C. Once moving ahead with T7 Endonuclease I assay the next day, samples were quantified by nanodrop.

2.5.4 T7 endonuclease I assay

T7 Endonuclease I assay has been used to determine genome placement. T7 Endonuclease I assay was conducted after gel extraction to see if any slicing had been done by Cas9. T7 Endonuclease I provides two aims; it acknowledges and cleaves paired DNA. The assay provided annealing and digestion reaction with T7 Endonuclease I. The protocol used T7 Endonuclease I (MO302) from New England Biolabs to determine genome targeting efficiency and Cas9 (MO641T) was used. Gel fragments have been evaluated.

2.5.4.1 Protocol

1. Annealing reaction

The first stage was the samples E1 & E2 & V1 and B2 annealing reaction. As outlined in Table 2.29, the statement was laid up.

Table 2.29. Annealing reaction with samples of E1,E2,V1 and V2

Reagents	Volume (µL)
DNA	200 ng
10x NEB Buffer	2 µL
ddH2O	Upto 19 µL
Total	19 µL

2. Hybridization condition (thermocycler)

Thermocycler annealing was accomplished as outlined in Table 2.30

Table 2.30. Thermalcycler procedure of annealing step

Step	Temperature	Ramp Rate	Duration
Initial Denaturation	95 Cº		5 min
Annealing	From 95 Cº to 85Cº	-2 Cº/ sec	
	From 85 Cº to 25Cº	-0.1 Cº/ sec	
Hold	4 Cº		Hold

3. Added T7 endonuclease and incubated

The last stage was to incorporate digestion T7 Endonuclease. Table 2.31 describes reagents. Two percent of agarose was conducted after this stage fragment assessment with gel electrophoresis. Samples could operate at 90 V for 50 minutes.

Table 2.31. Components for T7 Endonuclease I protocol

Reagents	**Volume**
Annealed PCR products	19 µL
T7 Endonuclease I	1 µL
Total	20 µL
Incubation Time	15 min
Incubation Temperature	37 Cº

CHAPTER 3

RESULTS

3.1 Growth and propagation of potato plants

Plants from both tissue and soil cultivation and propagated plants from PepsiCo potato crops were used to conduct the tests. Two techniques of sterilization have been tested; one with ethanol and one with Ca-hypochlorite. Ethanol sterilization seemed to destroy all the plants of potato seedlings. Thus, the ethanol sterilization method was dismissed. Ca-hypochlorite sterilized seeds were grown healthy. Figure 3.1 displays PepsiCo potato seedlings grown for 14 days in MS with sucrose medium.

Figure 3.1. After 14 days of growth; PepsiCo potato plants in MS medium with sucrose.

Throughout the experiments, the propagation of potato crops was carried out in agar containing MS medium with 2% sucrose after 15 days of growth. The crops have evolved as shown in the chart and the propagation was successful.

Figure 3.2. PepsiCo potato plants is shown in MS medium + agar 2 % sucrose after 15 days

Figure 3.2 cont'ed. PepsiCo potato plants is shown in MS medium + agar 2 % sucrose after 15 days

3.1.1 Potato plants on TM-1 medium

Plants were transmitted to the TM-1 medium after sowing potato seeds on MS medium (3% sucrose). Figure 3.3 demonstrates PepsiCo plant crops transmitted from MS intermediate with sucrose two weeks aged onTM-1 intermediate. In Figure 2.1 in Material and Methods chapter (Shepard 1977), plants were on TM-1 media for two to six weeks, where protocol with changes was pursued.

