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Employing CRISPR-Cas9 approach for DNA free editing of *Solanum lycopersicum* genome with pre-assembled CRISPR-Cas9 ribonucleoproteins and transcripts

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Master´s degree in Biological Chemistry

Hanna Rovik, June 2017

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Abstract

In recent years there has been an increase in new demands in the agriculture sector due to factors as an increasing population, climate changes and food supplies. Hence, producing more food on less space has become a huge industry. Development of new and more acceptable technologies for editing genomes of plants is under scope in order to preserve the environment while maintaining global and local regulations.

There are four genome-editing methods that are highly used: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein9 (Cas9). Several studies have shown that the CRISPR/Cas9 method has overcome other programmable nucleases techniques such as ZFNs and TALENs. The CRISPR/Cas9 system is based on a natural immune system found in bacteria and archaea that uses short ribonucleic acid (RNA) to direct degradation of foreign nucleic acids.

Deoxyribonucleic acid (DNA)-free editing of tomato genome could be of great value for agriculture. In this study, the aim was to investigate the ability to produce a DNA-free genetically modified organism (GMO), tomato, using CRISPR/Cas9 method. To achieve this, components needed to perform genome editing in tomato protoplasts was delivered, from which plants can be re-generated using the totipotency of plant cell. Two spacers were subcloned (20 bp; that can target the tomato reference gene; *phytoene desaturase* (*PDS*)) in order to prepare two single guide RNAs (sgRNAs) encoding genes.

Transcripts from sgRNAs and Cas9 were generated using *in vitro* transcription. To approve the setup, Cas9 protein and sgRNAs was combined *in vitro* and showed the ability of Cas9 protein to target and hence digest the *PDS* PCR product. To be able to perform these steps *in vivo*, editing with the low number of survived protoplasts using sgRNAs combined with Cas9 transcripts was attempted, but this did not succeed and needs further optimization.

Isolation of healthy tomato protoplasts was a challenge to achieve as a target of this study. However, using one-month-old plantlets from soil and the combination of long dark treatment (four days) and using lower centrifugation speed, resulted in high percent of intact and healthy tomato protoplasts. Overall, setting up CRISPR/Cas9 experiment *in vitro* was

achieved and the *in vivo* trials should be optimized with the use of higher amounts of healthy tomato protoplasts, which can be used for re-generation.

Table of Content

Figure List

Table List

Abbrevations

- Amp^R - antibiotic 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
- 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
- Kb - kilo base pair
- LB - Luria-Betani
- MCS - multiple cloning site
- MEGA - Molecular Evolutionary Genetics Analysis
- MES - 2-(N-morpholino)-ethanesulfonic acid
- Mg^{2+} - 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
- *SIPDS - phytoene desaturase* (Solyc03g123760.2.1)
- 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
- ZFNs - zinc finger nucleases

1 Introduction

1.1 DNA-free Editing

The world is changing rapidly due to an increasing population, climate changes, food supplies and other challenging conditions. These factors make new demands for the agriculture to produce more food on less space while preserving the environment and maintaining global and local regulations (Kanchiswamy 2016). Genetically modified organisms (GMOs) have been available for over two decades and would be considered to achieve many of these goals, but due to strict regulations in many countries, GMOs are not allowed (Wunderlich and Gatto 2015). This has led to an increase in finding new and more acceptable technologies, which both can meet these challenges for the future and satisfy regulations set by the authorities.

1.1.1 Cell Function

All cells have a copy of its genome. There are several components in the cell that contains genetic information. Most of the genes in the cell are found in the nucleus which is the control center of the cell. Deoxyribonucleic acid (DNA) in the nucleus is organized as chromosomes. These structures contain genetic information where each chromosome contain a long DNA molecule with several proteins which make up a complex called chromatin. In a dividing cell chromatin is visible as individual condensed chromosomes (Reece et al. 2011). Figure 1.1 shows an overview of a eukaryotic plant cell.

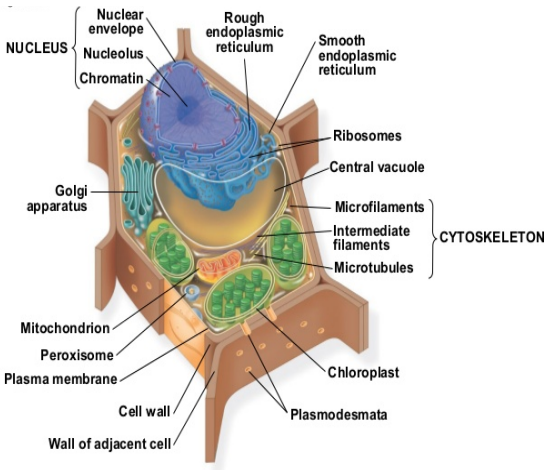

Figure 1.1: Eukaryotic Plant Cell *(Reece et al. 2011)*

Changing genes in living cells has several difficulties. To study gene functions, genetic mutants are essential. Due to natural mutants, many important biological mechanisms have been investigated. Random mutagenesis can however lead to many undesirable mutations and is therefore limited (Ma et al. 2016), but it can be applied for reverse genetics making it possible to target specific genes for studying its function. Reverse genetics works in the opposite direction as normal genetics, where the mutant phenotype is first found and ending up with protein sequence as the last step. The starting point for reverse genetics is to find the protein sequence and ending up with the mutant phenotype (Griffiths et al. 2000).

1.1.2 Genome Editing Methods

There has been a rapid increase in alternative technology to overcome the difficulties of random mutagenesis. Four genome-editing methods are highly used: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein9 (cas9) (Yin et al. 2017). These techniques take advantage of reverse genetics where random mutagenesis is performed *in vitro* or *in vivo*. Programmable nucleases such as ZFNs, TALENs and ribonucleic acid (RNA) guided endonucleases (RGENs) can facilitate genome-editing targeting by increasing the efficiency of homologous recombination. These methods are both time-consuming and have several difficulties of their optimization (Ma et al. 2016).

ZFNs were the first generation of programmable sequence-specific nucleases (SSNs) that provided a great breakthrough in the genome manipulation field. Double-stranded breaks (DSBs) could be induced by SSNs at chromosomal sites that could be repaired by either the error-prone non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway (Symington and Gautier 2011). ZFNs have been used to edit for example plant genomes, but due to difficulties when it comes to manipulation in various organisms and high costs, this technique is limited (Ramirez et al. 2008).

The other genome editing tool, TALENs, was adapted from the *Xanthomonas* bacteria (Moscou and Bogdanove 2009). In nature, TAL effector proteins will be secreted by *Xanthomonas* bacteria via the type III secretion system when the host is infected. Here they will alter host gene expression for the bacteria to resist the invasion (Malzahn et al. 2017).

Even though this technique was much more easy to use than ZFNs, it required complicated constructions of tandem repeat domains found in TAL proteins (Ma et al. 2016).

In recent years, the technology of CRISPR-Cas9 system has been under investigation in many different areas. This method has indicated to highly improve the ability to change genes in the cell for many different species (Kanchiswamy 2016).

1.2 CRISPR/Cas9

1.2.1 Natural Immune System in Bacteria

The CRISPR/Cas9 method is based on a natural immune system in bacteria and archea that uses short RNA to direct degradation of foreign nucleic acids. CRISPR has been known since 1987 where it was first identified in *Escherichia coli* genome (Ishino et al. 1987). CRISPR sequences are crucial components to the bacteria immune system. It is composed of short palindromic repeats of DNA that have so-called spacers between each repeat. These spacers are unique. The Cas genes are different genes that associate with CRISPR, and make Cas proteins (Jinek et al. 2012; Wiedenheft et al. 2012). These are both helicases that unwind DNA and nucleases that cuts the DNA.

When a bacterial phage attacks the bacteria, several responses in this system will occur. The mechanism for this process is shown in figure 1.2 (Lab 2012). The spacers will function as the genetic memory for previous attacks in the bacteria and will either recognize the bacterial phage or be activated. If the spacer is activated it will copy the foreign DNA into the CRISPR system. This will give a library of short CRISPR-derived RNA (crRNAs) that will contain the complementary sequence to that of the invading nucleic acid (Jinek et al. 2012; Wiedenheft et al. 2012). A more detailed description of this process is given in figure 1.3 (Reis et al. 2014).

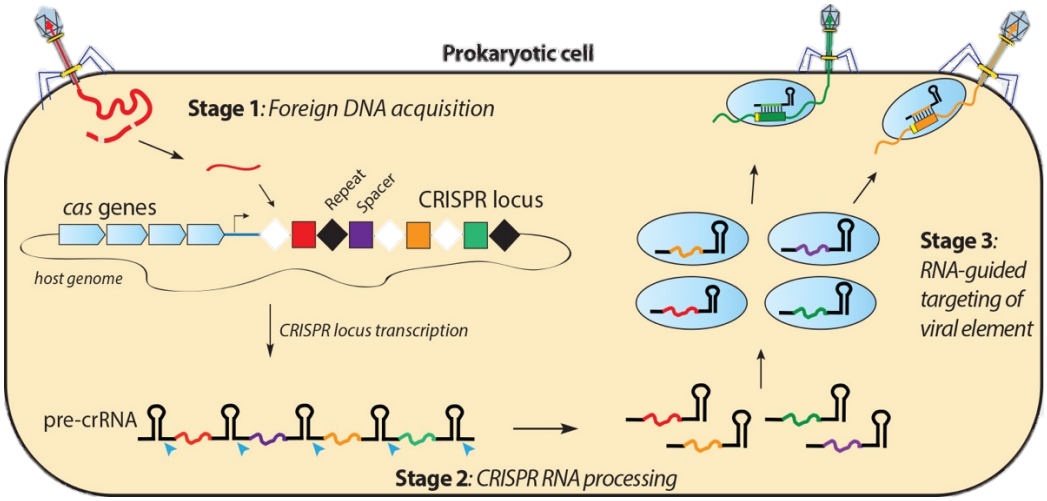

Figure 1.2: CRISPR system in a prokaryotic cell *(Lab 2012). When a bacterium gets attacked by a bacterial phage the CRISPR system responses. CRISPR is composed of short palindromic repeats of DNA that have so-called spacers between each repeat. These spacers are unique. The Cas genes are different genes that associate with CRISPR, and make Cas proteins. These spacers will function as the genetic memory for previous attacks in the bacteria and will either recognize the bacterial phage or be activated. If the spacer is activated it will copy the foreign DNA into the CRISPR. This will give a library of short CRISPR-derived RNA (crRNAs) that will contain the complementary sequence to that of the invading nucleic acid. A more detailed description of the mechanism is given in figure 1.3*

Three types of CRISPR have been identified, whereas type II is the most studied (Makarova et al. 2011). CRISPR type II was found in the bacterium *Streptococcus pyogenes* (Jinek et al. 2012), and is thought to be depended on only one protein (Cas9 (formerly Csn 1)) for gene silencing of foreign DNA (Sapranauskas et al. 2011). Type I and III is thought to be more complicated, and they also share some common features. They have specialized Cas endonucleases that will process the pre-crRNA until it is matured before each crRNA will form a large protein complex which will be able to both recognize and cleave nucleic acids complementary to the crRNA (Jinek et al. 2012). In type II systems this will occur by another mechanism shown in figure 1.3 (Reis et al. 2014).

1.2.2 CRISPR Type II System

The first step of CRISPR-mediated immunity is acquisition of foreign DNA at the CRISPR loci (Wiedenheft et al. 2012). Two types of short RNA will be produced when a bacterium gets attacked by a virus. This will happen when CRISPR loci is transcribed and processed into crRNA during crRNA biogenesis. crRNA will contain a sequence that will match the invading nucleic acid. During interference phase these two RNAs will form a complex with Cas9 protein, which is a nuclease that cuts the DNA. The complex between Cas9, crRNA and

a separate tracrRNA, that is partially complementary to the crRNA, is necessary to achieve site-specific DNA recognition and cleavage. The matching sequence known as a guide RNA (gRNA), which is a construct of crRNA and trans-activating CRISPR RNA (tracrRNA) composed of a 20 nucleotides sequence (spacer), will find its target within the viral genome (Jinek et al. 2012).

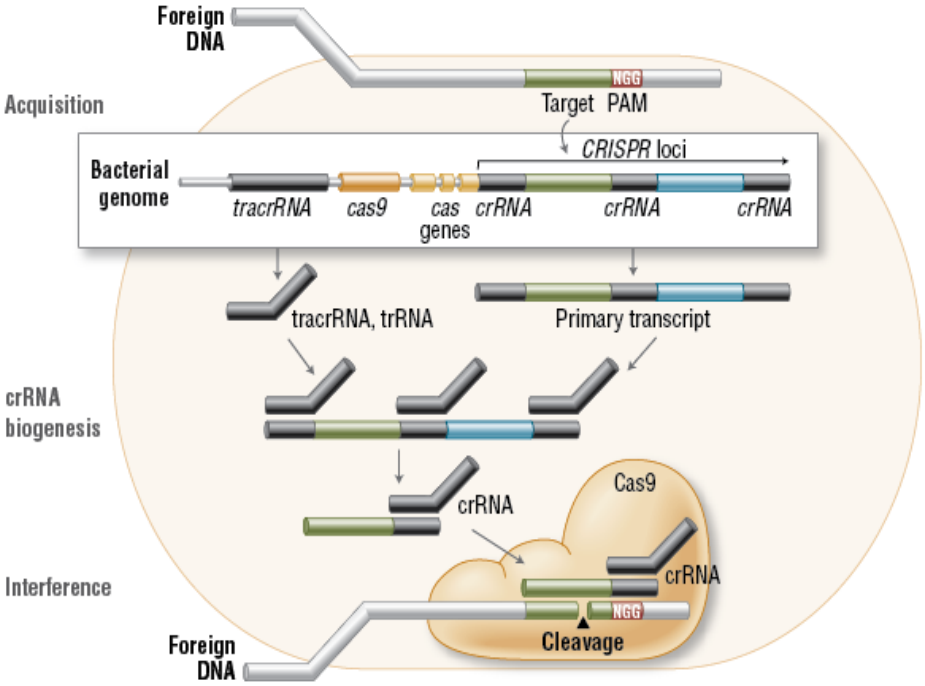

Figure 1.3: CRISPR prokaryotic defense mechanism. *If a bacterium gets attacked by a virus two different types of short RNA will be produced when CRISPR loci is transcribed and processed into CRISPR RNA (crRNA) during crRNA biogenesis. crRNA will contain a sequence that will match the invading nucleic acid. These two RNAs will form a complex with a protein called Cas9 during interference phase. When the matching sequence known as a guide RNA (gRNA) find its target within the viral genome, the Cas9 will cut the target DNA. This will disabell the virus (Reis et al. 2014)*

When the sequence is inside the nuclease, the 3´-end of the target sequence will lock on to a NGG protospacer adjacent motif (PAM), and be recognized by Cas9 (Jinek et al. 2012) as shown in figure 1.3 (Reis et al. 2014). Cas9 has two active sites, RucV and HNH domains, whereas each of these sites will cut one of the complementary DNA strands (Ma et al. 2016). The complementary strand will be cleaved by the HNH domain, while the non-complementary strand is cleaved by the RuvC domain (Jinek et al. 2012).

DSB can be repaired in two different ways; either by the NHEJ or HDR pathway. NHEJ is error-prone, and the common pathway. Usually this pathway leads to different mutations as small deletion, insertion or frameshift. These mutations can disable the virus. This mechanism is shown in figure 1.4 (Addgene 2017b).

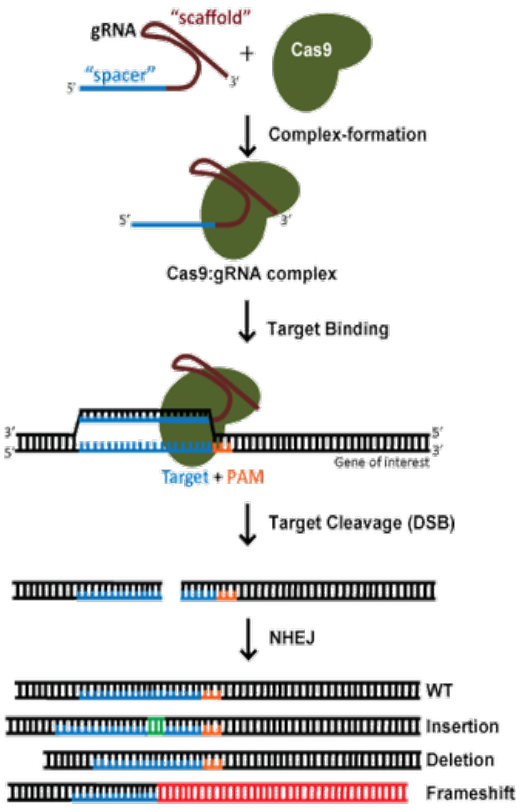

Figure 1.4: NHEJ pathway. *Guide RNA (gRNA) and Cas9 will form a complex which will lock on to a short protospacer adjacent motif (PAM). Cas9 has two active sites wheras each of these sites will cut one of the complementary DNA strands. The double-strand break will usually be repaired by the non-homologous end joining (NHEJ) pathway. This pathway will often lead to different mutations as small deletion, insertion or frameshift (Addgene 2017b)*

Studies have shown that this system can cut any DNA sequence at a precisely chosen location by changing the gRNA to match the target (Jinek et al. 2012; Ma et al. 2016). This can be performed by adding another piece of DNA that carries the desired sequence. The binding specificity of Cas9/sgRNA is affected by many different factors as both gRNA –DNA base pairing and the PAM region with sequence NGG immediately downstream to the target region (Lin et al. 2014). The domains of the Cas9 protein will cleave the DNA resulting in a

DSB, and then recombining and replacing the original sequence with the new version (Jinek et al. 2012; Jinek et al. 2014; Nishimasu et al. 2014; Sternberg et al. 2014). This has been done to perform gene editing in multiple organisms including bacteria, yeast, plants, animals and even in human cell lines (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).

Unlike previous methods, CRISPR/Cas9 system can target many genes at once (Pan et al. 2016) which has a great advantage in both research in plants and in complex human diseases that are caused by many genes acting together. This method can be used in many fields as for example in basic research, agriculture and in drug development.

1.2.3 CRISPR/Cas9 Experiment

There are different ways to set up a CRISPR/Cas9 experiment depended on the main goal. It is possible to use CRISPR/Cas9 for purposes as generation of a complete and permanent loss of gene expression or function (knock-out), expression of a mutant gene or increase or decrease of the expression of a target gene. These different genetic manipulation methods require different CRISPR components. In this study one of the overall targets was to edit genes by an expressing system composed of Cas9 protein (and/or Cas9 mRNA transcripts) with gRNA(s). Plasmids with Cas9 and gRNA are used for *in vitro* transcription to generate mature Cas9 mRNA and gRNA that will be delivered to target cells. This system can also be used to generate transgenic plants (Addgene 2017b).

Different delivery methods can be applied, as for example transfection of isolated protoplasts, which was performed in this study. There have been several studies that have used Cas9 protein with sgRNA for making a nuclease-gRNA complex in for example grapevine, apple, tobacco, lettuce, rice, *Arabidopsis thaliana* and *Petunia hybrida*. Some of these also used isolated protoplast for successful delivery of Cas9 and gRNA (Malnoy et al. 2016; Woo et al. 2015; Subburaj et al. 2016). Cas9 mRNA and gRNA have to the author´s knowledge not been applied in tomato plants yet, but it has been applied in wheat (Zhang et al. 2016) where both Cas9 DNA and RNA was used with sgRNAs.

Once the delivery method has been chosen, the next step is to select a target sequence and designing the gRNA(s). Figure 1.5 (Addgene 2017b) shows a chart over recommended steps

for this process. It is necessary to determine the cell line and genome sequence that are going to be used in the experiment. For reducing factors which can lead to reduced cleavage due to sequence variations, sequencing the region that is going to be used in the experiment before designing gRNA(s) is recommended (Addgene 2017b).

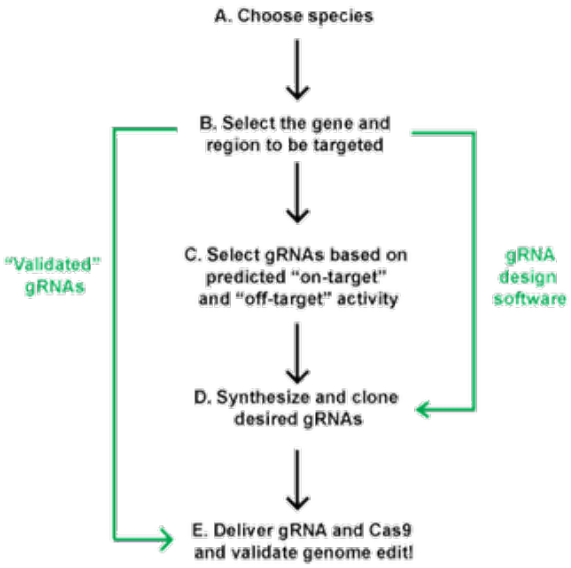

Figure 1.5: Selecting target sequence and designing gRNA(s)
(Addgene 2017b)

For manipulation of genes using CRISPR/Cas9 system, identification of the genome sequence to the target gene has to be performed before selecting gRNA(s). For selecting gRNA(s), all PAM sequences in the genetic region have to be targeted before selecting which site that most likely will result in on-target cleavage. There are different factors that are necessary to be aware of when it comes to choosing gRNA(s). One of the most crucial factors is that the gRNA sequence will match the target sequence. It is also important that the gRNA target sequence does not match other sites in the genome, leading to so-called "off-targets" (Xie et al. 2014).

There are different tools for constructing gRNA(s) as for example CRISPR-PLANT (Xie et al. 2014), which is a web tool where it is possible to select target sites for CRISPR/Cas9. Off-targets are not necessarily a critical concern in plant research since potential off-target mutations could be segregated away by crossing, leading to only specific mutants that can be used for further basic research and plant breeding (Zhang et al. 2016). In other fields, as for

example in clinical research, off-targets are a critical factor. The easiest way to avoid this is to construct the gRNA(s) carefully.