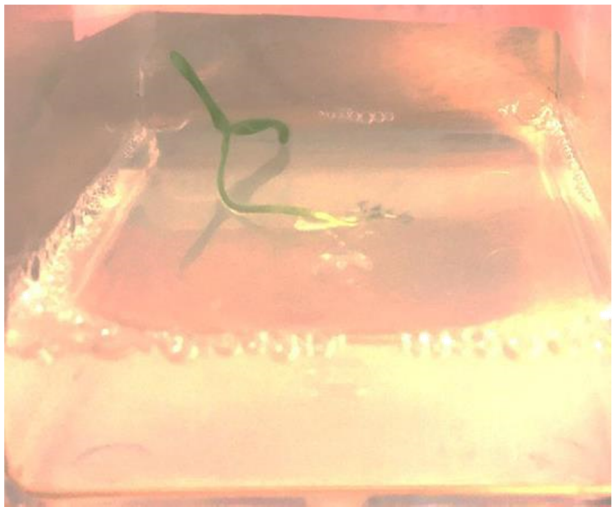

Figure 3.3:. Two weeks aged PepsiCo potato crops transmitted from MS medium with sucrose on TM-1 medium

3.1.2. Potato plants on soil

Infected with pathogens, potato crops from the MS medium were transmitted to soil. Figure 3.4 displays plant crops from MS intermediate with sucrose from two weeks old PepsiCo.

Figure 3.4. PepsiCo potato crops are 2 weeks aged and transmitted from intermediate MS medium + agar with sucrose to soil because of fungal infections.

3.2 gRNA, Cas9

Many studies have been conducted in this chapter on gRNAs and Cas9 as primers annealing, restriction and ligation reactions, conversion, encoding, plasmid digestion, gRNA and Cas9 in vitro transcription, and Cas9 nuclease in situ digestion. gRNA and Cas9 transcripts were planned to deliver in vivo with restricted potato protoplasts transfection before the last experiment was conducted with T7 Endonuclease I assay, but there are some troubleshoots that we couldn't conclude final products to finish transfection assay.

3.2.1 In vitro cloning

At first we started to produce our own target genes (EIF4E and Vlnv) according to CrispR / Cas9 procedure according to the overlapping PCR conditions with the primers (Sentegen Biolabs, Turkey) which we previously ordered from the gRNA fragment in the pUC119-gRNA plasmid. As shown in the figure 3.5, we were able to produce 4 gRNA fragments of the desired length (649 bps). We also wanted to repeat the experiment twice and make sure the result was correct.

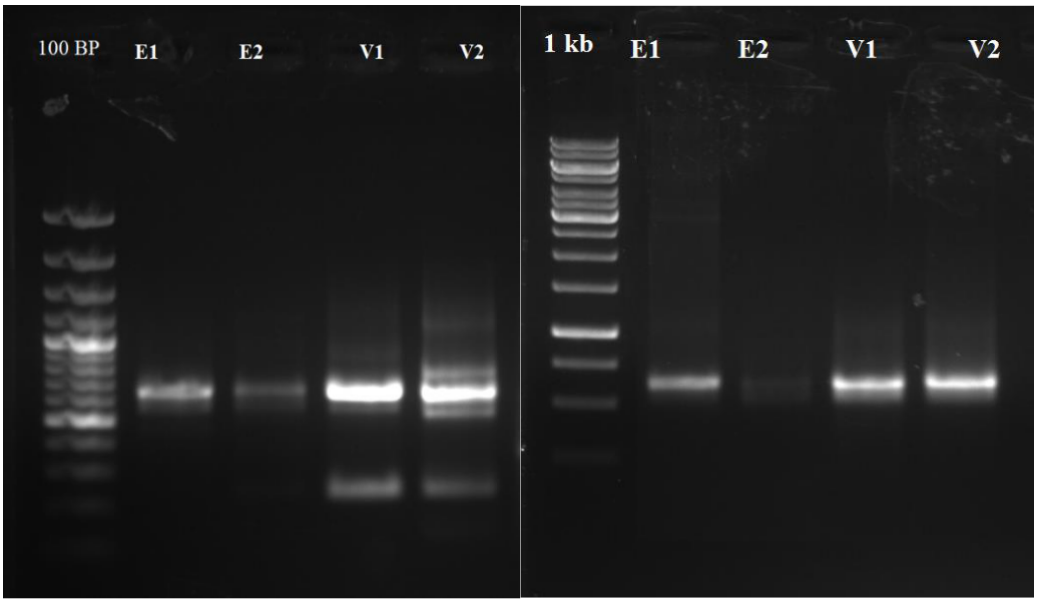

Figure 3.5. Overlapping PCR results of desired gRNA casettes (E1: EIF4E_1, E2: EIF4E_2, V1: Vlnv_1, V2: Vlnv_2)