For constructing gRNA(s) desired in the experiment, it is necessary to design oligos and clone these into a vector. This can be performed by for example synthesize, anneal and insert targeting oligos into plasmids where gRNA is present by standard restriction-ligation. Delivery of Cas9 and gRNA(s) will depend on the delivery method chosen for the experiment. It might be necessary to optimize the protocol for this purpose, for example for isolation of protoplasts in the specific target specie in the experiment (Addgene 2017b).

The last step in the CRISPR/Cas9 experiment will be to check if the genetic modification has been successful. Different methods can be applied for this purpose. In many cases the result will be a different possible genotype found in the resulting "mutant" cell population. Some of the cells might be wild-type after the experiment is performed due to lack of gRNA(s) and/or Cas9 expression or that the target cleavage is reduced in cells expressing gRNA(s) and Cas9. Cells that have been modified can either be homozygous, where both alleles have been modified, or heterozygous, where only one of the alleles have been modified. Methods that can be used to verify if the modification of cells have occurred will depend on the goal of the experiment. Methods that can be applied are mismatch-cleavage assay for NHEJ repaired DSB, PCR and restriction digest for HDR repaired DSBs, and for HDR or NHEJ PCR amplification and gel electrophoresis, PCR amplification, subcloning and Sanger sequencing or PCR amplification and next-generation sequencing can be used (Addgene 2017b).

1.2.4 Plasmids

Plasmids are small DNA molecules that are separate from chromosomal DNA and can replicate independently. They are most common in bacteria as small circular double stranded DNA, but can sometimes be found in archaea and eukaryotic organisms. Even though plasmids only contain a small number of genes, they have some beneficial features for their survival as antibiotic resistance (Reece et al. 2011).

In molecular cloning, artificial plasmids are often used as vectors where they will have an insert of DNA from another source. This will result in a recombinant DNA molecule. The plasmid is then returned to a bacterial cell where it will produce a recombinant bacterium and

replicate. When multiple copies of a single cell are produced, it is called gene cloning. A vector usually consists of different components which makes it very useful. Vectors often have in common that they contain an origin of replication (Ori) where it can be made many copies of the vector in the cell, a multiple cloning site (MCS) with many restriction sites where DNA fragments may be ligated, and a selectable marker. They are usually also antibiotic resistance (Amp^R) and the selectable marker is therefore often a antibiotic resistance gene which will give visible colonies where the recombinant plasmid is inserted (Reece et al. 2011).

1.3 Tomato (*Solanum lycopersicum*)

1.3.1 History and Description

Tomato, *Solanum lycopersicum,* belongs to the nightshade family, *Solanaceae*, and are usually recognized with its typically red phenotype (Perveen et al. 2015) as shown in figure 1.6 (Encyclopedia 2017). It is an important horticultural crop (Shahin 1985) that is grown all over the world. Tomato plants are thought to have great health benefits as they contain lot of vitamins and minerals as vitamin C, potassium, folic acid and carotenoids (Perveen et al. 2015). As the second most consumed vegetable in the word, tomatoes are an important part of the diet in many countries (Tomato Genome 2012; Pan et al. 2016), and it has for years been greatly enhanced through plant breeding (Shahin 1985).

Figure 1.6: Tomato, Solanum lycopersicum, is an important crop worldwide, and is known for its typical red phenotype *(Encyclopedia 2017)*

Although significant improvement have been done decades ago through wild germplasm when it comes to plant habitant, disease and insect resistance (Shahin 1985), newer technology can further improve crops, as for example tomato plants, to have stronger immunity against for example infection (Chaparro-Garcia et al. 2015). This will have a great impact on food security worldwide.

1.3.2 CRISPR/Cas9 Editing in Tomato

CRISPR/Cas9 has been used to strengthen for example *Nicotiana benthamiana* plants immunity against DNA virus infection (Ali et al. 2015). If this method of DNA-free editing can be used in an important crop as tomato plants, this can open the possibility for making tomato plants according to the strict regulations in many countries when it comes to GMOs. This has been done in crop improvement for wheat where CRISPR/Cas9 was used to knock out three MILDEW-RESISTANCE LOCUS (*MLO)* homolog alleles conferring heritably broad-spectrum resistance to powdery mildew in wheat plants (Wang et al. 2014).

Tomato has important traits which makes it an ideal plant to investigate for example plant reproductive development, functional genomics and quality improvement (Tomato Genome 2012). Previous studies have shown that CRISPR/Cas9 can induce mutations in tomato plants by *Agrobacterium tumefaciens*-mediated transformation (Brooks et al. 2014; Filler Hayut et al. 2017), or viral based delivery (Cermak et al. 2015). It has also been shown that CRISPR/Cas9 can be used to specifically induce heritable mutations in tomato plants with *phytoene desaturase* (*SIPDS*, Solyc03g123760.2.1) and phytochrome interacting factor PIF4 (SIPIF4, Solyc07g043580.2.1) genes with *Agrobacterium tumefaciens*-mediated transformation method (Pan et al. 2016).

1.3.3 *SIPDS* Gene

The tomato gene *SIPDS* encodes for phytoene desaturase, which is the key enzyme in carotenoid biosynthesis. Silencing this gene will cause photo bleaching or albino phenoptypes of tomato plants (Liu et al. 2002). Mutations and inheritance patterns of this gene have been investigated in previous studies (Pan et al. 2016). The study from Pan et al. (2016) showed an

albino phenotype in 54.54 % (12 out of 22) of the sgRNA1-*SIPDS* and 57.14 % (4 out of 7) of the sgRNA2-*SIPDS* transgenic plants. This indicated the complete loss or partial loss of function for the gene, see figure 1.7 (Pan et al. 2016).

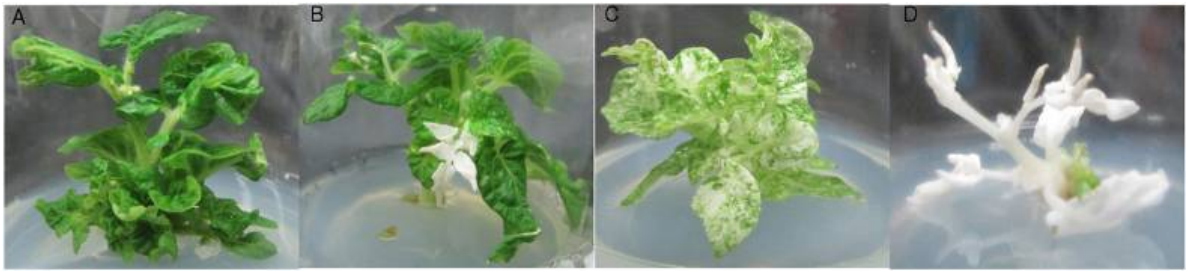

Figure 1.7: Albinism phenotype of SIPDS transgenic plants in T0 generations*. A) Wild type (containing T-DNA) B) Chimeric mutant C) Chimeric mutant D) Biallelic mutant. B, C and D shows mutants with varying degree of the albino phenotype (Pan et al. 2016)*

1.4 Tissue Culture

1.4.1 Growth of Plants *In Vitro*

Tissue culture is the *in vitro* growth of tissue, cells or the whole plant under controlled nutritional and environmental conditions, in a liquid, semi-solid or solid growth medium. It is often used to produce clones of a plant (Murashige and Skoog 1962; Thorpe 2007). Tissue culture makes it possible to grow plants independent on season and weather. It is also an important tool for biotechnology approaches like genetic engineering that depend on an efficient *in vitro* plant regeneration system (Hussain et al. 2012).

1.4.2 Important Crop Improvement Tool

Tissue culture has made it possible to produce a large number of secondary plant products, and it is considered to be the most efficient tool when it comes to crop improvement. It can be used for several purposes as for example plant propagation, genetic transformation, production of secondary metabolites, disease elimination and production of different varieties when it comes to salt tolerance, drought and heat stresses (Hussain et al. 2012). Figure 1.8 shows a chart for different tissue culture experiments.

One of the most recent aspects of plant cell and tissue culture is the possibility for genetic transformation that makes it possible to transfer genes with desirable traits into host plants and grow transgenic plants. For regenerating an entire plant the tissue culture technique exploits the totipotentiality of plant cells, meaning that a single cell can express the full genome by cell division, as well as the cells can alter their own metabolism, growth and development (Hussain et al. 2012).

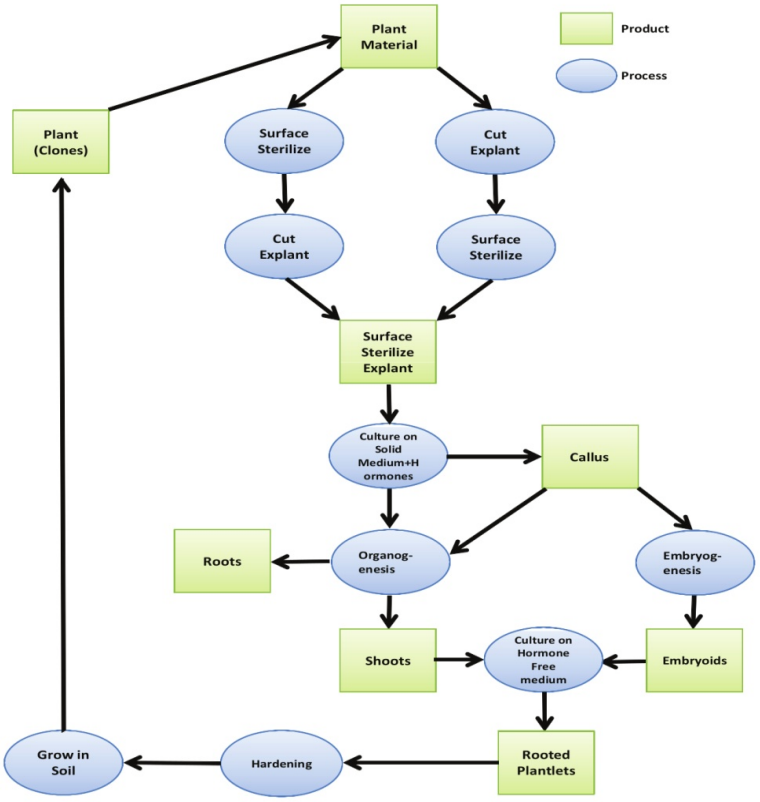

Figure 1.8: Chart for different tissue culture experiments *(Hussain et al. 2012)*

1.4.3 Optimization of Tissue Culture

Since 1902 when the German physiologist Gootleib Haberlandt for the first time tried to culture isolated cells from leaves in knop´s salt solution with sucrose, much improvement has been done when it comes to tissue culture (Hussain et al. 2012). For tissue culture to be optimized there are some compounds and conditions that needs to be fulfilled.

The medium should generally contain compounds as macronutrients, micronutrients, vitamins, amino acids or nitrogen source(s) of carbon, growth regulators, solidifying agents and in some cases undefined organic supplements. pH of the medium, temperature, proper gaseous and liquid in the environment is also important. pH of the medium is usually adjusted to 5.4-5.8 (Saad and Elshahed 2012; Hussain et al. 2012). For many plant species Murashige and Skoog medium (MS) is the most used medium for *in vitro* propagation (Hussain et al. 2012).

1.5 Objectivities of Present Study

If a method for DNA-free editing can be used in crops, as tomato plant, this can open the possibility for making tomato plants according to the strict regulations in many countries when it comes to traditionally GMOs that have different leftover foreign DNAs from delivery techniques (Graham and Root 2015; Ding et al. 2016; Khatodia et al. 2016).

To the author's knowledge DNA-free editing with CRISPR/Cas9 has not yet been performed in the important crop tomato. In this study, it was desired to use this new technology for editing specific genes for basic and applied research purposes. This has successfully been reported using a combination of Cas9 protein and gRNAs transcript and their subsequent transformation to plant protoplasts (Woo et al. 2015). In order to achieve this aim, the main objectives of this study were:

- Planting tomato seeds and making tissue culture propagation for tomato plants. Performing and establishing tissue culture system to re-generate plants from isolated tomato protoplasts.
- Cloning of guide RNA that can target one or more genes. *In vitro* transcriptions for cloned gRNA and Cas9.
- Performing *in vivo* CRISPR/Cas9 genetic modifications through the polyethylene glycerol (PEG) transfections of isolated protoplasts by Cas9 protein (and/or Cas9 transcripts) and transcribed gRNAs against the marker gene *PDS*.

2 Material and Methods

2.1 Materials

2.1.1 Kits

An overview of the different kits used in this study are given in table 2.1.

Table 2.1: Kits used

Kit	Function	Supplier	City, Country
GenElute™ Plasmid Miniprep kit	Plasmid isolation	Sigma Aldrich	St. Louis, MO, USA
MINIPrep NucleoSpin® Plasmid (NoLid)	Plasmid isolation	Macherey-Nagel	Düren, Germany
Gen Elute™ Gel Extraction Kit (NA1111)	Retrival of DNA after gel electrophoresis	Sigma-Adrich	St. Louis, MO, USA
Nucleospin® Gel and PCR Clean-up	Retrival of DNA after gel electrophoresis	Macherey-Nagel	Düren, Germany
MEGAclear™ Kit Purification for Large Scale Transcription Reactions (AM1908)	Purification of sgRNA transcripts	Thermo Fisher Scientific	Carlsbad, CA, USA
RNA clean and concentrator™-25 (R1017)	Purification of Cas9 mRNA transcript	Zymo Research	Irvine, CA, USA
HiScribe™ T7 Quick Yield RNA synthesis kit (NEB#E20505)	*In vitro* transcription of sgRNAs from plasmid	New England BioLabs	Ipswitch, MA, USA
In vitro digestion of DNA with Cas9 Nuclease, *S. pyogenes* (M0386)	*In vitro* digestion of DNA with Cas9	New England Biolabs	Ipswitch, MA, USA
HiScribe™ T7 ARCA mRNA kit (with tailing) (#E2060S)	*In vitro* transcription of Cas9 mRNA	New England Biolabs	Ipswitch, MA, USA

2.2 Methods

The experiments were set up in three different sections; Tomato plants, gRNA and Cas9, and isolation of protoplasts and transfection.

Tomato seeds were sterilized and grown in both tissue culture and on soil. Propagation of tomato plants was also performed. In the gRNA and Cas9 part there were many experiments performed as annealing of primers, restriction-ligation, transformation, sequencing, digestion of plasmids, gRNA and Cas9 *in vitro* transcription and *in vitro* digestion with Cas9 nuclease. The delivery of gRNA and Cas9 *in vivo* were performed with transfection of isolated tomato protoplasts before T7 Endonuclease I assay was performed as the last experiment.

2.3 Tomato Plants

Tomato seeds from Heinz and Germini were used during this study. Germini seeds were supplied by Prof. Lillo. Heinz seeds were supplied by Særheim Research Station.
S. lycopersicum cultivar Heinz 1706 was sequenced and assembled by the International Tomato Genome Sequencing Consortium. It is the only line where the genome is fully sequenced.

2.3.1 Sterilization of Tomato Seeds

Two methods for sterilization of Tomato seeds were performed. The first method was with ethanol while the other one was with Ca-hypochlorite. Both methods are described.

2.3.1.1 *Sterilization with Ca-hypochlorite*

25 ml one % (w/v) Ca-hypochlorite + one drop of Tween was mixed and left to settle. One ml of solution (supernatant) was taken into nine ml 96 % ethanol. Seeds were placed in Eppendorf tubes (five seeds in each tube) and one ml of the ethanol/hypochlorite solution were added, shaken and left to stand for maximum five minutes (longer time may kill the seeds). Supernatant was removed and one ml 95 % ethanol was added. Ethanol was then removed and the washing steps were repeated twice. Seeds were left to dry overnight in sterile hood, or washed with two ml autoclaved water and sown the same day. Each seed was placed in autoclaved Magenta box with ½ MS medium.

2.3.1.2 *Sterilization with Ethanol*

One ml 70 % ethanol + 0.01 triton were added to seeds in an Eppendorf tube, and incubated for 15 minutes on a shaker. The solution was removed in sterile hood, and one ml pure ethanol was added and incubated for 10 minutes on a shaker. The ethanol was removed, and one ml pure ethanol was added and the tube was inverted for a few times before the ethanol was removed again. The Eppendorf tube was left in sterile hood with the lid open for drying. After drying seeds were planted or stored at four °C

2.3.2 Sowing of Tomato Seeds

There were used different methods for sowing tomato seeds and propagating tomato plants.

2.3.2.1 *Tomato Seeds on ½ MS + Agar (+/- two % sucrose)*

45 ml of ½ MS (see table 2.2) + agar with either two % sucrose or minus sucrose were added to Petri dishes and left to dry for approximately 30 minutes. Seeds were sown with toothpick, and placed in 16 hours day light and eight hours dark room for 25 °C.

Table 2.2: MS medium. *Table shows content for MS medium. Vitamins were not included in the medium*

Stock solutions:	***g/L with exceptions***	***500 ml ½ MS medium***
KNO_3	95	5 ml
NH_4NO_3	120	3.25 ml
$MgSO_4•7H_2O$	37	2.5 ml
KH_2PO_4	17	5 ml
$CaCl_2•2H_2O$	44	2.5 ml
Fe/EDTA solution		12.5 ml
Na_2•EDTA	0.373	
$FeSO_4•7H_2O$	0.278	
Minor I for 1 l		2.5 ml
$ZnSO_4•7H_2O$	0.920	
H_3BO_3	0.620	
$MnO_4•4H_2O$	2.230	
Minor II for 1 l		2.5 ml
$Na_2MoO_42H_2O$	0.025	
$CuSO_4•5H_2O$	0.003	
$CoCl_2•6H_2O$	0.003	
KI	0.083	
Sucrose	2 %	2 %
Agar	7	3.5 g

Note. *pH was adjusted to 5.8 and autoclaved.*

2.3.2.2 *Propagation of Tomato*

Propagation of tomato seeds was performed by cutting of a little part of the stem from already grown Germini plants. These were placed in Magenta boxes with ½ MS + 0.8 % agar-agar and two % sucrose, and placed in 16 hours day light and eight hours dark room for 25 °C.

2.3.2.3 *Tomato Plants on Soil*

After sowing tomato seeds on ½ MS + agar (+/- two % sucrose), some of the tomato plants were infected by fungus. These plants were transferred to soil after 14 days so that surface sterilization could be performed later.

Soil was mixed with vermiculite in 3/1 proportion and placed in plant boxes. Water was placed in the tray for one to two hours until soil was wet from top and down. Tomato plants were placed in each plant box and placed in 16 hours day light and eight hours dark room for 25 °C.

2.3.3 Seed Germination

Seed germination was performed according to the chart for totipotency of tomato protoplasts (Appendices figure 4), from the protocol described in ”Totipotency of tomato protoplast” (Shahin 1985).

Tomato plants from ½ MS + agar (+/- two % sucrose) 3-12 days after germination were taken to Magenta boxes with 50 ml TM-1 medium (Appendices table 1). Roots were cut of with a portion of the hypocotyl and transferred to the TM-1 medium. Magenta boxes were then placed in 16 hours day light and eight hours dark at 25 °C for two to six weeks until protoplast isolation was performed.

2.4 gRNA, Cas9

2.4.1 Making gRNAs and Primers

Spacers for sgRNA 1 and 2 was found from (Pan et al. 2016). They could also be identified by using web tool "CRISPR-PLANT" (Xie et al. 2014). Query was: Solyc03123760 in chromosome three from 64554061 to 64561664. In order to find flanking primers for Polymerase Chain Reaction (PCR) amplification around the CRISPR target, sgRNA was aligned against tomato genome in Ensemble where the target place was found. This was used to design the primers. Steps for the construction of the gRNAs and primers are given in the Appendices.

2.4.2 Annealing of Reverse and Forward Primer
(Complementary oligo nucleotides)

Annealing of reverse and forward primer was performed to get complementary oligo nucleotides. Two protocols were used for this purpose with some modifications (Ran et al. 2013; Liu et al. 2015). CRISPR 1 and CRISPR 2 primers were used to make gRNA 1, and CRISPR 3 and CRISPR 4 were used to make gRNA 2. An overview over primer sequence, restriction enzyme and other information is given in table 2.3.

Table 2.3: Primers used to make gRNA 1 and gRNA 2. *Primers CRISPR 1 and CRISPR 2 were used to make gRNA 1, and CRISPR 3 and CRISPR 4 were used to make gRNA 2. Sequence, restriction enzyme, subcloning vector and checking for primers are given*

Primers	Sequence (5´----3´)	Restriction Enzyme	Subcloning vector	Checking
CRISPR 1 *(Forward)*	TAGGTAGTTGGGCGCGGAGAAGCA	BsaI	pUC-57-sgRNA	AcII
CRISPR 2 *(Reverse)*	AAACTGCTTCTCCGCGCCCAACTA	BsaI		
CRISPR 3 *(Forward)*	TAGGTAACGATCGATTGCAATGGA	BsaI	pUC-57-sgRNA	BsrD
CRISPR 4 *(Reverse)*	AAACTCCATTGCAATCGATCGTTA	BsaI		

2.4.2.1 *Protocol A and B for Annealing of Reverse and Forward Primer*

Two different protocols were followed, protocol A and protocol B, respectively. Protocol A was with T4 PNK (phosphorylated) while protocol B is without T4 PNK (non-phosphorylated). Solution for protocol A and protocol B was made in Eppendorf tubes according to table 2.4. Annealing buffer was made according to (Huang 2014). Dilution of oligos was performed to achieve 100 µM.

Table 2.4: Protocol A and protocol B for annealing of reverse and forward primer. *Protocol A was with T4 PNK while protocol B is without T4 PNK. CRISPR 1 and CRISPR 2 primers were used to make gRNA 1, and CRISPR 3 and CRISPR 4 was used to make gRNA 2. Annealing buffer was made according to (Huang 2014)*

Protocol A	**Volume**	**Protocol B**	**Volume**	**Reaction 1**	**Reaction 2**
Forward CRISPR Oligo 1 (100 µM)	1 µl	Forward CRISPR Oligo 1 (100 µM)	1 µl	CRISPR 1	CRISPR 3
Reverse CRISPR Oligo 2 (100 µM)	1 µl	Reverse CRISPR Oligo 2 (100 µM)	1 µl	CRISPR 2	CRISPR 4
T4 ligation buffer (Promega, 10X)	1 µl	Annealing buffer, 10X: 10 mM Tris, 5 mM NaCl, 1 mM EDTA	5 µl		
T4 PNK (NEB, 10 000 u/ml)	1 µl				
ddH_2O	6 µl	ddH_2O	43 µl		
Total	10 µl	**Total**	50 µl		

2.4.2.2 *Polymerase Chain Reaction (PCR)*

PCR is a technique to amplify a DNA fragment. To perform PCR it was necessary to have components as DNA-primer, DNA polymerase, nucleotides, deoxynucleotides triphosphates (dNTP), buffer and magnesium (Mg^{2+}). Reaction components in table 2.4 were mixed together before double-stranded DNA was warmed to 95 ºC for 5-10 minutes for the strands to be denatured and activate the polymerase. After this step the temperature will usually be set to 60 ºC or lower. For 55 ºC it will bind better, but the end product will be less specific. At this temperature, the single strands can react and attach to short DNA fragments (primers) with around 20 bases which is complementary to the sequence that will be amplified. The temperature will then be raised to around 70 ºC, which is optimal for the polymerase to replicate DNA. DNA polymerase will only make DNA in 5´-3´direction. This process will be repeated until enough copies of DNA are made. PCR was performed according to two different programs as shown in table 2.5.

Table 2.5: Thermocycler conditions for annealing of complementary oligos with protocol A and protocol B. *Protocol A was with T4 PNK (phosphorylated) while protocol B is without T4 PNK (non-phosphorylated)*

Protocol A		**Protocol B**	
37 ºC	30 min	95 ºC	5 min
95 ºC	5 min	Ramp down	3 ºC/min
Ramp down	5 ºC/min		
		∞	25 ºC
∞	25 ºC		

2.4.3 Restriction-ligation

After PCR, restriction-ligation reaction was performed in order to ligate the gRNAs into the plasmid. Figure 2.1 shows pUC57-sgRNA expression vector (Shen et al. 2014), and was a gift from Xingxu Huang (Addgene plasmid # 51132). Restriction-ligation solution was set up according to table 2.6. Restriction enzyme BsaI is used, shown in table 2.7. Content was mixed well and incubated for three hours at 37 ºC, and then for 30 min at 70 ºC.

Table 2.6: Restriction-ligation solution

Content	***Amount***
Diluted oligos	2 µl
Plasmid	100 ng (1 µl)
10 x T4 ligase buffer	2 µl
10 x BSA	2 µl
T4 ligase	0.75 µl
Bsa I	1 µl
H_2O	11.25 µl
Total	***20 µl***

Table 2.7: BsaI restriction enzyme used in restriction-ligation solution. *Restriction site is given as well as buffer and source*

Restriction enzyme	*Restriction site*	*Buffer*	*Source*
BsaI	5′… G G T C T C $(N)_1$▾… 3′ 3′… C C A G A G $(N)_5$▾… 5′	CutSmart Buffer	*Bacillus stearothermophilus*

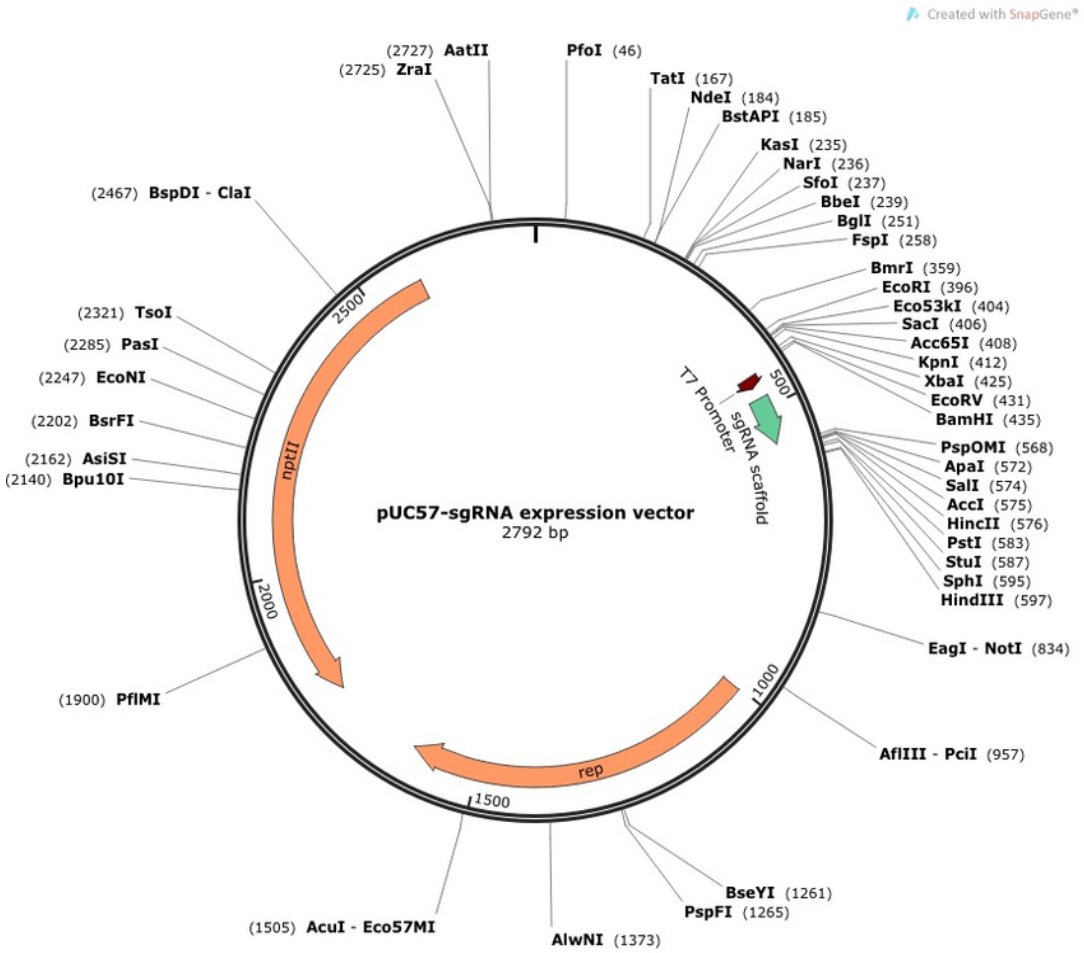

Figure 2.1: pUC57-sgRNA expression vector. *Plasmid used for in vitro transcription of sgRNA using T7 promotor with ampicillin resistance marker. pUC57-sgRNA expression vector was a gift from Xingxu Huang (Addgene plasmid # 51132)*

2.4.4 Transformation

To make competent cells transformation to *E.coli JM 109* was performed. The purpose for making competent cells is the ability for cells to take up free, extracellular genetic material, like a plasmid. The bacterial strain JM109 from *E.coli* is a useful host for several purposes as transformation of different vectors and production of single-stranded DNA from M13 or phagemid vectors (Promega 2017). In this experiment JM 109 strain from *E.coli* was used

from Promega. Transformation was performed several times in this experiment for both gRNAs and Cas9.

2.4.4.1 *Competent Cells*

Competent cells were left on ice to thaw for 20 min, ½ of product was mixed with competent cells (10 µl from each tube to competent cells) and left on ice for 30 min before heat shocking at 42 ºC for 50 seconds, and then placed back on ice for two minutes. LB broth (see table 2.8) 500 µl was added, shaken and incubated for 37 ºC for two hours and 20 minutes (at least 60 minutes). 500 µl of the product was then plated on agar plates, and incubated at 37 ºC overnight.

Table 2.8: LB broth medium with kanamycin/ampicillin. *For transformation of gRNA LB broth with kanamycin was used, and for transformation of Cas9 Lb broth with ampicillin was used*

gRNA		***Cas9***	
Content	***Amount***	***Content***	***Amount***
Agar	37 g/l		
LB broth	25 g/l	LB broth	25 ml
Kanamycin	50 mg/ml	Ampicillin	25 µl

2.4.4.2 *Colony PCR*

To check if transformed bacteria had received gRNA into the vector, colonies were tested with colony PCR using Dream Taq polymerase (Thermo Fisher Scientific). Spacer forward primers together with the reverse primer (M13 Thermo Fisher Scientific) shown in table 2.9 were used in the reaction. Solution for colony PCR is given in table 2.10.

Table 2.9: Reverse primer M13 and sequence

Primer	**Sequence (5´----3´)**
M13 Reverse	CAGGAAACAGCTATGAC

Table 2.10: Solution for colony PCR

Content	*Amount*	*Reaction 1*	*Reaction 2*
10 x Dream Taq Buffer	2 µl		
dNTP mix	2 µl		
Forward primer (10 µM)	2 µl	CRISPR 1	CRISPR 3
Reverse primer (10 µM)	2 µl	M13	M13
Enzyme (Dream Taq)	0.2 µl		
ddH_2O	11.8 µl		
Total	20 µl		

2.4.5 Loading of Colony PCR Product onto Two % Agarose Gel

After colony PCR, the products were loaded onto two % agarose gel. Agarose gel electrophoresis is a method that separates DNA macromolecules by their size. The negatively charged DNA molecules will move against the positive electrode, the anode. Small molecules will move against the anode in a higher speed than the bigger molecules. By comparing with a standard, a ladder, the sizes of the molecules can be determined. To make the bands visible in a UV-light a dye (GelRed™) is added. This dye will react with the DNA. Loading buffer and GelRed™ (from Biotium) are added to samples. Loading buffer provides both a visible dye that makes it possible to see how long the molecules have moved, and it contains a high percentage of glycerol that increases the density of the sample that will lead it to sink down into the well (Addgene 2017a).

For making two % agarose gel two gram agarose + 100 ml one x Tris-acetate-EDTA (TAE) buffer was warmed in microwave until totally dissolved and cooled down before it was poured into a leveled gel-frame with combs. Liquid was left for around 30 minutes until settled. After settling the gel, it was transferred to an electrophoresis-tub filled with one x TAE buffer. Master mix with loading buffer and GelRed™ was then made. For each sample two µl loading buffer + two µl 1/50 gel red was used. The first well was filled with ladder + two µl 1/50 gel red, before samples 10 µl were loaded with master mix in the rest of the wells.

Gel electrophoresis was run at 90 V for 35 minutes. After gel electrophoresis bands were made visible by using an UV-transilluminator.

2.4.5.1 *Overnight Culture*

To get a high amount of the plasmid, overnight cultures were made. One colony from positive colonies was transferred to five ml LB broth media with kanamycin in sterile hood. Cultures were incubated at 37 ºC for 16 hours.

2.4.6 Isolation of Plasmids

For isolation of plasmids, two commercial miniprep kits were used:
1. Mini-prep kit from GenElute^{TM} Plasmid Miniprep kit from Sigma Aldrich.
2. MINIPrep NucleoSpin® Plasmid (NoLid) from Macherey-Nagel.

Mini-prep kit from GenElute^{TM} Plasmid Miniprep kit from Sigma Aldrich is explained in detail.

Harvest and lyse bacteria

Bacteria culture was poured to Eppendorf tubes and centrifuged at full speed for one minute. Supernatant was discarded. This was then repeated. 200 µl resuspension solution was added to the pellet and vortexed until dissolved. 200 µl of lysis solution was added, inverted gently and cleared for five minutes.

Prepare cleared lysate

350 µl of neutralization was added to the solution and mixed by inversion four to six times. The solution was centrifuged for 10 minutes at maximum speed.

Prepare binding column

500 µl column preparation solution was added to binding column in a collection tube and centrifuged for four minutes at full speed. Flow-through was then discarded.

Bind plasmid DNA to column

Cleared lysate was transferred to binding column and centrifuged for one minute. Flow-through was discarded and centrifuged for one minute for column to dry.

Elute purified plasmid DNA

Column was transferred to new collection tube and 60 µl water was added and centrifuged for one minute and then freezed in -20 ºC.

Samples from freezer were then measured by nanodrop, and allowed to run on agarose gel for gel extraction.

2.4.7 Sequencing

The isolated plasmid with gRNA inserts was sent for sequencing with M13R primer to Seqlab-Microsynth Sequencing, Germany. See Appendices figure 1 for the whole sequence.

2.4.8 Digestion of Plasmids

For digestion of plasmids, restriction enzymes are required. Restriction enzymes are endonucleases from bacteria that recognize and cut DNA which is foreign to the bacteria. They take advantage of natural occurring enzymes that cleave the DNA at specific sequences. Restriction enzymes are commonly used in for example cloning. In this experiment Dra1 (New England Biolabs) restriction enzyme was used for gRNA and AgeI (New England Biolabs) was used for Cas9, see table 2.11.

Table 2.11: Restriction enzyme, restriction site, buffer and source used in the experiment

Restriction enzyme	*Restriction site*	*Buffer*	*Source*
DraI	5′… T T T▼A A A … 3′ 3′… A A A▲T T T … 5′	CutSmart	*Deinococcus radiophilus*
AgeI	5′… A▼C C G G T … 3′ 3′… T G G C C▲A … 5′	CutSmart	*Ruegeria gelatinovora*

Table 2.12: Samples for digestion and concentration. *Sample A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4, sample B1 and B2 are non-phosphorylated. Sample B1 has primers CRISPR 1 and CRISPR 2, and sample B2 has primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3)*

Samples	***Concentration***	***500 ng***
B1	164 ng/µl	3 µl
A2	224.7 ng/µl	2.2 µl
B210	214.8 ng/µl	2.3 µl
B211	124.5 ng/µl	4.0 µl

Digestion of successful plasmids containing gRNAs using Dra I was performed. Samples were made according to table 2.13 (table 2.12 shows the concentration and the calculation), and then sat to incubation at 37 ºC for one hour. Undigested samples were also allowed to run with the samples + MM. Afterwards; digested samples were allowed to run on one % agarose gel electrophoresis.

Table 2.13: Digestion of the samples, amount and content for gRNAs. *Sample A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4, sample B1and B2 are non-phosphorylated. Sample B1 has primers CRISPR 1 and CRISPR 2, and sample B2 has primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3)*

Content	***B1***	***A2***	***B210***	***B211***
DNA	3 µl	2.2 µl	2.3 µl	4.0 µl
Enzyme (DraI)	0.25 µl	0.25 µl	0.25 µl	0.25 µl
Buffer (CutSmart)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
H_2O	19.25 µl	20.05 µl	19.95 µl	18.25 µl
Total	***25.0 µl***	***25.0 µl***	***25.0 µl***	***25.0 µl***

Digestion of plasmids was performed several times. The second time it was performed with both gRNAs and Cas9 (pST1374-NLS-flag-linker-Cas9). A figure of pST1374-NLS-flag-linker-Cas9 (Shen et al. 2013) is given in figure 2.2, and was a gift from Xingxu Huang (Addgene plasmid # 44758). Five µg in each sample were used with two parallels. 50 units of

restriction enzyme were used for DraI, and a little less for AgeI. Table 2.14 shows all the components for the samples used for digestion of plasmids. Samples were incubated overnight at 37 ºC.

Table 2.14: Components for digestion of plasmids with samples from Cas9 (pST1374-NLS-flag-linker-Cas9) and gRNAs. *Sample A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4, and sample B1 has primers CRISPR 1 and CRISPR 2 (primers are shown in table 2.3). AgeI restriction enzyme are used for Cas9 samples while DraI are used for gRNA samples*

Components	Cas9 1	Cas9 2	gRNA A2.1	gRNA A2.2	gRNA B1.1	gRNA B1.1
AgeI/DraI	2 µl	2 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
DNA	7.8 µl	10.5 µl	17.6 µl	24.2 µl	24.8 µl	20.9 µl
10 x NEB buffer	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
H_2O	35.2 µl	32.5 µl	24.9 µl	18.3 µl	17.7 µl	21.6 µl
Total	*50.0 µl*	*50.0 µl*	*50.0 µl*	*50.0 µl*	*50.0 µl*	*50.0 µl*

Plasmids were stored at -18 ºC.

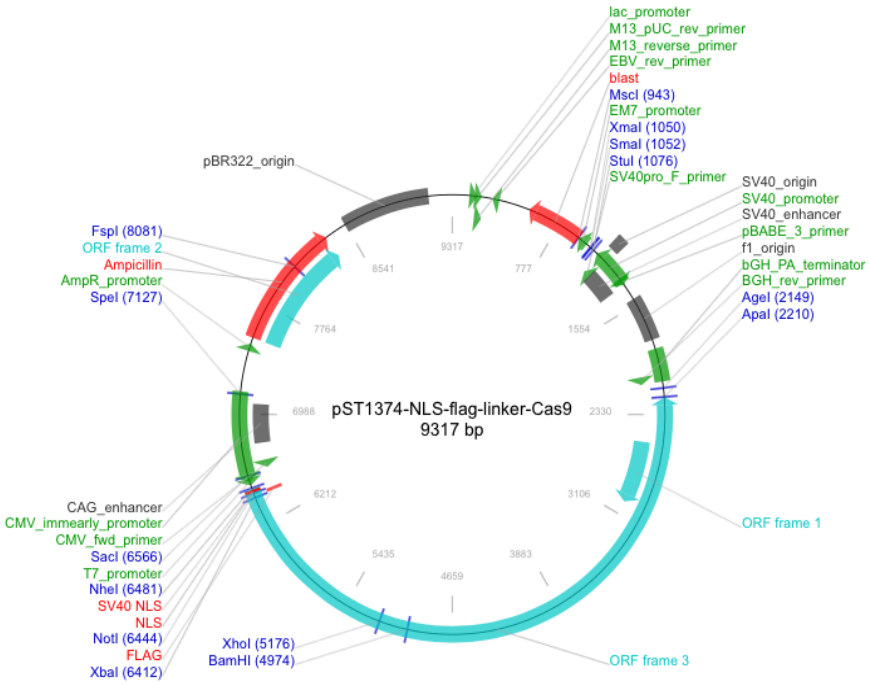

Figure 2.2: pST1374-NLS-flag-linker-Cas9 plasmid. *Plasmid is 9317 bp, and has ampicillin resistance. The plasmid (Shen et al. 2013) was a gift from Xingxu Huang (Addgene plasmid # 44758)*

2.4.9 Glycerol Stocks

Glycerol stocks to samples A2, B1 and Cas9 were made. 500 µl glycerol + 900 µl of the bacteria. These were then placed in -80 ºC.

2.4.10 Gel Extraction

Bands were cut in UV light, and measured. Gen Elute™ Gel Extraction Kit (NA1111) from Sigma-Adrich was used according to the protocol. 900 µl of solubilize gel was used. After gel extraction kit experiment was performed, samples were measured by nanodrop.

2.4.11 gRNA *In Vitro* Transcription

For *in vitro* transcription of gRNAs from plasmid HiScribe™ T7 Quick Yield RNA synthesis kit from New England BioLabs (NEB#E20505) was used. To get higher concentration in tubes from nanodrop, gRNAs tubes were opened and sat in heat block for 60 ºC for 45-75 minutes. This will result in higher concentration due to the fact that water will evaporate. Tubes were flipped, centrifuged and put on ice before they were measured with nanodrop again. RNA synthesis protocol was then set up. One µg of each sample was used. RNA synthesis protocol, reaction for short transcripts, was set up as shown in table 2.15. An extra control was made containing water (18 µl). Samples were then measured with nanodrop.

Table 2.15: RNA synthesis reaction with samples A2 and B1. *Sample A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4, and sample B1 has primers CRISPR 1 and CRISPR 2 (primers are shown in table 2.3)*

Reaction components	**A2**	**B1**
Nuclease free water	12 µl	10.4 µl
NTP buffer mix	10 µl	10 µl
Template DNA	6 µl	7.6 µl
T7 RNA polymerase mix	2 µl	2 µl
Total reaction volum	30 µl	30 µl

2.4.12 Cleaning RNAs

For cleaning RNAs Megaclear™ kit Purification for large scale transcription reactions kit from Thermo Fisher Scientific was used. Centrifuge users method was chosen (elution option 1). Samples were measured with nanodrop after step six and then freezed at -20 ºC.

2.4.13 Cas9 *In Vitro* Transcription

For making Cas9 mRNA, kit from "RNA Enzymes and gene analysis HiScribe™ ARCA mRNA kit with tailing was used from New England Biolabs. One µg template DNA was used. Reaction was set up as shown in table 2.16, and experiment was performed according to the protocol with some exceptions. Step three and four were skipped, and were replaced with cleansing with kit from "RNA clean and concentrator™-25" from Zymo Research (R1017). After cleansing steps, the rest of the protocol from step five was followed. Samples were after this measured by nanodrop.

Table 2.16: Reaction components for in vitro transcription of Cas9 DNA template

Reaction components	*Cas9*
Nuclease free water	3.4 µl
2 x 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

Isolation of plant DNA was performed according to the protocol (Springer 2010). Cetyltrimethyl ammonium bromide (CTAB) plant extraction buffer was prepared and sterilized according to table 2.17. 10 ml CTAB + 100 µl B-mercaptoethanol were used.

Table 2.17: CTAB solution

Reagent	*Amount*	*Final concentration*
Tris (1 M, pH 7.5)	100 ml	100 mM
NaCl (5 M)	140 ml	700 mM
EDTA (0.5 M)	20 ml	10 mM
CTAB	10 g	1 %
ddH_2O	Ut to 990 ml	
B-mercaptoethanol		1 %

500 µl of protoplasts were used and centrifuged for one minute at 700 rpm before discarding the supernatant. A leaf from Heinz was used as a control. Plant tissue was collected from small Petri dishes into Eppendorf tubes (two tubes per sample with 1.5 ml in). Samples were centrifuged at 500 rpm for one minute. Supernatant was removed to another Eppendorf tube to make sure that not all the protoplasts followed. 700 µl of CTAB solution (CTAB plant extraction buffer) was added and mixed by inversion and incubated for 30 minutes at 65 ºC (mixed by inversion after 15 minutes). The two Eppendorf tubes were transferred to one tube for each sample after this step. Mixed 400 µl of 24:1 chloroform:isoamyl alchol and mixed by vortexing until there was no "phases" in the hood. Centrifuged at 10 000 g for five minutes. Transferred the aqueous phase (upper phase) to a new Eppendorf tube. 300 µl isopropanol was added, mixed by inversion and centrifuged at 10 000 g for five minutes. Liquid was poured off. 500 µl 70 % ethanol was added, mixed by vortexing and centrifuged at 10 000 g for two minutes. Liquid was then poured off. Pellet was air-dried and resuspended in 100 µl one mM Tris (pH 8). Samples were measured by nanodrop and prepared for PCR.