3.2.2 Digestion of plasmids

After producing the desired gRNA fragments, we cut plasmid pFGC_pcoCas9 with the desired enzymes (EcorI and XmaI) to construct them into plasmid Cas9. As you can see in the figure 3.6, you can see that plasmid is divided into two parts after cutting with the desired enzymes (13kb and 216bp). These two fragments are of the desired length and we tried to insert the gRNA fragments that we had previously produced by ligation. However, as a result of long efforts did not get any positive results. Therefore, significant disruptions occurred in other targeted and planned parts of the experiments.

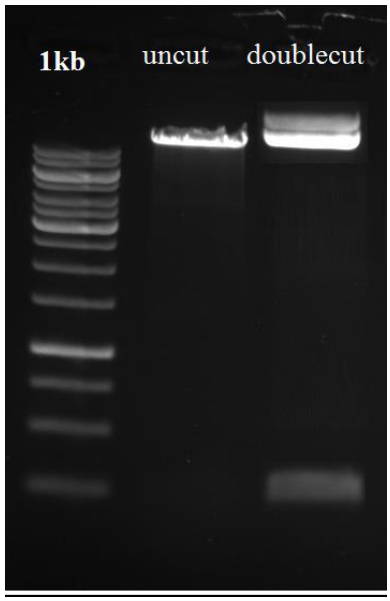

Figure 3.6. Double restriction enzyme cut with EcoRI and XmaI of pFGC_pcoCas9 plasmid and uncut control.

3.3 Isolation of Protoplasts

3.3.1 Potato plants for protoplast isolation

For protoplast separation, potato crops from PepsiCo from the MS medium were used. There were one month of plants. Figure 3.7 demonstrates the two crops used to isolate protoplast.

Also used were PepsiCo leaves from TM-1 press. Plants were put for three to four days in the dusk for the preparing of protoplast confinement.

Figure 3.7. PepsiCo potato plants. These plants were one month old. Plants were placed in dark for protoplast isolation preparation for three-four days

3.3.2 Preparation for protoplast isolation

Leaves were put in the solution of PET and/or enzyme after the dark procedure. Protoplast isolation was first conducted, overnight PET solution procedure was used before the enzyme solution as mentioned in the original protocol (Shepard 1977). Forward to testing distinct techniques for protoplast isolation, three distinct techniques were tried; the first was leaf slicing in PET solution, the second was leaf carving in enzyme solution, and the third was leaf carving in PET solution before enzyme treatment. For overnight treatment, leaves sliced straight into PET solution were put in a shaker, while leaves sliced into enzyme solution were put in dark for overnight treatment.

Leaves from PepsiCo were sliced into enzyme solution and put in the dark for overnight therapy as seen in figure 3.8.