Samples (100 µg) were then allowed to run on agarose gel, before PCR was performed. Diluted primers to 100 µmol/L. Primers were first centrifuged before water was added. Samples were mixed by vorting. Primers used are shown in table 2.18.

Table 2.18: Primers from gene SIPDS, sequence, PCR amplicon size and sgRNA

Primers	Sequence (5´----3´)	PCR amplicon size	sgRNA
CRISPR 59 *(Forward)*	AGTTAATCAGGCATGTACAGGTACAA	396	TAGTTGGGCGCGGAGAAGCACGG
CRISPR 60 *(Reverse)*	TAAAGATTCGTACTCCCCATGCC		
CRISPR 61 *(Forward)*	CATATAGTGACTCATACAAATTGGTGC	198	TAACGATCGATTGCAATGGAAGG
CRISPR 62 *(Reverse)*	ATTTAAAGGAGCGGGTAAAGCTTC		

PCR reaction was set up as shown in table 2.19.

Table 2.19: PCR reaction for protoplasts and control

Components	Amount	Protoplast	Control
10 x Dream Taq Buffer	5 µl	5 µl	5 µl
dNTP mix, 2mM each	5 µl	5 µl	5 µl
Forward primer	0.1-1.0 µM	1 µl	1 µl
Reverse primer	0.1-1.0 µM	1 µl	1 µl
Template DNA	10 pg – µg	20.83 µl	5.18 µl
Dream Taq poly (50/µl)	1.25 U	0.25 µl	0.25 µl
Water		0.25 µl	32.57 µl
Total	50 µl	50 µl	50 µl

Samples were then allowed to run on gel electrophoresis two % agarose. Loading buffer DNA II from AppliChem was used. Samples were allowed to run at 90 V for 40 minutes. Samples were after this allowed to run on two % agarose gel for gel extraction for 35 minutes at 80 V. Bands were cut and kit from Macherey-Nagel: "DNA, RNA and protein purification. DNA extraction from agarose gels" was used.

For each 100 mg agarose gel > 2 % 400 µl of NT1 buffer was added.
Exceptions from protocol:

- Centrifuged for one minute instead of 30 seconds (step two and three).
- Elution step performed twice (last step in kit before nanodrop)
- Step five: Eluted DNA → added 20 µl ddH_2O (not buffer NE as in protocol)

2.4.15 Cas9 mRNA Gel (one % agarose gel)

To check the quality on Cas9 mRNA transcripts these were allowed to run on formaldehyde denaturing agarose gel. Table 2.20 shows buffer and gel for Cas9 mRNA gel. To make the gel and buffers protocol from (Kataya 2011) was used. For sample preparation protocol "Preparation of Denaturing Agarose Gels" from National Diagnostics was used.

Table 2.20: 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 acetat	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
	GelRed™	5 µl
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 0.001 M EDTA)	2 µl (from 0.5 M EDTA)
	Bromophenol blue	3 µl (0.003 g)
	Xylene cyanol	3 µl (0.003 g)
	ddH_2O	492 µl

*Note. * = dimetylformamide was used instead of formamide*

Agarose and water were heated in microwave until dissolved, and cooled down to approximately 60 ºC. Warmed 10 x MOPS buffer and formaldehyde to 55 ºC and added GelRed™ at last. Gel was left to settle for one hour. Sample buffer No.1 was prepared and heated to 55 ºC for 15 minutes with samples. Took out 10 µl of sample buffer No.1 + 2.5 µl (two µg sample) of Cas9 and heated it for 15 minutes 55 ºC in heat block. After heating sample buffer No.2 2.5 µl was added. Gel electrophoresis was then allowed to run for 50 minutes at 90 V. This was performed in hood.

2.4.16 gRNA Gel (denaturing PAGE/Urea Gel)

To check the quality of gRNA transcripts these were allowed to run on denaturing PAGE/Urea gel. gRNA gel was set up according to (Summer et al. 2009). Buffers for the gRNA gel are listed in table 2.21.

Table 2.21: 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)	27.82 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 instead of formamide*

Gel was pre-run with one x TBE buffer to two gels. It was checked if there was any leakage. Combs were then removed. Wells were rinsed with running buffer to remove UREA before running the gel. Samples were taken directly from the ice. Added three µl sample and two µl ddH_2O + five µl loading buffer. This was heated at 80 ºC for a few minutes on heat block and added two µl GelRed™ into the samples. This was performed in hood.

2.4.17 *In Vitro* Digestion of DNA with Cas9

In vitro digestion of DNA with Cas9 was performed according to kit "*In vitro* digestion of DNA with Cas9 Nuclease, *S. pyogenes* (M0386)" from New England Biolabs. Cas9 MO641T was used. Cas9 nuclease from *Streptococcus pyogenes* was used in this kit for the purpose of

digestion of double-stranded DNA *in vitro* with Cas9 and gRNA. Reaction set up and reaction solution are shown in table 2.22 and 2.23.

Table 2.22: Reaction set up of in vitro digestion of DNA with Cas9 and gRNA

	Cas9	***gRNA***	***DNA***
Relationship	10	10	1
Concentration	1 µM	1 µM	0.05 µM
Volume	2 µl	2 µl	4 µl
Final concentration	100 nM	100 nM	10 nM

Solutions from Cas9, gRNAs (A2 and B1.1) and DNA (protoplasts) were made:

- Cas9 final concentration one µM (from 20 µM)
- gRNAs final concentration one µM (B1.1 had concentration 74.14 µM and A2 had concentration 37.64 µM)
- DNA (Protoplasts) final concentration 0.05 µM (Protoplast 1 with primers 59+60 had concentration 0.0786 µM and protoplast 2 with primers 61 + 62 had concentration 0.11751 µM.) Due to these concentrations, samples with 0.05 µM were made and four µl in total sample instead of two µl were taken.

Table 2.23: In vitro digestion reaction of DNA with Cas9 nuclease

Components	***Volume***
Nuclease free water	10 µl
10 x Cas9 nuclease reaction buffer	2 µl
300 nM sgRNA (A2 and B1.1)	2 µl
1 µM Cas9 nuclease	2 µl
Pre-incubate for 10 minutes at 25 ºC (To make complex)	
100 nM substrate DNA	4 µl
Total	20 µl

Samples were mixed thoroughly and centrifuged before incubation for one hour at 37 ºC. Proceeded then with fragment analysis with gel electrophoresis (two % agarose gel).

2.5 Isolation of Protoplasts and Transfection

Isolation of protoplasts and transfection were performed for proper delivery of gRNAs and Cas9 mRNA transcripts.

2.5.1 Preparation for Isolation and Transfection of Protoplasts

The following solutions listed in table 2.24 and table 2.25 were made for isolation of protoplasts and transformation. Some solutions that are listed in table 1 and 2 in Appendices were also used for isolation of protoplasts.

Table 2.24: Solutions used for protoplast isolation

Solutions	*Components*	*Amount*	*Filter sterilization and storage*
Enzyme solution (50 ml)	Cellulase RIO (1.0 %)	0.5 g	
	Maceroenzyme RIO (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 500 mM solution)	
	$CaCl_2$ (1 M)	500 µl	
	Bovine Serum Albumin (BSA)	0.05 g	
PEG solution (10 ml)	Polyethylene Glycerol (PEG) 4000	4 g	Filter sterilized.
	$CaCl_2$ (1 M)	1 ml	
	Mannitol (0.5 M)	4 ml	
	Water	Until volum reached 10 ml	
W5 (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 $CaCl_2$	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
	$MgCl_2$ x $6H_2O$ (15 mM)	0.152 g	
	MES (0.1%)	0.100 g	
B5 (50 ml)	1 x B5	500 µl of all stock solutions*	
	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 2.25*

Table 2.25: Stock solutions in Gamborg medium used to make B5 for transformation of protoplasts

	Concentration in Gamborg medium (1x)		Stock solutions (100x)	Volume (ml) of stock to make Gamborg (1x) 1L
Macro elements	**mM**	**Mg/L**	**g/100 ml**	
$CaCl_2$ x $2H_2O$	1.02	150	1.5 g	10 ml
KNO_3	24.73	2500	25 g	10 ml
$MgSO_4$ x $7H_2O$	1.01	250	2.5 g	10 ml
NaH_2PO_4 x $2H_2O$	1.09	170	1.7 g	10 ml
$(NH_4)2SO_4$	1.01	134	1.34 g	10 ml
FeNaEDTA	0.100	36.7	0.367 g (10 mM)	10 ml
Micro elements	**µM**	**Mg/L**	**g/1000ml**	
$CoCl_2$ x $6H_2O$	0.11	0.025	0.0025 g	
$CuSO_4$ x $5H_2O$	0.10	0.025	0.0025 g	
H_3BO_4	48.52	3.00	0.30 g	
KI	4.52	0.75	0.075 g	10 ml
$MnSO_4$ x $1H_2O$	59.16	10.00	1.0 g	
Na_2MoO_4 x $2H_2O$	1.03	0.25	0.025 g	
$ZnSO_4$ x $7H_2O$	6.96	2.00	0.20 g	

2.5.2 Isolation of Protoplasts

2.5.2.1 *Surface Sterilization of Leaves*

Two solutions were made to perform surface sterilization of tomato leaves: Calcium hypochlorite 0.5 % and 50 ml 70 % ethanol. Leaves were cut and taken to Falcon tubes with 70 % ethanol in maximum 60 second. Tubes were inverted. Leaves were then transferred to calcium hypochlorite 0.5 % solution in a Petri dish and dipped for 20 minutes. Leaves were then transferred to a Petri dish with sterilized water, removed, dipped in new sterilized water and repeated once more. After this sterilization, leaves were ready to be transferred to pre-enzyme treatment (PET) solution. PET solution is given in table 2 in Appendices.

2.5.2.2 *Preparation for Protoplast Isolation*

Aluminum foil was placed around propagated Germini plants and then placed back into the same room for three days. Leaves were cut into small fragments and 30 ml PET solution was taken into big Petri dishes and placed overnight in sterile hood (15 °C). PET solution was taken out with pipette in sterile hood. For digestion of cell wall enzyme solution 30 ml was placed inside and placed at 40 rpm for 28 °C with parafilm over in shaker overnight.

2.5.2.3 *Collection of Protoplasts*

Collection of protoplasts was performed according to protocol with modifications (Woo et al. 2015). Plates with leaves in enzyme solution were taken out after overnight treatment. Enzyme solution was taken out by cut pipette tips and protoplasts were gently filtered with funnel and filter, which were pre-sterilized with ethanol. Protoplasts were collected in 15 ml Falcon tubes. Protoplasts were then centrifuged at 600 rpm for one min at 10-11 ºC. Supernatant was removed and five ml W5 solution was added in each Falcon tube. Centrifuged at 600 rpm for one min at 12 ºC before supernatant was removed. Added 2.5 ml washing solution in each Falcon tube and took 100 µl from this to two Eppendorf tubes and calculated protoplasts by a hemocytometer.

2.5.2.4 *Microscopy*

During this study two different microscopies were used for calculation and examination of protoplasts: light,- and epi-fluorescence microscopy. Light microscopy is a well known biology tool and is used to employ light for detection of small objects not visible to the naked eye. In this microscopy a hemocytometer was used for calculation of protoplasts.

Epi-fluorescence microscopies are normal to use in laboratories that are working with life science. This microscopy was used for examination of the protoplasts. The microscope usually consists of these essential components: a light source, a filter for incoming light, a dichroic beam splitter or mirror, a filter for emitted light and a change-coupled device (CCD) camera (Webb and Brown 2013; Scientific 2017).

In epi-fluorescence microscopy both the illuminated and the emitted light will go through the same objective lens. Through the objective lens light of the excitation wavelength will be focused on the sample. The filter for incoming light will narrow the wavelength for the light coming in so that only the wavelengths used to excite the sample will go through. The dichroic beam splitter or mirror will reflect the excitation light and only transmit emitted light from the sample back to the detector. Filter for emitted light will transmit wavelengths of the emitted light from the sample and block all the light that has passed through the excitation filter. For detecting the emitted light, fluorescence imaging normally uses a CCD camera that is connected to a computer screen (Webb and Brown 2013; Scientific 2017).

2.5.3 Transfection

After isolation of protoplasts, transfection was performed. W5 was removed and protoplasts were resuspended in MaMg. DNA was added to Petri dishes (overview in table 2.26) and protoplasts resuspended in MaMg were also added. This was then mixed well by pipetting. 500 µl PEG was added drop wise before lid was put on and left for 30 minutes. W5 was then added to the Petri dish drop wise and very slowly. First 0.5 ml, then one ml, two ml and three ml with a total of 6.5 ml. Solution was then transferred to a Falcon tube and centrifuged for 500 rpm for one minute at 14 ºC. Resuspended solution in three ml B5 and transferred to small Petri dishes and incubated them over night at 25 ºC in dark. Overview of content in Petri dishes for protoplast transfection are given in table 2.26.

Table 2.26: Petridishes, content and amount for protoplasts transfection

Petridishes	Content	Amount
1	Cas9 mRNA	42 µl
	A2 gRNA	33 µl
	B1 gRNA	35 µl
	Protoplasts	300 µl (250 000 protoplasts per 300 µl MaMg)
2	Protoplasts	300 µl

After transfection, isolation was performed according to the protocol (Springer 2010). Exceptions from protocol were that 300 µl of CTAB solution (CTAB plant extraction buffer) was added and mixed by inversion and incubated for 30 minutes at 65 ºC (mixed by inversion after 15 minutes). Samples were measured by nanodrop and prepared for PCR. 100 ng of each sample was prepared. PCR was set up according to table 2.27.

Table 2.27: PCR upset. *PCR was set up for samples Cas9 mRNA with gRNA A2 (Forward primer CRISPR 61, reverse primer CRISPR 62), Cas9 mRNA with gRNA B1 (Forward primer CRISPR 59, reverse primer CRISPR 60)*

Reagent	***Cas9 mRNA gRNA A2***	***Cas9 mRNA gRNA B1***
Template DNA	35.71 µl	35.71 µl
Water	2.04 µl	2.04 µl
Forward primer	CRISPR 61 1 µl of 50 µM	CRISPR 59 1 µl of 50 µM
Reverse primer	CRISPR 62 1 µl of 50 µM	CRISPR 60 1 µl of 50 µM
10 x Dream taq buffer	5 µl	5 µl
dNTP Mix, 2 mM each	5 µl	5 µl
Dream Taq Poly (5 u/µl)	0.25 µl	0.25 µl
Total	50 µl	50 µl

PCR products were then allowed to run on gel electrophoresis two % agarose. Two gels were made; one for cutting and one for checking. Samples were allowed to run for 50 minutes at 80 V. Bands were cut in UV light and measured. Gel extraction was then performed.

2.5.3.1 *DNA Extraction from Agarose Gels*

For DNA extraction, protocol from Nucleospin Gel and PCR Clean-up from Macherey-Nagel was followed with some exceptions. Centrifugation was used for one minute instead of 30 seconds. For drying silica membrane centrifuged for two minutes instead of one minute, and incubated columns for three minutes at 50 ºC. For elution of DNA water was used instead of

buffer, NE. Samples were then freezed at -20 ºC overnight. Samples were measured by nanodrop before proceeding with T7 Endonuclease I assay the following day.

2.5.4 T7 Endonuclease I Assay

For determination of genome targeting, T7 Endonuclease I assay was used. After gel extraction, T7 Endonuclease I assay was performed to see if Cas9 had made any cutting. T7 Endonuclease I serves two purposes; it recognizes matched DNA and cleaves it. The assay provided an annealing reaction and digestion with T7 Endonuclease I. Protocol was used from Determining Genome Targeting Efficiency using T7 Endonuclease I (MO302) from New England Biolabs, and Cas9 (MO641T) was used. Fragments were analyzed with gel electrophoresis after step three.

2.5.4.1 *Protocol*

1. Annealing reaction

The first step was the annealing reaction with sample A2 and B1. The reaction was set up as described in table 2.28.

Table 2.28: Annealing reaction with sample A2 and sample B1

Reagents	**A2**	**B1**
DNA (200 ng)	9.4 µl	9.7 µl
10 x NEB buffer 2	2 µl	2 µl
ddH_2O	7.6 µl	7.3 µl
Total	19 µl	19 µl

2. Hybridization condition (thermocycler)

Annealing was achieved with thermocycler as described in table 2.29.

Table 2.29: Annealing step with thermocycler

Step	*Temp*	*Ramp rate*	*Time*
Initial denaturation	95 ºC		5 min
Annealing	95-85 ºC 85-25ºC	-2.0 ºC/sec -0.1 ºC/sec	
Hold	4 ºC		Hold

3. Added T7 Endonuclease and incubated

The last step was to add T7 Endonuclease for digestion. Reagents are described in table 2.30.

Table 2.30: Reagents for digestion of T7 Endonuclease I

Reagents	*Volume*
Annealed PCR products	19 µl
T7 endonuclease I (MO302)	1 µl
Total	20 µl
Incubation time	15 min
Incubation temp	37 ºC

After this step fragment analysis with gel electrophoresis two % agarose was performed. Samples were allowed to run for 50 minutes at 90 V.

3 Results

3.1 Tomato Plants

Tomato plants from Heinz and Germini were used to perform the experiments. Plants from both tissue culture and soil were used as well as propagated plants.

3.1.1 Sterilization of Seeds and Growing of Tomato Plants

Two sterilization methods were tried out; one with ethanol and one with Ca-hypochlorite. Sterilization with ethanol seemed to have killed all the tomato seeds. None of the seeds sterilized with ethanol grew at all. Seeds sterilized with Ca-hypochlorite grew as normal.

3.1.1.1 *Tomato Plants on ½ MS Medium Minus Sucrose*

Figure 3.1 shows Germini plants on ½ MS medium minus sucrose after 14 days. Most of the seeds have grown. These seeds were sterilized with Ca-hypochlorite.

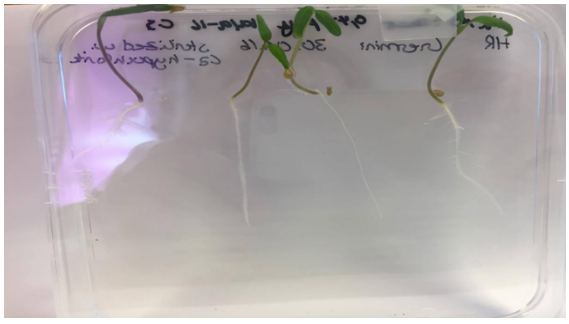

Figure 3.1: Germini plants on ½ MS medium minus sucrose after 14 days*. Seeds were sterilized with Ca-hypochlorite*

3.1.1.2 *Propagated Tomato Plants*

Propagation of tomato plants was performed several times throughout the experiment. Figure 3.2 shows propagated Germini tomato plants on ½ MS medium + agar with two % sucrose after 15 days. As shown in the figure the plants have grown and the propagation seems to have worked.

Figure 3.2: Germini tomato plants is shown in ½ MS medium agar 2 % sucrose after 15 days

3.1.1.3 *Tomato Plants on TM-1 Medium*

After sowing tomato seeds on ½ MS medium (+/- two % sucrose), plants were transferred to TM-1 medium. Figure 3.3 shows Heinz tomato plants two weeks old on TM-1 medium transferred from ½ MS medium minus sucrose. Plants were on TM-1 media for two to six weeks, where protocol with modifications was followed, shown in figure 4 in Appendices (Shahin 1985).

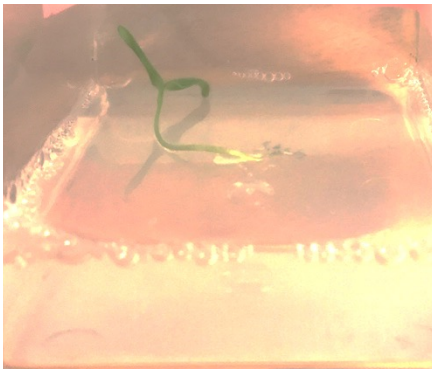

Figure 3.3: Two weeks old Heinz tomato plants on TM-1 medium transferred from ½ MS medium minus sucrose

3.1.1.4 *Tomato plants on soil*

Tomato plants from ½ MS medium that were infected by fungus were transferred to soil. Figure 3.4 shows two weeks old Heinz tomato plants from ½ MS medium minus sucrose.

Figure 3.4: Two weeks old Heinz tomato plants transferred to soil due to fungus infection from ½ MS medium minus sucrose

3.2 gRNA, Cas9

In this section about gRNAs and Cas9 there were many experiments performed as annealing of primers, restriction-ligation, transformation, sequencing, digestion of plasmids, gRNA and Cas9 *in vitro* transcription and *in vitro* digestion with Cas9 nuclease. The delivery of gRNA and Cas9 transcripts *in vivo* were performed with transfection of isolated tomato protoplasts before T7 Endonuclease I assay was performed as the last experiment.

3.2.1 Transformation

Transformation was performed by making competent cells for the ability to take up free, extracellular genetic material, like a plasmid. This was performed in the beginning of the study with samples from A1, A2, B1 and B2 as well as a control. Sample A1 and A2 were phosphorylated, while B1 and B2 samples were non-phosphorylated. A1 had primers CRISPR 1 and CRISPR 2 and A2 had primers CRISPR 3 and CRISPR 4. Sample B1 had primers

CRISPR 1 and CRISPR 2, and sample B2 had primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3 in the material and methods section).

Figure 3.5 shows colonies after transformation. The control contained colonies which can indicate that plasmids in the transformed cells could have closed without inserting oligos. The control should have been without any colonies, and would indicate that oligos most likely was inserted in the plasmid.

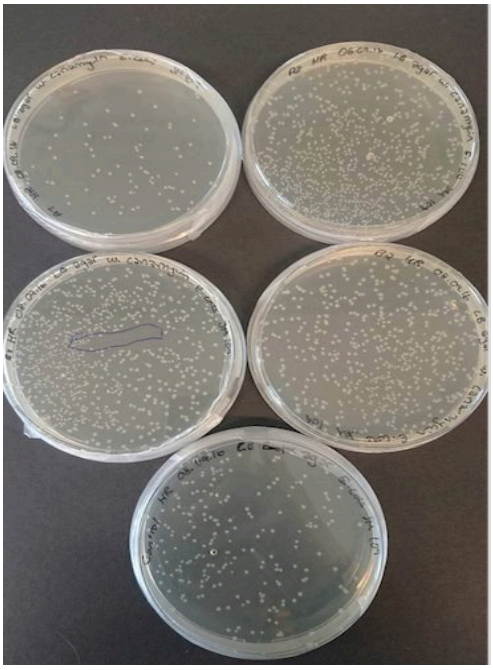

***Figure 3.5: Overnight culture**. Transformated cells on LB agar with kanamycin. Samples from A1, A2, B1 and B2 as well as a control was used. Sample A1 and A2 were phosphorylated, while B1 and B2 samples were non-phosphorylated. A1 had primers CRISPR 1 and CRISPR 2 and A2 had primers CRISPR 3 and CRISPR 4. Sample B1 had primers CRISPR 1 and CRISPR 2, and sample B2 had primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3 in the material and methods section). Control shows colonies which can indicate that plasmids in the transformated cells could have closed without inserting oligos*

3.2.1.1 *Colony PCR Products After Gel Electrophoresis*

To confirm the transformation, colony PCR was performed as well as gel electrophoresis with PCR samples.

Figure 3.6 shows Colony PCR product from gRNA 1 (oligo 1 + oligo 2) product after gel electrophoresis. Three colonies were taken from A1 and three colonies from B1, one colony from the control and 0.5 µl from plasmid. A1 had been phosphorylated, while B1 had not been phosphorylated. There is one band in sample from B1 on approximately 200 bp, and no bands are shown from A1. Band on approximately 200 bp seems to be the right size.

Restriction enzyme BsaI restriction sites are given in table 2.7 in the material and methods section and are shown in figure 3.10, as well as sequence for reverse primer M13 (table 2.9 in the material and methods section). When the spacer forward primer is replaced with the sequence part that is cut by BsaI it will give a sequence for 174 nucleotides (a figure over sequence and cutting places is given in Appendices figure 2). This band shown from B1 200 bp indicated that colonies from sample could be used for further investigation. 100 bp DNA ladder from New England Biolab was used (figure 5 in Appendices).

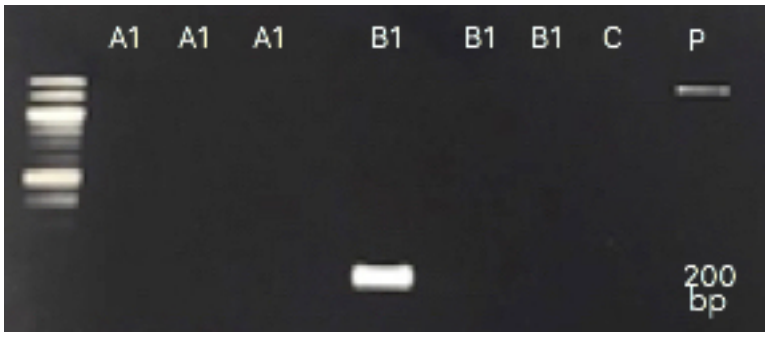

Figure 3.6: Colony PCR product from gRNA 1 (oligo 1 + oligo 2) product after gel electrophoresis*. There is one band on approximatly 200 bp from B1, no bands are shown from A1. A=phosphorylated, B=non-phosphorylated. C= control and P= plasmid. 100 bp DNA ladder from New England Biolab was (figure 5 in Appendices)*

Figure 3.7 shows Colony PCR product from gRNA 2 (oligo 3 + oligo 4) product after gel electrophoresis. There are five bands in total on approximately 200 bp each. Three bands were from A2 and two from B2. These results are the same as for B1 shown in figure 3.6 and give

an indication that the experiment has been successful. Colonies from these samples can be used for further investigations. 100 bp DNA ladder from New England Biolab was used (figure 5 in Appendices).

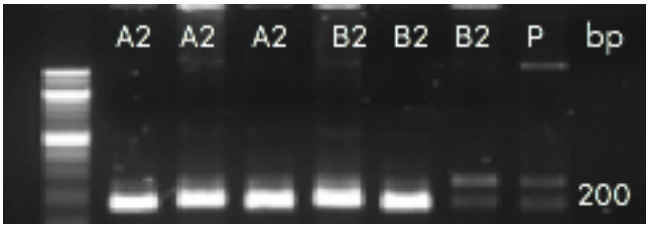

Figure 3.7: Colony PCR product from gRNA 2 (oligo 3 + oligo 4) product after gel electrophoresis. *There are five bands in total on approximatly 200 bp each. Three bands from A2 and two from B2. A=phosphorylated, B=non-phosphorylated, P=plasmid. Ladder used was 100 bp DNA ladder from New England Biolabs (figure 5 in Appendices)*

3.2.2 Sequencing

The isolated plasmid (puc57) with gRNA insert for sample A2, B1 and B2 was sent for sequencing with M13R primer to Seqlab-Microsynth Sequencing, Germany. The sequencing for all constructs proved to be successful, which indicated that the transformation has worked. Sequences was aligned with Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al. 2016) and boxshade. This is shown in figure 1 in Appendices.

3.2.3 Digestion of Plasmids

In order to prepare for the *in vitro* transcription, linearization of the plasmid puc57 including T7 promotor and the gRNA region was necessary. Digestion of the plasmids was therefore performed and the products were allowed to run on gel electrophoresis after the digestion. Samples of both digested and undigested were used. The samples were from B1, A2 and B2 (two samples) with DraI restriction enzyme. Sample A2 was phosphorylated, while B1 and B2 samples were non-phosphorylated. A2 had primers CRISPR 3 and CRISPR 4. Sample B1 had primers CRISPR 1 and CRISPR 2, and sample B2 had primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3 in the material and methods section).

Figure 3.8 shows the bands from these samples. Bands are from B1, A2 and B2. There are three bands in the samples which were digested (to the left beside the ladder). This indicates that DraI has cut on three sites which it should (see figure 3.10). The first band seems to be approximately 1700 bp and the second 1200 bp. Band on approximately 3000 bp is partly digested plasmid (size of vector is 2792 bp). Band on 1700 bp is the band that contains the gRNA, which was used for further gel extraction. One-kilo base pair (kb) hyper-ladder from Bioline (figure 6 in Appendices).

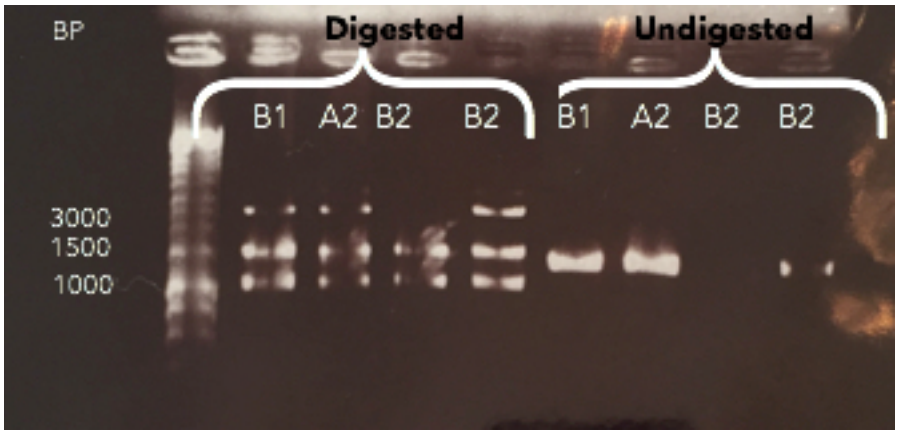

***Figure 3.8: Digestion of plasmids**. Samples from B1, A2 and B2 (two samples) were allowed to run on gel electrophoresis after digestion of plasmids with Dra I restriction enzyme. There are three bands on the samples which were which indicates that Dra I have cut on three sites which it should.*

The first band seems to be on approximatly 1700 bp, second 1200 bp and the third 3000 bp. Band on approximately 3000 bp is partly digested plasmid (size of vector is 2792 bp). Band on 1700 bp is the band that contains the gRNA, which was used for further gel extraction. The other samples are undigested.

A=phosphorylated, B= non-phosphorylated. A2 has primers CRISPR 3 and CRISPR 4. Sample B1 has primers CRISPR 1 and CRISPR 2, and sample B2 has primers CRISPR 3 and CRISPR 4 (primers are shown in table 2.3 in the material and methods section). One kb hyperladder from bioline was used (shown in figure 6 in Appendices)

Digestion of plasmids was performed another time as well on samples from A2, B1 and Cas9. This time samples were incubated at 37 ºC overnight instead of just one hour as the previous samples. Figure 3.9 shows samples before digestion of plasmid. Bands from samples A2 and B1 were approximately 1700 bp, while sample from Cas9 had several bands. Figure 2.2 in the material and methods section shows pST1374-NLS-flag-linker-Cas9 plasmid. Since Cas9 is a circular plasmid it has many bands which seems to range from 6000 bp to 10 000 bp. The size of Cas9 plasmid is 9317 bp.

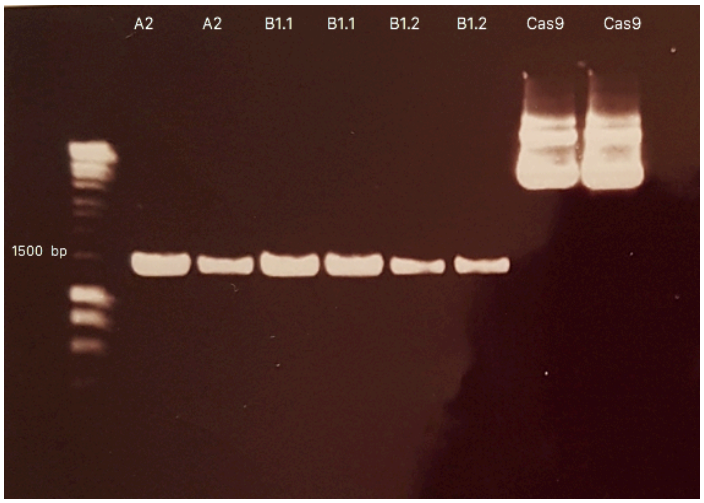

Figure 3.9: Samples from A2, B1 and Cas9 before digestion of plasmid. *Bands from samples A2 and B1 were approximatly 1700 bp, while Cas9 had several bands.*

A=phosphorylated, B= non-phosphorylated. A2 has primers CRISPR 3 and CRISPR 4. Sample B1 has primers CRISPR 1 and CRISPR 2. One kb hyperladder from Bioline was used (figure 6 in Appendices)

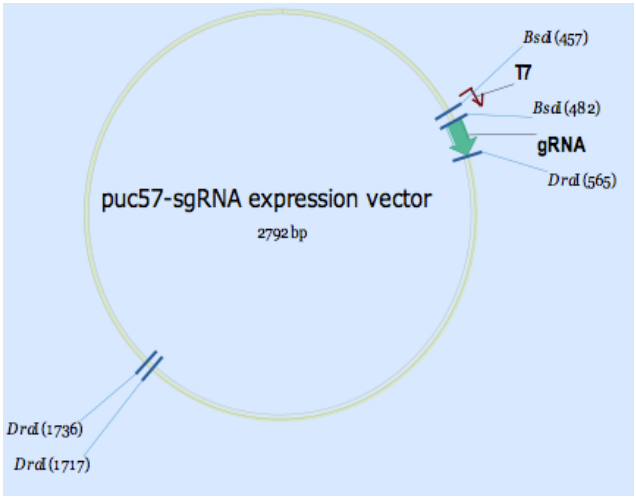

Figure 3.10: puc57-sgRNA expression vector. *Shows DraI restriction enzyme cutting on three different places in the vector puc57-sgRNA expression vector (2792 bp). Figure shows also where gRNA is, BsaI and DraI restriction enzyme, as well as the T7 promotor. DraI cuts on restriction sites 565 bp, 1717 bp and 1736 bp*

3.2.4 Protoplast isolation

Protoplast isolation was performed several times with modification to protocols (Shahin 1985). When the original protocol was followed (Shahin 1985) almost all protoplasts were dead. After changing the protocol and using solutions for other plants as *A. thaliana* protoplast isolation (Woo et al. 2015) as well as tomato plants from soil, the end results was many intact protoplasts.

3.2.4.1 *Tomato plants for protoplast isolation*

Tomato plants from Heinz and Germini from soil were used for protoplast isolation. Plants were three months old. Figure 3.11 shows both plants that were used for protoplast isolation. Leaves from Germini and Heinz from TM-1 media were also used. Plants were placed in the dark for protoplast isolation preparation for three to four days.

Figure 3.11: Heinz and Germini tomato plants. *These plants were three months old. Plants were placed in dark for protoplast isolation preparation for three-four days*

3.2.4.2 *Preparation for Protoplast Isolation*

After the dark treatment, leaves were placed in PET solution and/or enzyme solution. The first times protoplast isolation was performed, overnight treatment with PET solution was used prior to the enzyme solution as stated in original protocol (Shahin 1985). After trying out different methods for protoplast isolation three different methods were tested; the first one was cutting of leaves in PET solution, the second one was cutting of leaves in enzyme solution and the third was cutting of leaves in PET solution prior to enzyme solution. Leaves cut directly in PET solution were placed in a shaker for overnight treatment, while leaves cut in enzyme solution were placed in dark for overnight treatment.

Leaves from Germini and Heinz on soil were cut in enzyme solution and placed directly in the dark for overnight treatment shown in figure 3.12.

Figure 3.12: Leaves from Germini and Heinz on soil were cut in enzyme solution

3.2.4.3 *Surviving Protoplasts*

Tomato plants from Heinz and Germini from soil were used for protoplast isolation. Plants were three months old. Figure 3.11 shows both plants that were used for protoplast isolation. Leaves from Germini leaves and Heinz leaves from TM-1 media were also used. Plants were placed in the dark for protoplast isolation preparation for three to four days.

Leaves from Germini and Heinz on soil that had been treated with enzym solution used for Arabidopsis among others, showed surviving protoplasts. Figure 3.13 shows some intact protoplasts and many broken. Some of the intact protoplasts have a big and rounded shape. Leaves from Germini and Heinz on soil treated with PET solution prior to the same enzyme solution also showed surviving protoplasts. Leaves from Germini and Heinz treated with PET solution and original enzyme solution (table 2 in Appendices) showed almost no intact protoplasts.

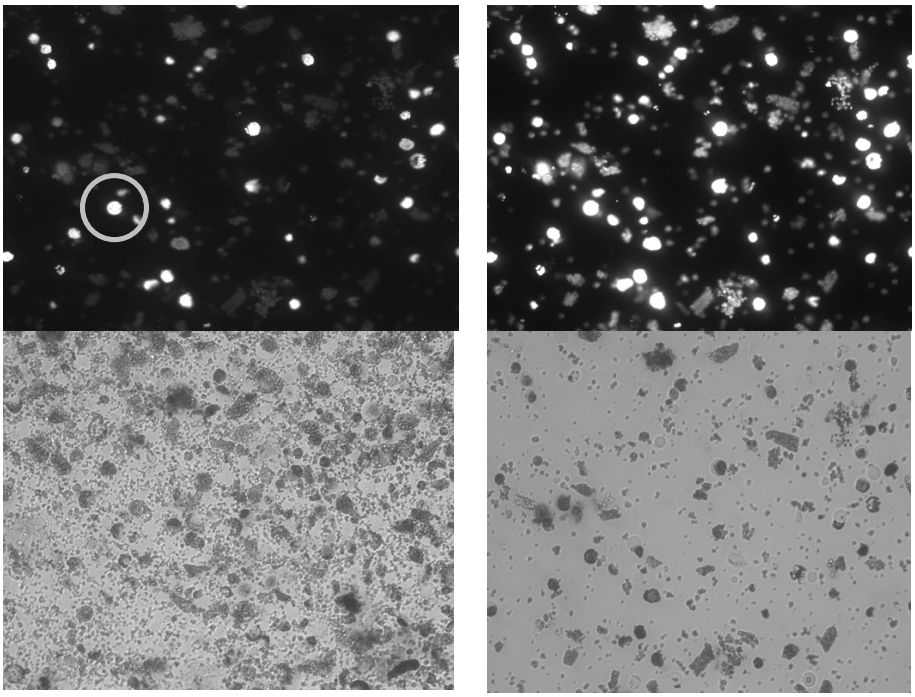

Figure 3.13: Protoplasts isolation. *Intact protoplasts showing plastids autofluorescence are highlighted. Leaves from tomato plants on soil, Gerimini and Heinz, were used to isolate protoplasts. There are some intact protoplasts, and many broken. Some of the intact protoplasts have big, rounded shape. In the background there are a lot of bacteria*

3.2.4.4 *Isolated Genomic DNA*

Isolation of genomic DNA from protoplasts as well as a tomato leaf from Heinz as a control was performed after isolation of protoplasts. Isolated genomic DNA samples were then allowed to run on gel electrophoresis one % agarose. Figure 3.14 shows isolated DNA samples from both protoplasts and control. Control sample shows a band which is approximately 8000 bp. There are no bands shown for protoplasts samples. Bands show the whole genome for the DNA. One kb hyperladder from Bioline was used (Figure 6 in Appendices).

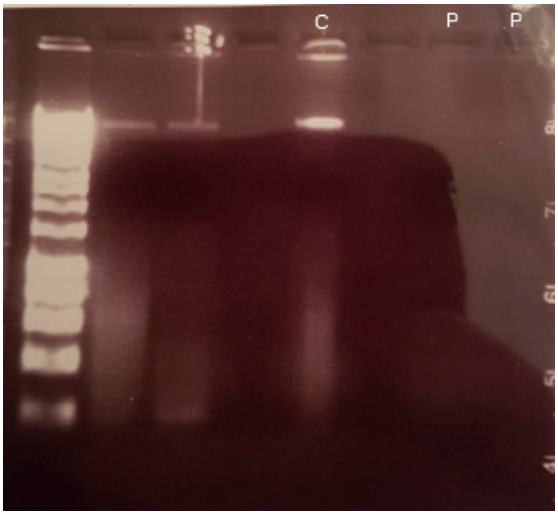

Figure 3.14: Isolated Genomic DNA from control leaves and protoplasts. *Samples marked C= control and P= protoplasts, samples in other wells are from a colleage. Control sample shows a band which is approximatly 8000 bp. There are no bands shown for protoplasts samples. One kb hyperladder from Bioline was used (figure 6 in Appendices)*

3.2.4.5 *Gel Electrophoresis after PCR*

PCR was performed on isolated DNA samples. After PCR, PCR products were allowed to run on gel electrophoresis two % agarose. 100 bp ladder was used. Figure 3.15 shows PCR products from P1, P2, C1 and C2. P1 are protoplasts with CRISPR 59 and CRISPR 60, while P2 are protoplasts with CRISPR 61 and CRISPR 62. Both C1 and C2 are controls from Heinz leaves. C1 had primers CRISPR 59 and CRISPR 60, and C2 had CRISPR 61 and CRISPR 62. Sequences for primers are given in table 2.18 in the material and methods section.

Bands are bigger for the control probably because there were more protoplasts present there. Samples with same primers have cut same places. P1 and C1 have bands on approximately 400 bp, 200-250 bp and 100 bp, and P2 and C2 have around five bands where the highest is approximately 200 bp and the lowest is approximately 100 bp. Band on approximately 400 bp for both P1 and C1 and band on approximately 200 bp are the bands where the gRNA binds in the gene (*PDS*). Figure 3 in Appendices shows *PDS* gene sequence and where in the sequence gRNAs will bind. The experiment seems to have been successful and these two bands were used for further gel extraction.

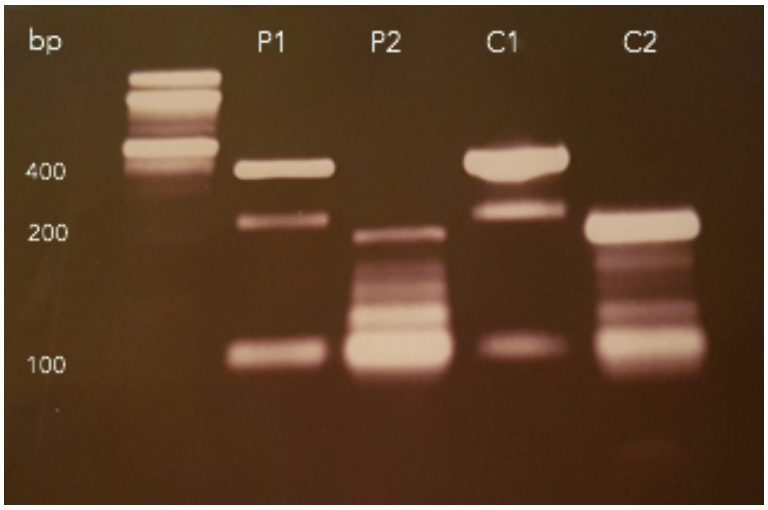

Figure 3.15: PCR products from isolated DNA. *P1 are protoplasts with CRISPR 59 and CRISPR 60, while P2 are protoplasts with CRISPR 61 and CRISPR 62. Both C1 and C2 are controls from Heinz leaves. C1 had primers CRISPR 59 and CRISPR 60, and C2 had CRISPR 61 and CRISPR 62. Sequences for primers are given in table 2.18 in the material and methods section. Samples with same primers have cut same places. 100 bp ladder was used (figure 5 in Appendices).*

P1 and C1 have bands on approximately 400 bp, 200-250 bp and 100 bp, and P2 and C2 have around five bands where the highest is approximately 200 bp and the lowest is approximately 100 bp. Band on approximately 400 bp for both P1 and C1 and band on approximately 200 bp are the bands where the gRNA binds in the gene (PDS). PDS gene sequence and where gRNAs will bind are shown in figure 3 in Appendices. The experiment seems to have been successfull and these two bands were used for furter gel extraction

3.2.5 gRNA and Cas9 Gel

To check quality on gRNA and Cas9 mRNA transcripts (section 2.4.15 and 2.4.16 in the material and methods) these were allowed to run on two different gels. For gRNA samples, denaturing PAGE/Urea gel was used, and for Cas9 samples, formaldehyde denaturing agarose gel was used. Before loading samples in wells for the denaturing PAGE/Urea gel, all the wells were rinsed several times with running buffer to remove urea. When samples were loaded on the gRNA gel, the content would not sink down into the wells. The wells were rinsed several times in between and loading again without any result. This experiment was therefore ended. Cas9 mRNA samples were allowed to run on the other gel. From figure 3.16 it is shown that the Cas9 mRNA is intact. One kb hyperladder from Bioline was used (figure 6 in Appendices).