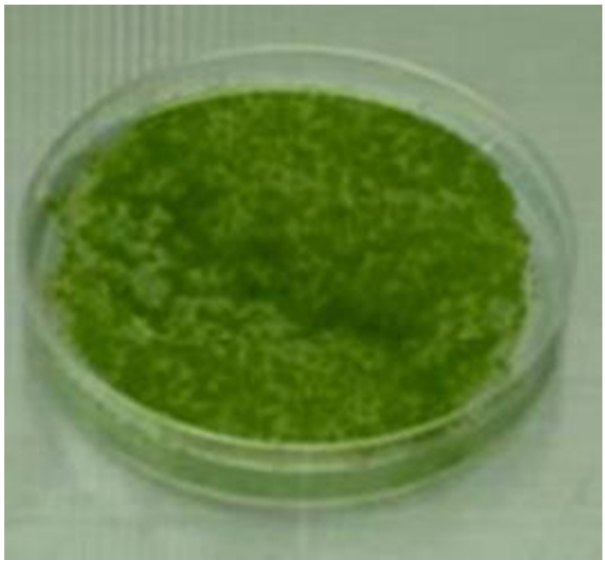

Figure 3.8. Leaves from PepsiCo were sliced in enzyme solution

Furthermore, TM-1 PepsiCo leaves were last conducted protoplast separation, were used. For three days, leaves were put in dark to wait for protoplast isolation. Whereupon leaves were sliced and put in, and many others, enzyme fluid used for Arabidopsis without leaves being sterilized. For two to three weeks, leaves from TM-1 crops in PepsiCo had been in TM-1 media. Propagated PepsiCo crops were several weeks present, and five days before protoplast isolation, PepsiCo crops on soil had been transmitted to MS medium. Leaves from soil crops had a bunch of yellowish and greenish colour after enzyme operation. Figure 3.9 displays filtered protoplasts from leaves of PepsiCo on MS medium and from propagated leaves of TM-1. Protoplasts from PepsiCo plants on the MS medium to the left in the image have a bunch of light greenish-colored protoplasts relative to leaves from propagated PepsiCo leaves from TM-1 where they do not appear to be as many protoplasts and have more dead cells . Less centrifugation rate was used when filtrating the protoplasts, then suggested in the following protocols.

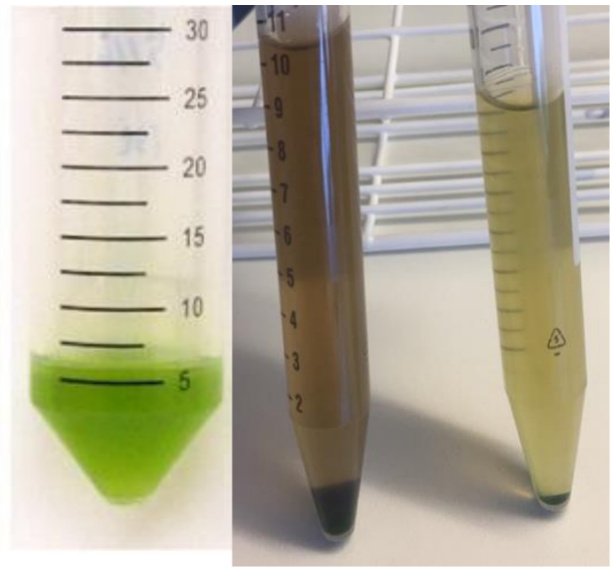

Figure 3.9. Protoplasts after mesh filtering. To the left protoplasts from PepsiCo on MS medium. To the right protoplasts from PepsiCo leaves from TM-1 medium. Dead cells are more in TM-1 medium filtration.

3.3.3 Surviving protoplasts

For protoplast isolation, 3-4 weeks old potato seedlings which were grown in the dark in TM-1 and MS media, were tested. The details of the isolation methods are presented in the Materials and Methods (Section 2.5). Figure 3.7 presents the potato seedlings used for protoplast isolations. Figure 3.10 demonstrates intact and fractured protoplasts.

The intact protoplasts appeared larger and spherical. The plants were sterilized prior to the PET treatment (Section 3.3.2) and followed by enzyme treatment overnight. Mesh filtered and centrifugated samples were analyzed under microscope for intactness of the protoplasts. The samples grown in the MS medium resulted in

approximately 90 % intact protoplasts ($5x10^5$ protoplasts/mL), nevertheless, the number of protoplasts were sufficient for PEG transfection (Figure 3.10 C & D).