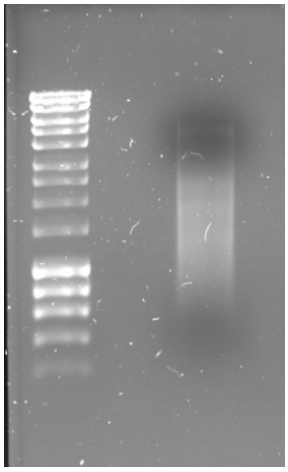

Figure 3.16: Cas9 mRNA sample were allowed to run on formaldehyde denaturing one % agarose gel. *One kb hyperladder from bioline was used (figure 6 in Appendices)*

3.2.6 *In vitro* Digestion of DNA with Cas9 Nuclease

In vitro digestion of DNA with Cas9 nuclease was performed. Cas9 nuclease was used to digest double-stranded DNA from protoplasts *in vitro* with Cas9 and gRNA. After the reaction was set up and incubated, fragment analysis was performed with gel electrophoresis two % agarose. Four samples were allowed to run on the gel as shown in figure 3.17.

3.2.6.1 *Fragment Analyzis*

P1/A2 contained protoplasts with primers CRISPR 59 and CRISPR 60 while A2 was phosphorylated and had primers CRISPR 3 and CRISPR 4. P1/B1 contained protoplasts with primers CRISPR 59 and CRISPR 60 while B1 was non-phosphorylated and had primers CRISPR 1 and CRISPR 2. P2/A2 contained protoplasts with primers CRISPR 61 and CRISPR 62 while A2 was phosphorylated and had primers CRISPR 3 and CRISPR 4. P2/B1 contained protoplasts with primers CRISPR 61 and CRISPR 62 while B1 was non-phosphorylated and had primers CRISPR 1 and CRISPR 2. Only band from P1/B1 and P2/A2 is relevant because it is only on these combinations it will contain gRNA 1 and gRNA 2.

P1/B1 have bands that seems to be 100 bp, 200-250 bp and one that is 396 bp. P2/A2 seems to have bands that are 100 bp and 198 bp. P1 will bind to gRNA 1 (with CRISPR 1 and

CRISPR 2), and P2 will bind to gRNA 2 (CRISPR 3 and CRISPR 4). gRNA 1 has PCR amplicon size 396 bp, and 198 bp for gRNA 2 as seen in table 2.18 in the material and methods section. These two bands from each gRNA were used for further experiments. Gene ruler 50 bp (Thermo Fisher Scientific) ladder was used (figure 7 in Appendices).

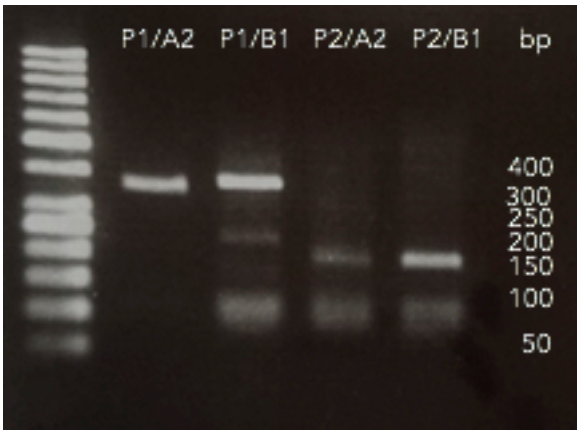

Figure 3.17: In vitro digestion of DNA with Cas9 nuclease. *Bands from P1/B1 and P2/A2 is relevant because it is only on these combinations there will be a binding. P1/B1 has bands that seems to be 100 bp, 200-250 bp and one that is 396 bp. P2/A2 seems to have band that is 100 bp and 198 bp. P1 will bind to gRNA 1 (with CRISPR 1 and CRISPR 2), and P2 will bind to gRNA 2 (CRISPR 3 and CRISPR 4). gRNA 1 has PCR amplicon size 396 bp, and 198 bp for gRNA 2 as seen in table 2.18 in the material and methods section. These two bands from each gRNA were used for further experiments. Gene ruler 50 bp (Thermo Fisher Scientific) ladder was used (figure 7 in Appendices).*

P1/A2 contains protoplasts with primers CRISPR 59 and CRISPR 60 while A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4. P1/B1 contains protoplasts with primers CRISPR 59 and CRISPR 60 while B1 is non-phosphorylated and has primers CRISPR 1 and CRISPR 2. P2/A2 contains protoplasts with primers CRISPR 61 and CRISPR 62 while A2 is phosphorylated and has primers CRISPR 3 and CRISPR 4. P2/B1 contains protoplasts with primers CRISPR 61 and CRISPR 62 while B1 is non-phosphorylated and has primers CRISPR 1 and CRISPR 2

3.2.6.2 *Comparing*

Samples from P1, P2, B1 and A2 were allowed to run on gel electrophoresis two % agarose. This was performed to check and compare results with the previous experiment. It was assumed that gRNA samples would not be seen or that it would be folded so that it would not go straight in a DNA gel with no treatment before. Figure 3.18 shows the results for this

experiment. Samples for gRNAs (B1 and A2) were seen, as well as bands for protoplasts. P1 shows band on size 396 bp and P2 shows band on 198 bp. gRNA samples shows band for 106 bp. The sizes of the bands were correct and the experiment seems to have been successful even with gRNA samples. Gene ruler 50 bp (Thermo Fisher Scientific) ladder was used (figure 7 in Appendices).

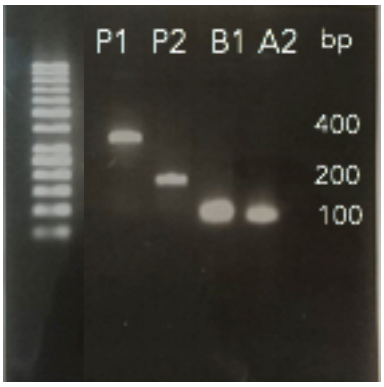

Figure 3.18: P1, P2, B1 and B2 after gel electrophoresis two % agarose. *P1 shows band on size 396 bp and P2 shows band on 198 bp. gRNA samples shows band for 106 bp. The sizes for the bands were correct and the experiment seems to have been successfull. Gene ruler 50 bp (Thermo Fisher Scientific) ladder was used (shown in figure 7 in Appendices)*

3.2.6.3 *Reaction with RNase*

To compare results from the previous gel electrophoresis shown in figure 3.17 and 3.18, gel electrophoresis was allowed to run again with the following samples listed in table 3.1. Reaction was set up the same way as the previous experiment with RNase (from 10 mg/ml).

Table 3.1: Samples C1/B1 and C2/A2 were set up according to the table

	C1/B1			**C2/A2**		
	1	*2*	*3*	*4*	*5*	*6*
DNA	+	+	+	+	+	+
Cas9	-	+	+	-	+	+
gRNA	-	-	+	-	-	+

Controls with leaves were used instead of protoplasts in this experiment since there was nothing left of the protoplasts. Controls had same vectors as the protoplasts. Three µl RNase was used to make sure it was cleansed. Fragment analysis was performed with gel electrophoresis (two % agarose) shown in figure 3.19. One kb plus DNA ladder from Thermo Fisher Scientific was used (figure 8 in Appendices).

All three samples from C1/B1 have bands on approximately 400 bp. Band from sample one with DNA has high intensity. Only sample four from C2/A2 with DNA gives a visible band on approximately 200 bp. Bands with very low intensity could be seen when gel was in the UV-illuminator as well for the other two samples (five and six). Both these bands seem to be the right sizes for where the gRNAs binds in *PDS* gene (396 bp and 198 bp). It seems to have been something wrong with the gel since samples with different content should have given more bands.

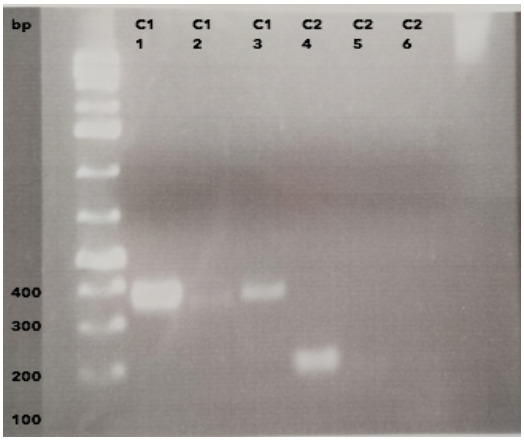

Figure 3.19: Gel electrophoresis with RNase.
Samples from C1/B1 gives band on approximately 400 bp. Band from sample one with DNA has high intensity. Only sample four from C2/A2 with DNA gives a visible band on approximately 200 bp. There could be seen band with very low intensity when gel was in the UV-illumuniator as well for the other two samples (five and six). Both these bands seems to be the right sizes for where the gRNAs binds in PDS gene (396 bp and 198 bp). One kb plus DNA ladder from Thermo Fisher Scientific was used (figure 8 in Appendices).

Samples named C1 contains also B1. Samples named C2 contains also A2. C1 1 and C2 1 contains only DNA, C1 2 and C2 2 contains DNA and Cas9 while C1 3 and C2 3 contains DNA, Cas9 and gRNA

3.3 Isolation of Protoplasts and Transfection

3.3.1 Protoplasts Isolation

The last time protoplast isolation was performed TM-1 Heinz plants, propagated Germini plants and Heinz plants from soil were used. To prepare for protoplast isolation plants were placed in dark for four days. Leaves were then cut and placed in enzyme solution used for Arabidopsis among others, without any sterilization of leaves. Leaves from TM-1 Heinz plants had been in TM-1 media for two to three weeks. Propagated Germini plants were several months old, and Heinz plants on soil had been transferred to soil one month prior to the protoplast isolation. After enzyme treatment leaves from soil plants had a lot of greenish and purple color. The purple color indicated that there are a lot of anthocyanins present, a class of pigments that are responsible for purple and blue color in many fruits.

Figure 3.20 shows filtrated protoplasts from Heinz leaves on soil and from propagated Germini leaves and Heinz leaves from TM-1. Protoplasts from Heinz leaves on soil to the left on the figure have a lot of protoplasts with dark greenish color compared to leaves from propagated Germini and Heinz leaves from TM-1 where it does not seem to be as many protoplasts. When filtrating the protoplasts there was used less centrifugation speed then recommended in the protocols that were followed.

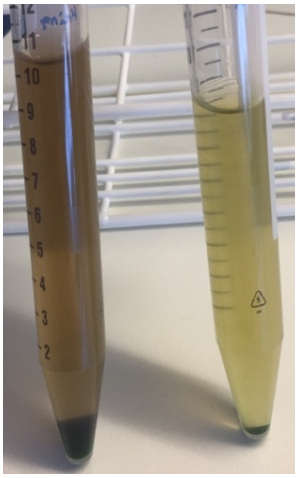

Figure 3.20: Filtering protoplasts. *To the left protoplasts from Heinz on soil. To the right protoplasts from Heinz leaves from TM-1 and propagated Germini*

After filtration and washing the protoplasts, both samples were taken to the microscope for calculation. In the sample with leaves from both propagated Germini and Heinz protoplasts from TM-1 there was just a few protoplasts. Many of the protoplasts were intact but because of the low number it was decided not to proceed with these for further transfection. Samples with protoplasts from Heinz leaves on soil showed a lot of protoplasts whereas approximately 90 % of these were intact. There was also a lot of anthocyanin present as shown in figure 3.21.

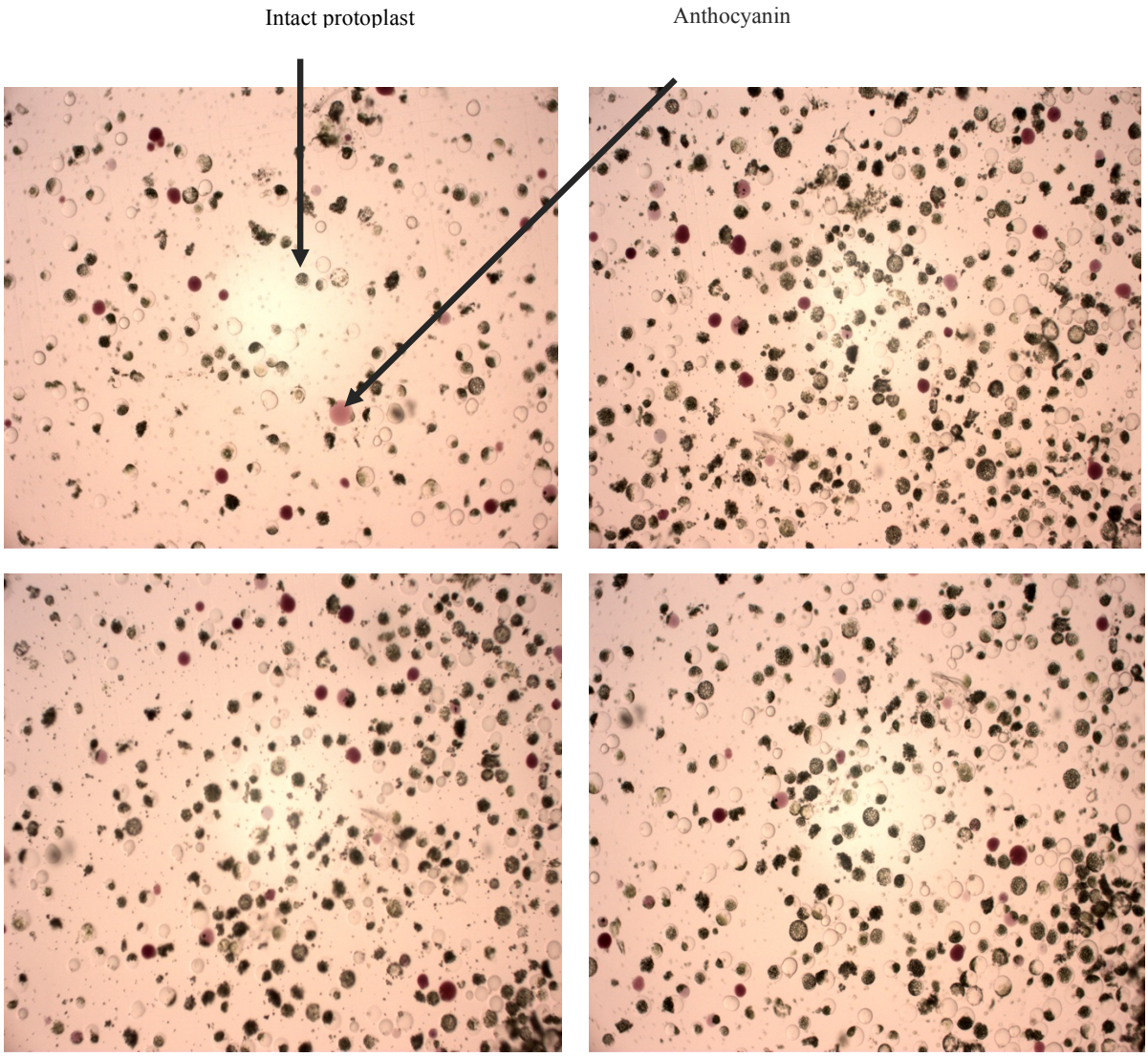

Figure 3.21: Heinz tomato protoplasts from leaves on soil. *Intact protoplasts are highlighted in figure. The purple color indicated that there are a lot of anthocyanin present.*

The next step after calculation of protoplasts was transfection. There were around 50 protoplasts in each chamber (0.1 µl), and 1.5 000 000 protoplasts per 1.5 ml MaMg solution. It was difficult to get all the protoplasts up from the Petri dishes. Some were sticking to the plate when we performed the transfection. Due to this reason, some of the protoplasts might have been lost as the transfection took place.

3.3.2 T7 Endonuclease I Assay

After the successfully isolation of tomato protoplasts, transfection with Cas9 mRNA and gRNAs transcripts were performed on the protoplasts. DNA was then isolated from the protoplasts, before PCR program was set up and samples were allowed to run on gel electrophoresis for gel extraction. DNA extraction was also performed before T7 endonuclease I assay was set up as the last experiment. T7 assay should confirm if the transfection had been successful. Figure 3.22 shows bands after gel electrophoresis two % agarose from sample A2 and B1 with transfected DNA from protoplasts.

Band for sample A2 has low intensity and seems to be around 200 bp, while sample B1 has high intensity and seems to be around 400 bp. This is the same size for both of the gRNAs binding in *PDS* gene (396 bp and 198 bp). There should have been more bands which indicated that it has not cut as it should. 50 bp ladder from New England Biolabs was used (figure 9 in Appendices).

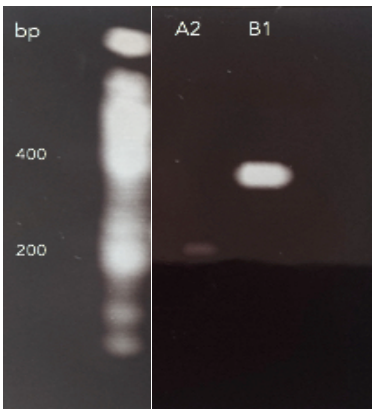

Figure 3.22: Gel electrophoresis of sample A2 and B1 with transfected DNA from protoplasts after T7 endonuclease I assay.
50 bp DNA ladder from New England Biolabs was used (figure 9 in Appendices)

4 Discussion

The CRISPR/Cas9 system has offered scientists all over the world the possibility to do precise gene editing at almost any laboratory in an easy and low-cost way. CRISPR/Cas9 system can be applied to cells in any species and can most likely be used in agriculture, medicine and for future human therapy. Successful DNA-free editing with CRISPR/Cas9 system in important crops as tomato, can open up great advantages in the agriculture field and industry, where GMOs in many countries are forbidden due to different leftover foreign DNAs (Khatodia et al. 2016).

If the CRISPR/Cas9 technique can gain social acceptance and still follow the strict regulations set by global and local authorities, it can further develop crops in agriculture which are more adapted to factors as climate changes, stress and viruses. Other techniques used to gene editing, as random mutagenesis approaches, were not able to control every gene in the process because of the random nature of the gene integration. With the precise gene targeting the CRISPR/Cas9 technique uses, it is possible to change genes in crops without introducing a transgene (Khatodia et al. 2016).

Even though the CRISPR/Cas9 system seems to be promising in many areas, and might even be omitted from the GMO legislation, there are some factors that need to be outlined. For gaining social acceptance for DNA-free genome editing the society needs to understand properly how the system works. It will also be important that the final products from this technique will not be considered GMOs. Uncertainty of potential side effects with the modified crops could be a hurdle for this. The potential this system has for the World's food supply, will largely depend on the societies perception of the crops that have been modified with CRISPR/Cas9.

In this study, the main objectives were to set up a CRISPR/Cas9 system for tomato plants by the use of tissue culture for both propagation and re-generation of plants from isolated tomato protoplasts. Cloning of gRNAs that could target different parts of the marker gene was performed as well as *in vitro* transcriptions for cloned gRNA and Cas9. In addition, *in vivo* CRISPR/Cas9 genetic modifications were also performed through PEG transfections of isolated protoplasts with gRNA and Cas9 mRNA transcripts against the marker gene *PDS*.

CRISPR/Cas9 technique has been tried out in tomato plants with both *Agrobacterium tumefaciens*-mediated transformation (Brooks et al. 2014; Filler Hayut et al. 2017; Cermak et al. 2015) and viral based delivery (Cermak et al. 2015). When performing a CRISPR/Cas9 experiment to target plants, it is necessary to generate a nuclease-gRNA complex. To accomplish this, it is important to prepare all components for the experiment correctly. Cas9 will only cut if the gRNA spacer matches the target DNA. When gRNAs are designed correctly, the CRISPR/Cas9 system is very specific. gRNAs in this study were chosen by using correct prediction sites. Sub cloning with restriction-ligation was performed. The isolated plasmid (puc57) with gRNA insert was sent for sequencing with M13R primer. The sequencing for all constructs proved to be successful, which indicated that the transformation had worked, and that the gRNAs, chosen in this study, seems to have been accurate and correct.

To perform the CRISPR/Cas9 experiment, both Cas9 mRNA transcripts and Cas9 protein were used to form a complex with gRNA. Using Cas9 protein is the fastest way to perform a CRISPR/Cas9 experiment for editing genes as neither transcription nor translation is necessary, and it is also the most common one. There have been several studies that has used Cas9 protein with sgRNA for making a nuclease-gRNA complex, as in for example grapevine, apple, tobacco, lettuce, rice, *A. thaliana* and *Petunia hybrida*. Some of these also used isolated protoplast for successful delivery of Cas9 and gRNA (Malnoy et al. 2016; Woo et al. 2015; Subburaj et al. 2016). To the author's knowledge this has not yet been performed in tomato plants.

Buying Cas9 protein is expensive and might be a limitation for some laboratories. On the contrary, making a Cas9 protein is time consuming and can have some difficulties to perform in the laboratory. *In vitro* digestion with Cas9 nuclease was performed during this study. Cas9 nuclease was used to digest double-stranded DNA (PCR product that was amplified from isolated genomic DNA from protoplasts) *in vitro* with Cas9 and gRNA. The results from these experiments showed that bands were approximately 396 bp and 198 bp, the same size as the PCR amplicon size for gRNA 1 and gRNA 2 respectively. These results indicated that the *in vitro* digestion of DNA from the marker gene with Cas9 nuclease were successful, and proved the functionality of selected spacers and gRNAs.