On the other hand, TM-1 grown seedlings did not yield functional protoplasts (Figure 3.10 A & B).

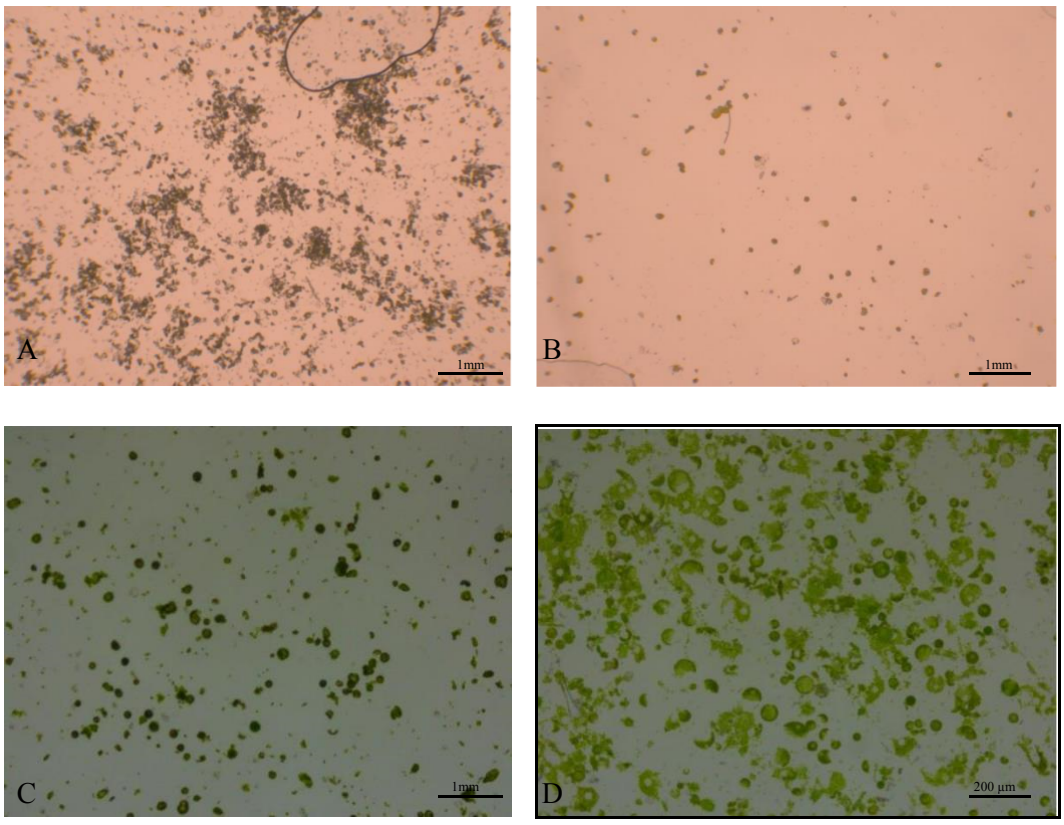

Figure 3.10. Potato protoplasts isolated from MS medium. (Leica DM4000, 40x to 1000x)

CHAPTER 4

CONCLUSION

The CRISPR / Cas9 system has afforded researchers the opportunity to easily and minimal-cost accurate gene editing in nearly any facility. CRISPR / Cas9 scheme can be implemented in any species to cells and can most probably be used in agriculture, medicine and potential animal treatment. Effective DNA-free processing in significant plants such as potatoes with the CRISPR / Cas9 scheme can create excellent benefits in agriculture and manufacturing, where GMOs are prohibited in many nations owing to various overseas DNA leftovers (Hirsch et al., 2014).

Whether the method of CRISPR / Cas9 can achieve personal recognition and still obey the rigid laws laid down by worldwide and local councils, it can further create plants in agriculture that are more suited to variables such as climate change, stress and viruses. Due to the spontaneous existence of gene inclusion, other methods used for gene editing as discrete mutagenesis methods were unable to regulate every gene in the system. With either the specific gene promoting the use of the CRISPR / Cas9 method, genes in plants can be changed without the introduction of a transgene (Khatodia et al. 2016).

While the CRISPR / Cas9 scheme appears bright in many fields and may even be excluded from the GMO legislation, some variables need to be described. The community requires to correctly comprehend how the scheme operates in order to gain public recognition for DNA-free genome processing. It will also be essential that GMOs are not deemed to be the ultimate products of this method. The conflict with the altered plants of future side effects could be a barrier to this. The capacity

that this scheme has for the water consumption of the world will rely mainly on the perception of communities of the plants that have been altered with CRISPR / Cas9.

The primary goals in this research were to establish a CRISPR / Cas9 model for potato crops using tissue culture to propagate and regenerate crops from intact potato protoplasts. As well as in vitro transcriptions for cloned gRNA and Cas9 casettes, cloning of gRNAs that could affect distinct components of the marker genes was conducted.

In fact, At first we were very focused on producing and combining gRNA fragments and Cas9 plasmid backbone fragments, but the fact that the experiments had consistently negative results prevented us from moving to PEG transformation in vitro. Therefore, we sent it to another company to test the accuracy of the parts we had, but when they failed, we began to question the reality of the plasmids we had and sent them to sequencing. They indicated that the gRNA fragments were produced correctly as a result of the sequence, but the plasmid pFGC_pcoCas9 was defective. Therefore, we have found the reason why cloning processes cannot be completed.

Then we requested a new plasmid from AddGene, but we couldn't have any positive plasmids due to customs problems. Therefore, we have not been able to finish the cloning process.

In this research, isolated protoplasts from potato crops were planning to used to deliver Cas9 mRNA and gRNA in vivo. Massive sections of the test were focused on tissue-growing plant crops, spread of plant crops through this scheme, and isolation from tissue-growing crops to regenerate plant crops. Many of these studies were focused on the renowned paper (Shepard 1977) "Mesophyll Cell Protoplasts of Potato." These procedures have been pursued several occasions as outlined in the potato protoplasts isolation method, but have not resulted in protoplasts remaining.

It was difficult to isolate potato protoplasts from tissue culture. Different methods have been tested after several failed efforts to detect protoplasts. In order to optimize protoplastic confinement, several parameters such as plantlet durations, light therapy, distinct alternatives, centrifugation velocity, and crops on tissue culture versus plants on soil were tested.

A few of these variables were likened to the initial protocol based on recent research that effectively isolated potato protoplasts (Yoo, Cho, & Sheen, 2007). Observations submitted in this research indicate that several intact isolated protoplasts originated from the use of one month old MS medium growth plantlets with light therapy for four days that were sliced straight into enzyme water usually used for Arabidopsis and other crops, W5 washing water and reduced centrifugation velocity.

The tissue culture model used for the basis of (Shepard 1977) did not operate as expected resulting in a great deal of energy consumption. TM-1 media, that was the media the crops were relocated from MS media, includes a lot of varying macro,-and micronutrients, vitamins, hormones and enzymes, some of which may have harmed the potato leaves.

There were also a number of other additives in the enzyme solution than that used for Arabidopsis and other crops that could have an impact on the potato leaf. Use of the potato crops from soil appears to be a stronger solution for isolating intact potato protoplasts, as well as using alternatives with fewer chemicals such as those used for Arabidopsis and other crops.

During cloning process troubleshooting, huge sections of the research were used to optimize the procedure for isolation of potato protoplasts. Due to time constraints, optimization procedure were undertaken just once into PEG transfections of isolated protoplasts with only plasmids which includes original non processed pFGC_pcoCas9.