Since the combination of Cas9 mRNA and gRNA had not been tried out in other studies in tomato plants, this was therefore investigated in this study. *In vitro* transcription of both Cas9 mRNA and gRNA were performed. As has been done in mammalian studies (Albadri et al. 2017; Hwang et al. 2013; Song et al. 2017), *in vivo* editing using the capped and tailed transcripts of Cas9 mRNA and gRNAs was attempted. Cas9 mRNA and gRNA complex was used for *in vivo* transfection in tomato protoplast. A recent study from 2017, Albadri et al. compared sgRNAs with mRNA versus protein Cas9 injection in zebrafish. Their study showed that the cutting efficiencies were largely improved with Cas9 protein and that the survival rate was enhanced, probably due to lower toxicity of protein compared to the Cas9 mRNA. However Cas9 mRNA yielded better results when it came to NHEJ-based insertion than Cas9 protein (Albadri et al. 2017). Since Cas9 mRNA have not previously been tried out in tomato plants to the author's knowledge, it is difficult to say if these results would relate to tomato plant cells. Though, the results might indicate that there likely will be a difference in the end results when comparing Cas9 protein and Cas9 mRNA.

Another study published by Zhang et al. (2016) expressed CRISPR/Cas9 in wheat plant cells through Cas9 DNA or RNA with sgRNA. Their study showed that Cas9 mRNA based genome editing had lower frequency than using Cas9 DNA. A reason for this could be that RNA is less stable than DNA and could therefore be easily degraded. Their findings showed that it was easier to use Cas9 DNA since there was no need for *in vitro* transcription, and there was a higher mutagenesis frequency. On the other hand, the use of Cas9 mRNA resulted in no transgene compared to Cas9 DNA that were not transgene free (Zhang et al. 2016). The reason for this is probably that RNA molecules under normal conditions are unlikely to become integrated into the nuclear DNA in plant cells.

Therefore, it was suggested that the Cas9 DNA method was used to develop and optimize a genome-editing protocol with callus cells for a given plant species, and that the mutant obtained from the experiments would be used only for basic research. When a protocol had been established and gene function had been validated, the use of Cas9 mRNA might be better to use for producing transgene-free mutants for further production development (Zhang et al. 2016). This might also be applied for tomato plants, where it could be easier to establish a protocol for genome-editing with CRISPR/Cas9 first with Cas9 protein and gRNAs through

transfection of isolated protoplasts, and then compare results with Cas9 mRNA and gRNAs after this has been performed.

For delivery of Cas9 mRNA and gRNA *in vivo* in this study, isolated protoplasts from tomato plants were used. Big parts of the experiment were based on tomato plants growing on tissue culture, propagation of tomato plants through this system and isolating protoplasts from tissue culture plants for re-generating tomato plants. Much of these experiments were based on the famous article "Totipotency of tomato protoplasts" (Shahin 1985). A figure over the experiments that were planned from this article is given in figure 2 in Appendices (see table 1 and 2 in Appendices for buffer and solutions). These steps were followed as described in the protocol for isolation of tomato protoplasts several times, but did not result in surviving protoplasts.

Isolation of tomato protoplasts from tissue culture was challenging. After several unsuccessful attempts to isolate protoplasts, different approaches were tried out. Several parameters as weeks of plantlets, dark treatment, different solutions, centrifugation speed, and plants on tissue culture versus plants on soil were tried out for optimization of isolation of protoplasts. Based on previous studies that have successfully isolated tomato protoplasts, some of these parameters were compared to the original protocol (Ray et al. 2015; Horváth 2009). Findings presented in this study show that using one month old plantlets from soil with dark treatment for four days that were cut directly in enzyme solution normally used for Arabidopsis and other plants, W5 washing solution and lower centrifugation speed, resulted in many intact isolated protoplasts.

The tissue culture system that were used based on (Shahin 1985) did not work as assumed leading to much time consumption. TM-1 media which was the media that the plants were transferred to from ½ MS media contained a lot of different macro,- and micronutrients, vitamins, hormones and enzymes where some of these might have affected the tomato leaves. The enzyme solution also contained a lot of other chemicals than the one used for Arabidopsis and other plants that can have effect on the tomato leaves. Using tomato plants from soil seems to be a better alternative for isolation of intact tomato protoplasts, as well as using solutions with less chemicals as those used for Arabidopsis and other plants.

Big parts of the study were used on optimization of the protocol for isolation of tomato protoplasts. Because of time limitation, *in vivo* CRISPR/Cas9 genetic modifications through the PEG transfections of isolated protoplasts with gRNA and Cas9 transcripts against marker gene *PDS,* were only performed once. The end results for this experiment with T7 endonuclease assay were also negative. If these experiments could be performed several times, optimization of the amount of both Cas9 mRNA (or protein) and gRNA under the transformation could be tried out. This could have resulted in better results with T7 endonuclease assay.

By trying the experiment several times, it could be possible to compare results between Cas9 mRNA and Cas9 protein, as well as optimization of amounts used for both Cas9 and gRNA. Since Cas9 protein and mRNA have not been used in tomato plants previously there might be unknown limitation to its use. With better time, transgenic plants could also be re-generated so that the phenotype for plants with successful genetic transformation would be visible with white color (Pan et al. 2016).

5 Future Research

Successful *in vitro* work with all the components for the CRISPR/Cas9 experiment constructed, would allow future work resulting in transgenic plants. An optimized delivery method for Cas9 and gRNA, as protoplast isolation for tomato plants, will be time saving for future work in this important crop. Results presented in this study showed that using one month old plantlets from soil with dark treatment for four days that were cut directly in enzyme solution normally used for Arabidopsis and other plants, W5 washing solution and lower centrifugation speed resulted in higher percentage of intact protoplasts.

The next step for editing specific genes tomato plants, will be to perform *in vivo* CRISPR/Cas9 modifications through isolated and transfected protoplasts before re-generating tomato plants. Using Cas9 protein (and/or Cas9 transcripts) and transcribed gRNA against the marker gene *PDS* will result in white phenotype if the modification has been successful as figure 1.7 shows in the introduction section (Pan et al. 2016). Further investigations for both nuclease-gRNA complex as well as Cas9 mRNA/gRNA should be investigated. Optimization of a protocol for tomato plants could be tried out first with Cas9 protein with gRNAs through transfection of isolated protoplasts, before comparing results with Cas9 mRNA with gRNA.

In the future, the CRISPR/Cas9 system will most likely have a great impact on a lot of different areas, not only in the research field. In some years, it might be normal to eat meals where crops have been genetically modified by CRISPR/Cas9 approach. It will most likely also have a great impact in other fields besides agriculture, as medicine and human therapy. The revolutionary effect that the CRISPR/Cas9 system yields, will depend on the social acceptance for its use, as well as the regulations set by the authorities. If crops modified by CRISPR/Cas9 technique will be omitted from the GMO legislation this will have great impact in the World's food supply.

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Appendices

Making gRNAs and primers

Spacers for sgRNA 1 and 2 were found from (Pan et al. 2016), but could also be found in the web tool "CRISPR-PLANT" (Xie et al. 2014). A description for the steps is given below. A detailed description of how to use the web tool is given in the web page at the instruction section.

1. Enter web tool "CRISPR-PLANT".
2. Put in query. In this case: Solyc03g123760 in Chr3 from 64554061 to 64561664

Class0.0 gRNA

SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr3:64554870-64554890:c	NA	4	TAGTTGGGCGCGGAGAAGCA	CGGAACGTTG	-	exon

gRNA (Spacer was shown in upper-case):
5'-
TAGTTGGGCGCGGAGAAGCAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttttt-
3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TAGTTGGGCGCGGAGAAGCA-3'
5'-TGCTTCTCCGCGCCCAACTA-3'

GC content of Spacer sequence: 0.6

Potential Pol III terminator (TTTTT): null

2 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
AclI cut AACGTT
HpyCH4IV cut ACGT

Class 1.0 gRNA 1

Chr3:64556691-64556711	3	4	TAACGATCGATTGCAATGGA	AGGAACATTC	+	exon

gRNA (Spacer was shown in upper-case):
5'-
TAACGATCGATTGCAATGGAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TAACGATCGATTGCAATGGA-3'
5'-TCCATTGCAATCGATCGTTA-3'

GC content of Spacer sequence: 0.4

Potential Pol III terminator (TTTTT): null

3 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BsrDI cut GCAATG
Nb.BsrDI cut GCAATG
HpyCH4V cut TGCA

Class 1.0 gRNA 2

Chr3:64557090-64557110	4	4	GGACTCTTGCCAGCAATGCT	TGGAGGGCAA	+	exon

gRNA (Spacer was shown in upper-case):
5'-
GGACTCTTGCCAGCAATGCTgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GGACTCTTGCCAGCAATGCT-3'
5'-AGCATTGCTGGCAAGAGTCC-3'

GC content of Spacer sequence: 0.55

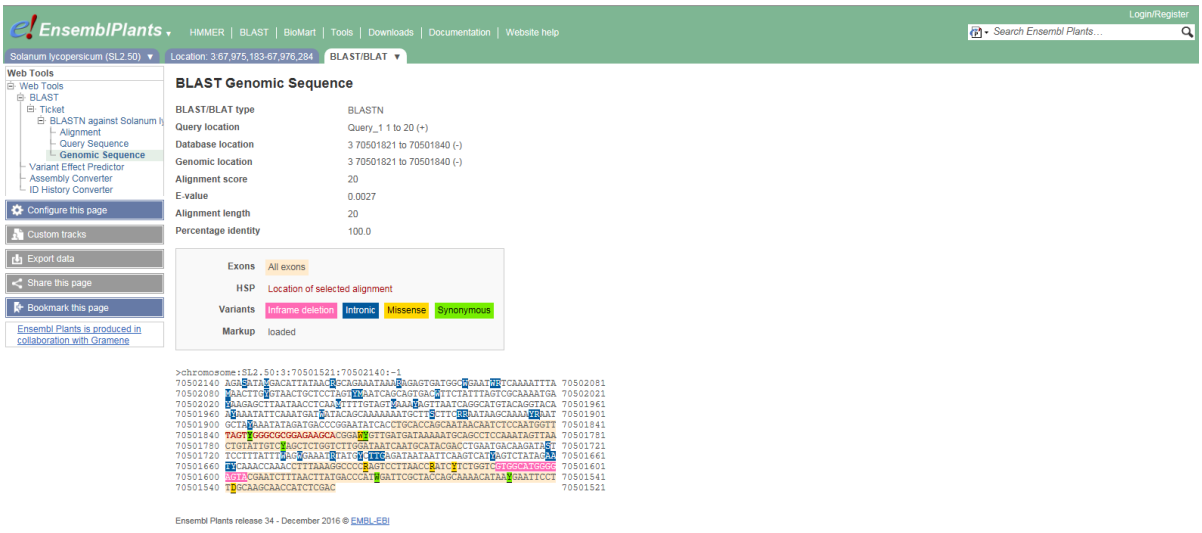
Potential Pol III terminator (TTTTT): null

2 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BsrDI cut GCAATG
Nb.BsrDI cut GCAATG

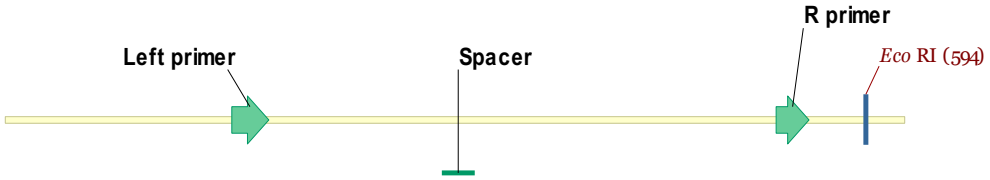
3. To find flanking primers for PCR amplification around the CRISPR target, sgRNA was aligned against tomato genome in Ensemble where the target place was found. This was used to designing primers.

BLAST Genomic Sequence

For the first spacer:




```
>chromosome:SL2.50:3:70501521:70502140:-1
70502140 AGASATAMGACATTATAACRGCAGAAATAAARAGAGTGATGGCWGAATWRTCAAAATTTA
70502081
70502080 MAACTTGYGTAACTGCTCCTAGTYMAATCAGCAGTGACWTTCTATTTAGTCGCAAAATGA
70502021
70502020 YAAGAGCTTAATAACCTCAAMTTTTGTAGTMAAAYAGTTAATCAGGCATGTACAGGTACA
70501961
70501960 AYAAATATTCAAATGATWATACAGCAAAAAAATGCTTSCTTCRRAATAAGCAAAAYRAAT
70501901
70501900 GCTAYAAATATAGATGACCCGGAATATCACCTGCACCAGCAATAACAATCTCCAATGGTT
70501841
70501840 TAGTYGGGCGCGGAGAAGCACGGAWYGTTGATGATAAAAATGCAGCCTCCAAATAGTTAA
70501781
70501780 CTGTATTGTCYAGCTCTGGTCTTGGATAATCAATGCATACGACCTGAATGACAAGATAST
70501721
70501720 TCCTTTATTTWAGWGAAATRTATGYCTTGAGATAATAATTCAAGTCATYAGTCTATAGAA
70501661
70501660 TYCAAACCAAACCTTTAAAGGCCCCRAGTCCTTAACCRATCYTCTGGTCGTGGCATGGGG
70501601
70501600 AGTACGAATCTTTAACTTATGACCCATWGATTCGCTACCAGCAAAACATAAYGAATTCCT
70501541
70501540 TDGCAAGCAACCATCTCGAC
70501521
```

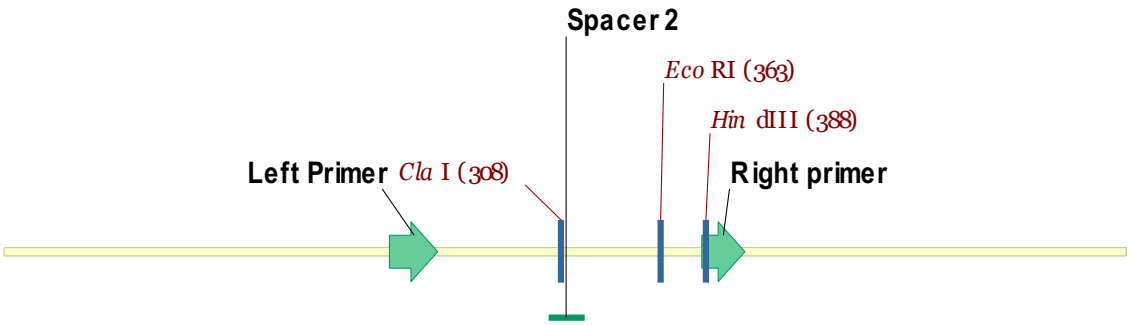


SLPDS-SPACER!

620 bp

For the first spacer:


```
>chromosome:SL2.50:3:70503342:70503961:1
70503342 TGGTTTRCATATATTCTGTAAGTTTGACCYCTCATTGTTRTATGTTTACGTTAATCTTCT
70503401
70503402 WTATWCTGTCATTGTATTTTTTTTTTTGATCTCTAGYCAATTAGACATCTCCTATCCTYG
70503461
70503462 TTTGTCRTTTATCGTTTATCTTTTACAAAAATRGCCTATYATTGTMAGTAAATCTGTATT
70503521
70503522 WTGTCTWGYTTCTYCTTTCTCATCTTATYATTCATATAGTGACTCATACAAATTGGTGCT
70503581
70503582 TGATCTMTTTTAAGTTGGGGCTTACCCAAATATTCAGAACCTRTTTGGAGAATTAGGGAT
70503641
70503642 TAACGATCGATTGCAATGGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGG
70503701
70503702 AGAATTCAGCCGCTTTGATTTCTCCGAAGCTTTACCCGCTCCTTTAAATGGTGAGCTAAT
70503761
70503762 CAYGAGTAAATTTCTCCCTCTTGTAGTYATKTTGTTAAACTTCYCTAATWARCTGTAAAG
70503821
70503822 TTGATTARAATTCTMAAAAAAAAAATCTGTAAAATTGAYAAGTCAATYACACCTATRGGAC
70503881
70503882 TTYACTAACCTTARAAGAGCATAAAAGTTCAYTACTTCYTCATTGGMCCTTTTGTGTGCA
70503941
70503942 GCTAAAATRTTAAATTCTTT
```



SLPDS1-Spacer 2

620 bp

The isolated plasmid (puc57) with gRNA insert was sent for sequencing with M13R primer Seqlab-Microsynth Sequencing. The sequencing for all constructs proved to be successful, which indicated that the transformation has worked. Sequences was aligned with MEGA7 (Kumar et al. 2016) and boxshade. Figure 1 shows successful sequencing in puc57-sgRNA-expression vector with gRNA from samples A1, B1 and B2. Sequences was aligned with MEGA7 (Kumar et al. 2016) and boxshade.

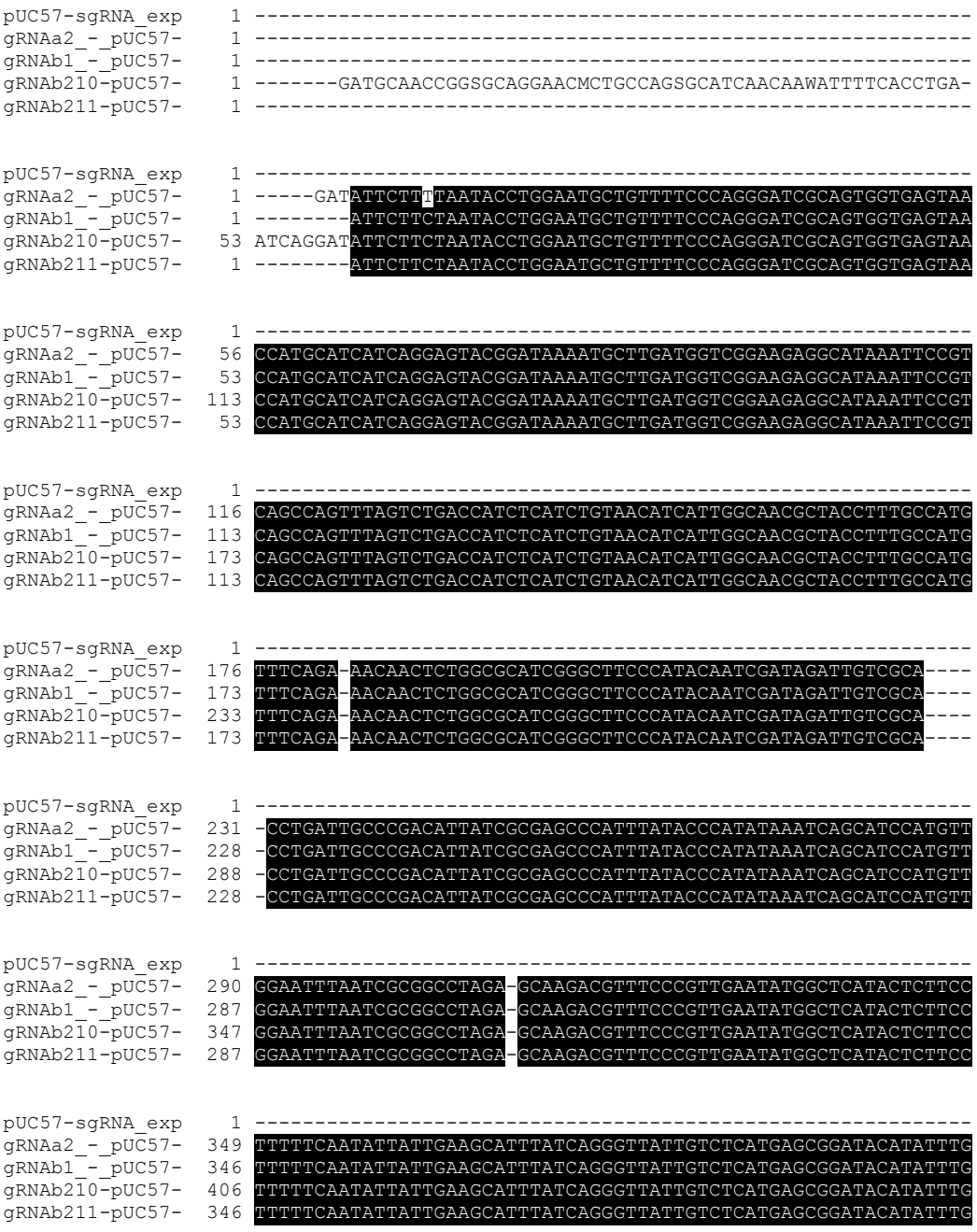


Figure 1: Successful sequencing in puc57-sgRNA-expression vector with gRNA from samples A2, B1 and B2. *The isolated plasmid (puc57) with gRNA insert was sent for sequencing with M13R primer. The sequencing for all constructs proved to be successful, which indicated that the transformation has worked. Sequences was aligned with MEGA7 (Kumar et al. 2016) and boxshade*