Moreover, successful in vitro job with all the elements built for the CRISPR / Cas9 test would allow transgenic plants to be worked in the future. Time-saving for

potential job in this significant plant will be an optimized delivery technique for Cas9 and gRNA as protoplast isolation for potato crops. Results described in this research indicated that W5 washing solution and reduced centrifugation velocity led in a greater proportion of untouched protoplasts using one month old plantlets with light therapy for four days that were sliced straight into enzyme solution usually used for potato and other crops.

The next stage to edit particular genes of potato crops will be to conduct changes in vivo CRISPR / Cas9 through extracted and transfected protoplasts before regenerating potato crops. The use of Cas9 protein (and/or Cas9 transcripts) and written gRNA against the marker genes will lead in virus resistance and deep fry colour change if the change is effective as shown in the overview chapter in figures 1.8 and 1.9 (Hameed et al. 2018). Further studies should be explored for both nuclease-gRNA complex and Cas9 mRNA / gRNA.

The CRISPR / Cas9 mechanism will most probably have a major effect in many distinct fields in the future, not just in the area of science. It may be ordinary in a few years to consume foods where CRISPR / Cas9 method has genetically modified plants. In addition to agriculture, it will most probably also have a major effect in other areas, such as medicine and animal treatment.

The groundbreaking impact produced by the CRISPR / Cas9 scheme will rely for its use on cultural recognition, as well as on the laws established by the regulators. If plants altered by the CRISPR / Cas9 method are excluded from the GMO legislation, this will have a major effect on the factory farming worldwide.

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APPENDICES

A - pUC119-gRNA plasmid sequence 3771 bps

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAA
TGCAGCTGGCACGACAGGTTCCCGACTGGAAAGCGGGCAGTGAGCGCAA
CGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACT
TTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTT
CACACAGGAAACAGCTATGACCATGGGCGCGCCAAGCTTAGGCCTGTCG
ACGCCCGGGCGGTACCGCGATCGCTCGCGACCTGCAGGCATAAAGCCGT
CAGTGTCCGCATAAAGAACCACCCATAATACCCATAATAGCTGTTTGCCA
TCGCTACCTTAGGACCGTTATAGTTAATTACCCTGTTATCCCTATTAATTA
AGAGCTCAGAAATCTCAAAATTCCGGCAGAACAATTTTGAATCTCGATC
CGTAGAAACGAGACGGTCATTGTTTTAGTTCCACCACGATTATATTTGAA
ATTTACGTGAGTGTGAGTGAGACTTGCATAAGAAAATAAAATCTTTAGTT
GGGAAAAAATTCAATAATATAAATGGGCTTGAGAAGGAAGCGAGGGAT
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ATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA
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CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAA
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CTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAA
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CAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACG
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CAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTAC
CAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTT
CGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATA
CCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAG
GCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACG
AGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTT
TCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGC
GGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCT
GTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAG
CCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

B – pFGC-pcoCas9 plasmid sequence 13330 bps

GAATTCCTCGAGTACGTAGGATCCATTTAAATTCTAGAGGCGCGCCGATA
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TAACCTTAACAAGCTCATCAACAACCTTAACGGTCTGAAGGATTCCCTTC
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CCTCAACTCCAGAGATTTCAACAGAATCGAAGCACTCGATCTTCTTGAAG
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GAAGATCCTCTCTGTTCAACTTAACAAGAAGCTCCTCGGTTCCATCCATC
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ACCCGCGAAATTCAGGCCCGGTTGCCATGTCCTACACGCCGAAATAAACGACCA
AATTAGTAGAAAAATAAAAACTGACTCGGATACTTACGTCACGTCTTGCGCACT
GATTTGAAAAATCTCA

C – Sequencing Results of cloned gRNAs (uppercase section is target nucleotides)

i) EIF4E_1 : 647 bps

gaattcctcgagtacgtaggatccatttaaattctagaggcgcgccgatgggtaatgccaactttgtacaagaaagctggg
tctagaaaaaaagcaccgactcggtgccacttttcaagttgataacggactagccttattttaacttgctatttctagctctaa
aacCAAATACGACGCCGTATCATaatcactacttcgtctctaaccatatataaactcagctgctttctttac
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ii) EIF4E_2 : 647 bps

gaattcctcgagtacgtaggatccatttaaattctagaggcgcgccgatgggtaatgccaactttgtacaagaaagctggg
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iii) Vlnv_1 : 647 bps

gaattcctcgagtacgtaggatccatttaaattctagaggcgcgccgatgggtaatgccaactttgtacaagaaagctggg
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aacGAACCGCATGCAGCACTTCAaatcactacttcgtctctaaccatatataaactcagctgctttctttac
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gcccatttatattattgaatttttcccaactaaagattttatttcttatgcaagtctcactcacactcacgtaaatttcaaatataa
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iv) Vlnv_2 : 647 bps

gaattcctcgagtacgtaggatccatttaaattctagaggcgcgccgatgggtaatgccaactttgtacaagaaagctggg
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gtggttctttatgcggacactgacggctttatgcctgcaggtcgcgagcgatcgcggtaccgcccggg

D – PET, enzyme and washing solution. g/L except when notated. Solutions were made according to the original protocol (Shahin 1985)

	PET		Enzyme Solution		Washing Solution	
Nutrients	**1 L**	**250 mL**	**1 L**	**100 mL**	**1 L**	**250 mL**
KH_2PO_4	0.425	0.10625	1.7	0.17	0.85	0.2125
$CaCl_2$• 2 H_2O	1.1	0.275	4.4	0.44	2.2	0.55
KNO_3	0.375	0.09375	1.5	0.15	0.75	0.1875
$MgSO_4$•$7H_2O$	0.925	0.23125	3.7	0.37	1.85	0.4625
Sucrose	0.3 M	25.67	0.3 M	10.269	0.3 M	25.67
Nicotinic acid	0.0025	625 µL	0.0025	250 µL	0.0025	625 µL
Thiamine HCl	0.01	0.0025	0.01	0.001	0.01	0.0025
Pyridoxine HCl	0.001	250 µL	0.001	100 µL	0.001	250 µL
Folic acid	0.0005*	125 µL	0.0005	50 µL	0.0005	125 µL
Biotin	0.00005*	12.5 µL	0.00005*	5 µL	0.00005*	12.5 µL
D-Ca-Pantothenate	0.0005*	125 µL	0.0005*	50 µL	0.0005*	125 µL
Choline chloride	0.0001*	25 µL	0.0001*	10 µL	0.0001*	25 µL
Glycine	0.0005*	125 µL	0.0005*	50 µL	0.0005*	125 µL
Casein hycrolysate	0.05	0.0125	0.15	0.015	0.15	0.0125
L-Cysteine	0.001	250 µL	0.001	100 µL	0.001	250 µL
Malic acid	0.01	0.0025	0.01	0.001	0.01	0.0025
Ascorbic acid	0.0005*	125 µL	0.0005*	50 µL	0.0005*	125 µL
Adenine sulfate			0.04	0.004	0.04	0.01
L-Glutamine			0.1	0.01	0.1	0.025
Myo-inositol	0.1	0.025	4.6	0.46	4.6	1.15
Riboflavin	0.00025*	62.5 µL	0.00025*	25 µL	0.00025*	62.5 µL
2,4-D	0.001	250 µL				
BAP	0.0005*	125 µL				
Maceroenzyme			% 0.1	0.1		
Cellulysin			% 0.75	0.75		
Polyvinylpyrolidine			% 1.0	1		
MES			5 µM	9.76×10^{-5}*	0.05 M	2.5 mL
pH	5.80		5.60		5.80	
						Filter sterilize

Note * = Stock solutions 10 mg/10 mL were made.

E –Gel result of in vitro enzymatic digestion of Cas9/gRNA fragments