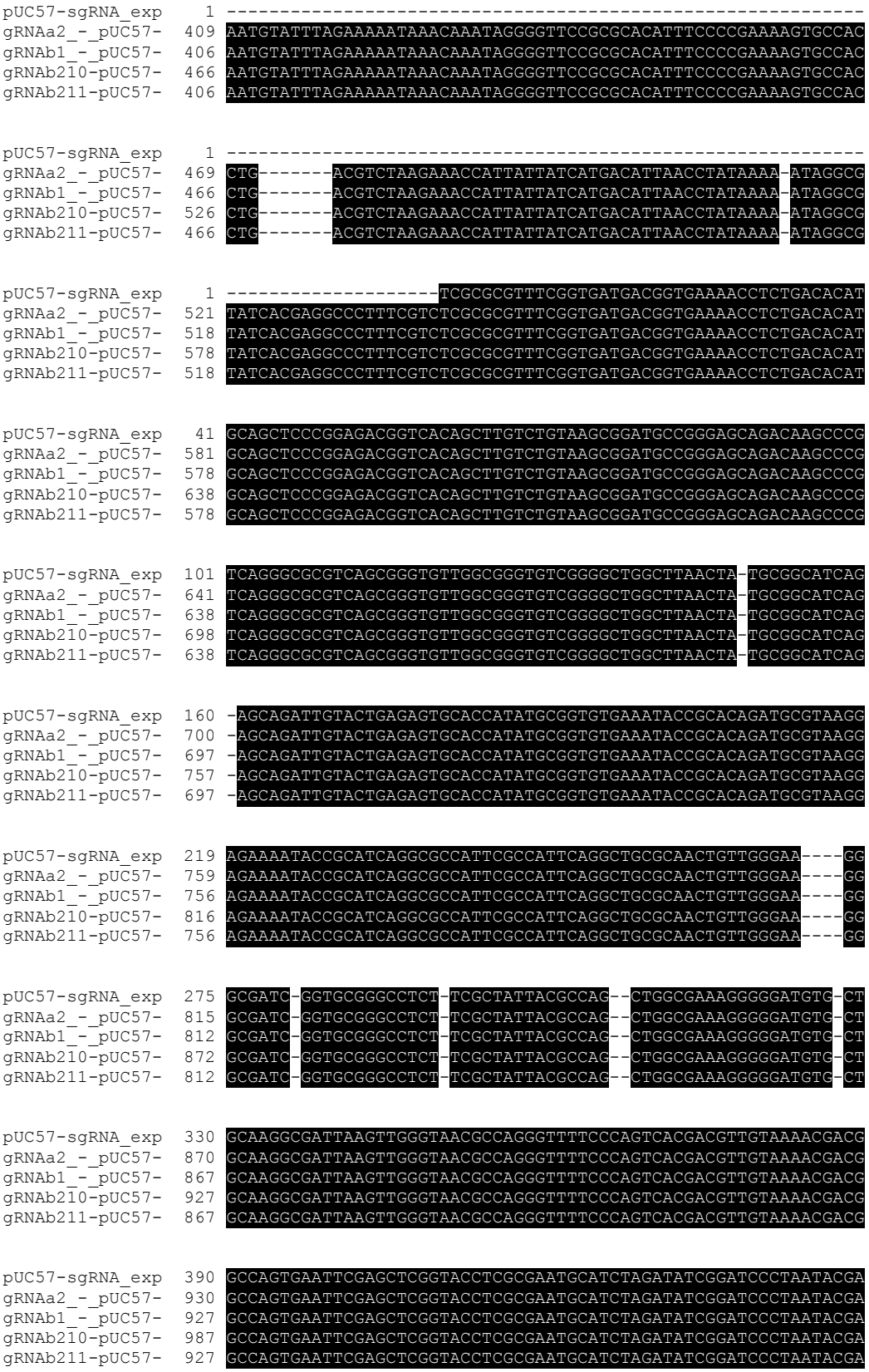


Figure 1 continues: Successful sequencing in puc57-sgRNA-expression vector with gRNA from samples A2, B1 and B2. *The isolated plasmid (puc57) with gRNA insert was sent for sequencing with M13R primer. The sequencing for all constructs proved to be successful, which indicated that the transformation has worked. Sequences was aligned with MEGA7 (Kumar et al. 2016) and boxshade*

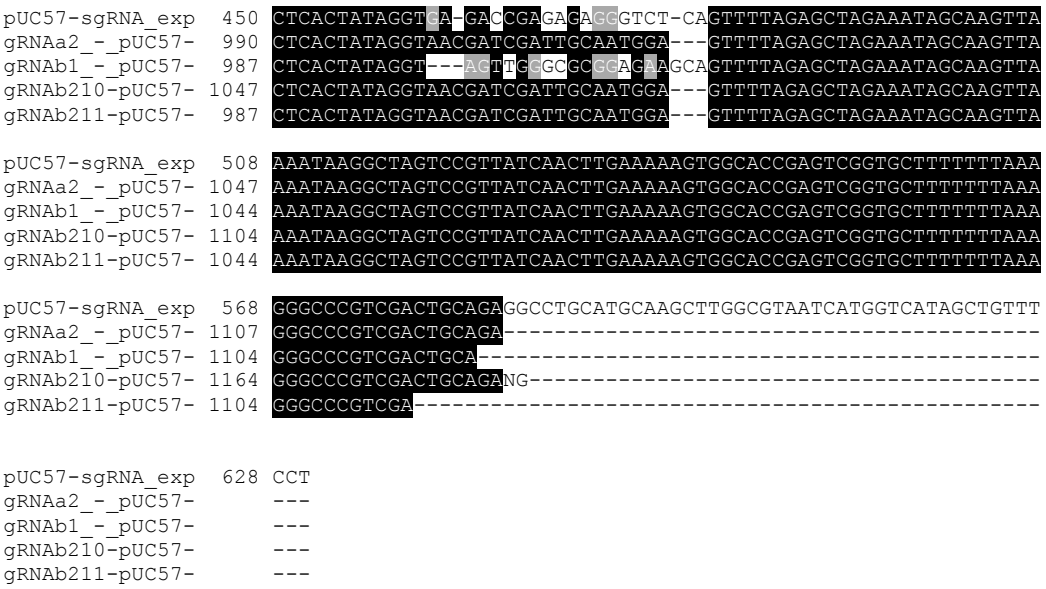


Figure 1 continues: Successful sequencing in puc57-sgRNA-expression vector with gRNA from samples A2, B1 and B2. *The isolated plasmid (puc57) with gRNA insert was sent for sequencing with M13R primer. The sequencing for all constructs proved to be successful, which indicated that the transformation has worked. Sequences was aligned with MEGA7 (Kumar et al. 2016) and boxshade*

Figure 2 shows puc57-sgRNA expression vector sequence. Red= restriction enzyme BsaI (and where it cuts). Yellow= M13 complement reverse primer. Spacer forward (CRISPR 1 or CRISPR 3) will attach where the restriction enzyme cuts (spacer forward has 20 + 4 nucleotides). If the spacer is replaced where the restriction enzymes cutting place (GGTCTCA) it will be 174 nucleotides from beginning to end (where M13 reverse is).

>pUC57-sgRNA expression vector

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcaca
gcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttgg
cgggtgtcggggctggcttaactatgcggcatcagagcagattgtactgagagtgcaccata
tgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgccattcgcca
ttcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagct
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aatattttcacctgaatcaggatattcttctaatacctggaatgctgttttcccagggatcg

Figure 2: pUC57-sgRNA expression vector sequence. *Red= restriction enzyme BsaI (and where it cuts) Yellow= M13 complement reverse primer. Spacer forward (CRISPR 1 or CRISPR 3) will attach where the restriction enzyme cuts (spacer forward has 20 + 4 nucleotides). If the spacer is replaced where the restriction enzymes cutting place (GGTCTCA) it will be 174 nucleotides from beginning to end (where M13 reverse is)*

cagtggtgagtaaccatgcatcatcaggagtacggataaaatgcttgatggtcggaagaggc
ataaattccgtcagccagtttagtctgaccatctcatctgtaacatcattggcaacgctacc
tttgccatgtttcagaaacaactctggcgcatcgggcttcccatacaatcgatagattgtcg
cacctgattgcccgacattatcgcgagcccatttatacccatataaatcagcatccatgttg
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tcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgta
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taagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggccctttcg
tc

Figure 2 continues: pUC57-sgRNA expression vector sequence. *Red= restriction enzyme BsaI (and where it cuts) Yellow= M13 complement reverse primer. Spacer forward (CRISPR 1 or CRISPR 3) will attach where the restriction enzyme cuts (spacer forward has 20 + 4 nucleotides). If the spacer is replaced where the restriction enzymes cutting place (GGTCTCA) it will be 174 nucleotides from beginning to end (where M13 reverse is)*

Figure 3a shows the whole sequence for *Solanum lycopersicum PDS* genome sequence on chromosome 3. Figure 3b shows copied gene 198 bp. Protoplast 2 (CRISPR 61 + CRISPR 62) binds to gRNA 2 (CRISPR 3 and CRISPR 4). Sample A2 (phosphorylated). Yellow=gRNA 2, red = PAM site. Figure 3 c shows copied gene 396 bp. Protoplast 1 (CRISPR 59 + CRISPR 60) binds to gRNA 2 (CRISPR 1 and CRISPR 2). Sample B1 (non-phosphorylated). Yellow=gRNA 1, red = PAM site.

>NC_015440.2:70500860-70508537 Solanum lycopersicum cultivar Heinz 1706 chromosome 3, SL2.50, whole genome shotgun sequence
ATTAAAGAAAGAAAAGGGTTTATCTCGCAAGTGTGGCTATGGTGGGACGTGTCAAATTTTGG
ATTGTAGCCAAACATGAGATTTGATTTAAAGGGAATTGGCCAAATCACCGAAAGCAGGCATC
TTCATCATAAATTAGTTTGTTTATTTATACAGAATTATACGCTTTTACTAGTTATAGCATTC
GGTATCTTTTTCTGGGTAACTGCCAAACCACCACAAATTTCAAGTTTCCATTTAACTCTTCA
ACTTCAACCCAACCAAATTTATTTGCTTAATTGTGCAGAACCACTCCCTATATCTTCTAGGT
GCTTTCATTCGTTCCGAGGTAAGAAAAGATTTTTGTTTCTTTGAATGCTTTATGCCACTCGT
TTAACTTCTGAGGTTTGTGGATCTTTTAGGCGACTTTTTTTTTTTTTTGTATGTAAAATTTGT
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AGTAAAATGCCTCAAATTGGACTTGTTTCTGCTGTTAACTTGAGAGTCCAAGGTAGTTCAGC
TTATCTTTGGAGCTCGAGGTCGTCTTCTTTGGGAACTGAAAGTCGAGATGGTTGCTTGCAAA
GGAATTCGTTATGTTTTGCTGGTAGCGAATCAATGGGTCATAAGTTAAAGATTCGTACTCCC
CATGCCACGACCAGAAGATTGGTTAAGGACTTGGGGCCTTTAAAGGTTTGGTTTGAATTCTA
TAGACTGATGACTTGAATTATTATCTCAAGGCATATATTTCTCTAAAATAAAGGAACTATCT
TGTCATTCAGGTCGTATGCATTGATTATCCAAGACCAGAGCTGGACAATACAGTTAACTATT
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CATTTGCATTTAGGTGCAAGTTGGTATTTGCATACTTAATAATGCATGTTTTAATTTTACTT
TTCTGCCTATTCTATGTCCTTTAATCAAGAATCGATTTGCACGCTATTTCTTCCTCGTATTT
TATGCATCTATATGCTTTCAAGTTGAAATGGCTAGAATGGATTATCTTGCTTATGGAGTCAC

Figure 3a: SI_PDS genome sequence

>NC_015440.2:70500860-70508537 Solanum lycopersicum cultivar Heinz 1706 chromosome 3, SL2.50, whole genome shotgun sequence
CAAGAATTCTAGTTGTATAGGCAAACAATTGATTTAGTATTAGTCTTTATGTGTTCTACTGT
TGAGAATTTATATCTCCCTTTAGGTAGCTGCATGGAAAGATGATGATGGAGATTGGTACGAG
ACTGGTTTGCATATATTCTGTAAGTTTGACCTCTCATTGTTATATGTTTACGTTAATCTTCT
ATATACTGTCATTGTATTTTTTTTTTTGATCTCTAGCCAATTAGACATCTCCTATCCTCGTT
TGTCGTTTATCGTTTATCTTTTACAAAAATAGCCTATTATTGTCAGTAAATCTGTATTTTGT
CTAGCTTCTCCTTTCTCATCTTATTATTCATATAGTGACTCATACAAATTGGTGCTTGATCT
CTTTTAAGTTGGGGCTTACCCAAATATTCAGAACCTGTTTGGAGAATTAGGGATTAACGATC
GATTGCAATGGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATTCAGC
CGCTTTGATTTCTCCGAAGCTTTACCCGCTCCTTTAAATGGTGAGCTAATCACGAGTAAA
TTTCTCCCTCTTGTAGTTATTTTGTTAAACTTCCCTAATAAGCTGTAAAGTTGATTAGAATT
CTAAAAAAAAAATCTGTAAAATTGATAAGTCAATCACACCTATGGGACTTTACTAACCTTAA
AAGAGCATAAAAGTTCATTACTTCTTCATTGGACCTTTTGTGTGCAGCTAAAATATTAAATT
CTTTGATATAATTTGCAGGAATTTTAGCCATCTTAAAGAATAACGAAATGCTTACATGGCCA
GAGAAAGTCAAATTTGCAATTGGACTCTTGCCAGCAATGCTTGGAGGGCAATCTTATGTTGA
AGCTCAAGATGGGATAAGTGTTAAGGACTGGATGAGAAAGCAAGTGCGTAATCAATTATGTT
GCTTTTTAAGTGCATAAACCCTGGCTATATAGTTACCAGTTCACACACAAATTGTTCATTTG
AGGTGTTAGATTGTCTACCTGCCTCTCTTTTGCTAAGAGCATACATCTAGCAAATCTCAGCA
GCAGCAAGTTGCTTTGCAGGGTGTGCCGGACAGGGTGACAGATGAGGTGTTCATTGCTATGT
CAAAGGCACTCAACTTTATAAACCCTGACGAACTTTCAATGCAGTGCATTTTGATCGCATTG
AACAGGTTTCTTCAGGTTAGAATCCTGATCCCACATTCAAAACAAAGAGAGAGAGAGAAGTA
CTTTTGTCCCTTCCAGACTCTCACATAACATGAAATCTCTAACTAAGGAGCTTAAATGTGAT
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TGTAAATTATACCCTGAATCTCATGGATGAAGGACTACTTTACTGATTCACTTTTAAACAGC
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TTGCTGATTTTTGATGAACCAATTAGTGGATATTTTCAAGCTTCTATTGCCTGAAGACTGGA
AAGAGATTCCATATTTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCTGTGATAAATGTACAT
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AATTTCTGTACATATTTTAATGAGAAGAACTACCATCGTTCTGGCCATTAGTGGCTAATATT
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GCATTAGCTGGTGAGATTCAGTTGAGGATAGACATGACATGTCACGGCAACCACTTCTCCGT
GGCATGAAAAATGGACTAGAGATCCAAGTCGTCCACATTTCATCAGCTATATGTTAATTATG

Figure 3a continues: SI_PDS genome sequence

>NC_015440.2:70500860-70508537 Solanum lycopersicum cultivar Heinz 1706 chromosome 3, SL2.50, whole genome shotgun sequence
TTCCCTTTAGTTTTTAAATGACACCGCTGGTGGAGACAAGATAAATAGAGCTCCTTTTTCAA
TTCTTAAAATATTGTGTTGTTTATGATACTTCTATTGGTTCCTTTTCTTAATTTCTCTTTCA
TATTCATGCAGAAGCTCACTGCTCAGTGTGTATGCTGACATGTCTGTTACATGTAAGGTATT
CATATGTAACCATTTATATTGCTCTCAAATTATAATCTGTGGACTTGTGTATAATTTGCTGT
GAATTGCATAATGCTTGTTGGTAAGTGTATGAATTCCCGCTGTTGTAATCTTCATTTGGGAT
ATACAATGATATTGGTTTTGTATATATGGAATAGCCAGGGCTATGATCCTGTCTGTGCACGG
GCCCGGAGCCACCACATGAGGCAAGTTACACAGGACAAGTAAAGAAATTTTGACGAAACTTA
GCCGGAGATTTTTTTTTTCCATTTTAGCTAATTTTCTAAGAATTTAATGGGATAAAATTCTT
TTATTAGCTATCTAGGTTCTTGCTGCCTTGGGGGTGGAGGGGTAAGAAGAGAACTTAATTCA
GTGTTACCTGCAGTTTTGGAAAGAGTCTACAGATCTACTTCTCAGTTTTATTGGTTGTGGAA
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ATGTCGAACATGCCTATACTAATTCTGCCTAGCGTAATAGTTTCTCCAACCAAATTTAATAG
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GCTTCTGTACACTAAATTTATGATGCAAGAAGCGTTGTACACAACATATAGAAGAAGAGTGC
GAGGTGAAGCAAGTAGGAGAAATGTTAGGAAAGCTCCTATACAAAAGGATGGCATGTTGAAG
ATTAGCATCTTTTTAATCCCAAGTTTAAATATAAAGCATATTTTATGTACCACTTTCTTTAT
CTGGGGTTTGTAATCCCTTTATATCTTTATGCAATCTTTACGTTAGTTAATATC

Figure 3a continues: SI_PDS genome sequence

Copied gene 198 bp: Protoplast 2 (CRISPR 61 + CRISPR 62): binds to gRNA 2 (CRISPR 3 and CRISPR 4). Sample A2. Phosphorylated.

CATATAGTGACTCATACAAATTGGTGCTTGATCTCTTTTAAGTTGGGGCTTACCCAAATATT
CAGAACCTGTTTGGAGAATTAGGGATTAACGATCGATTGCAAT (107 bp)

GGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATTCAGCCGCTTTGAT
TTCTCCGAAGCTTTACCCGCTCCTTTAAATG (91 bp)

Yellow = gRNA 2
Red = PAM site

TAACGATCGATTGCAATGGAAGG

Figure 3b: Copied gene 198 bp. *Protoplast 2 (CRISPR 61 + CRISPR 62) binds to gRNA 2 (CRISPR 3 and CRISPR 4). Sample A2 (phosphorylated). Yellow=gRNA 2, red = PAM site*

Copied gene 396 bp. Protoplast 1 (CRISPR 59 + CRISPR 60) binds to gRNA 1 (CRIPSR 1 og CRISPR 2). Sample B1. Non-phosphorylated.

TAAAGATTCGTACTCCCCATGCCACGACCAGAAGATTGGTTAAGGACTTGGGGCCTTTAAAG
GTTTGGTTTGAATTCTATAGACTGATGACTTGAATTATTATCTCAAGGCATATATTTCTCTA
AAATAAAGGAACTATCTTGTCATTCAGGTCGTATGCATTGATTATCCAAGACCAGAGCTGGA
CAATACAGTTAACTATTTGGAGGCTGCATTTTTATCATCAACGTTCCTGC (239 bp)

TTCTCCGCGCCCAACTAGAACCATTGGAGATTGTTATTGCTGGTGCAGGTGATATTCCGGGT
CATCTATATTTGTAGCATTCGTTTTGCTTATTTCGAAGGAAGCATTTTTTTGCTGTATTATC
ATTTGAATATTTATTGTACCTGTACATGCCTGATTA (163 bp)

Yellow = gRNA 1. Reverse.
Red = PAM site

ATCAACCCGCGCCTCTTCGTCCT

Figure 3c: Copied gene 396 bp. *Protoplast 1 (CRISPR 59 + CRISPR 60) binds to gRNA 2 (CRISPR 1 and CRISPR 2). Sample B1 (non-phosphorylated). Yellow=gRNA 1, red = PAM site*

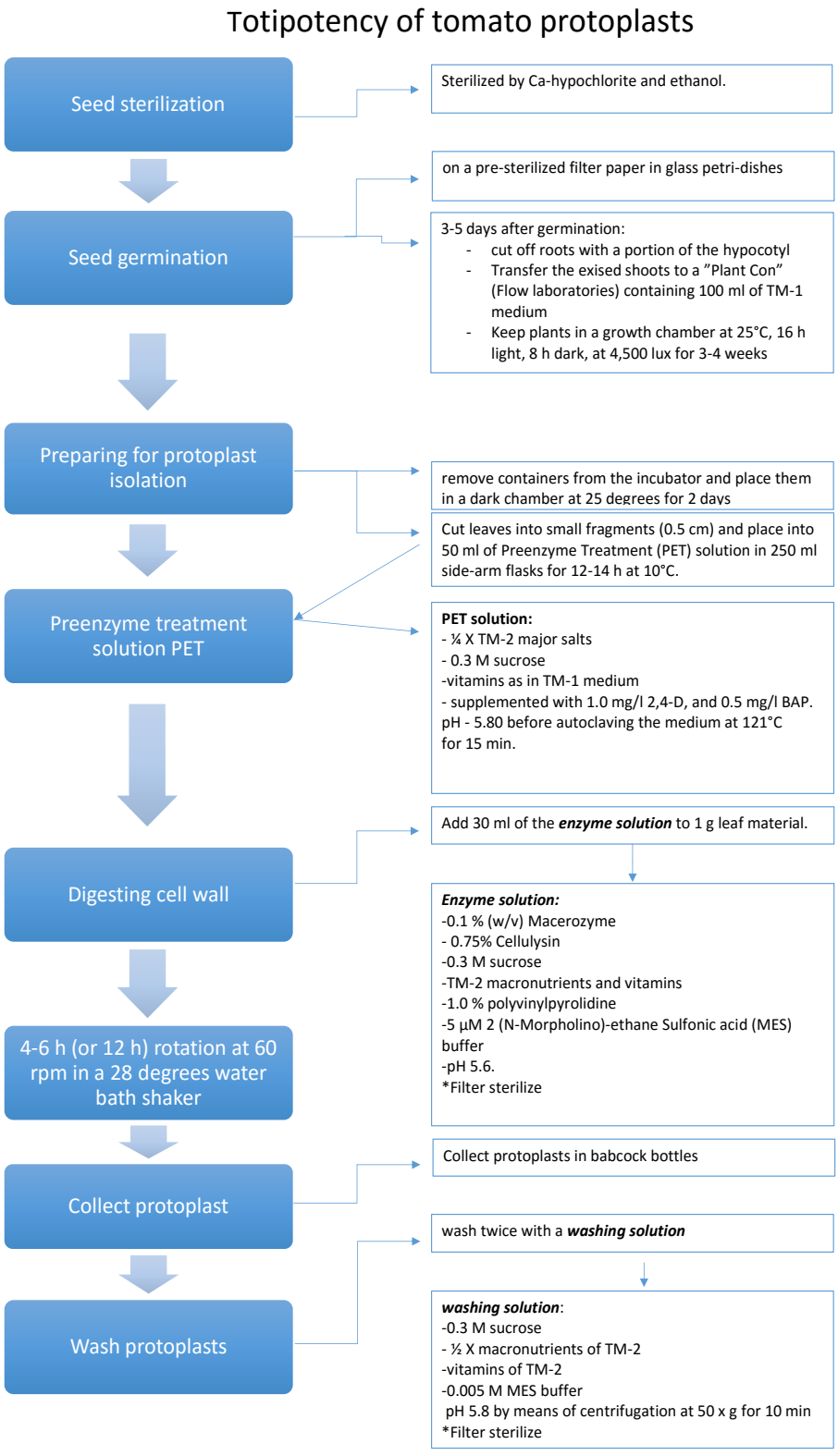

Figure 4: Totipotency of tomato protoplasts. Shows the whole protocol (Shahin 1985) that was planned to follow during this study

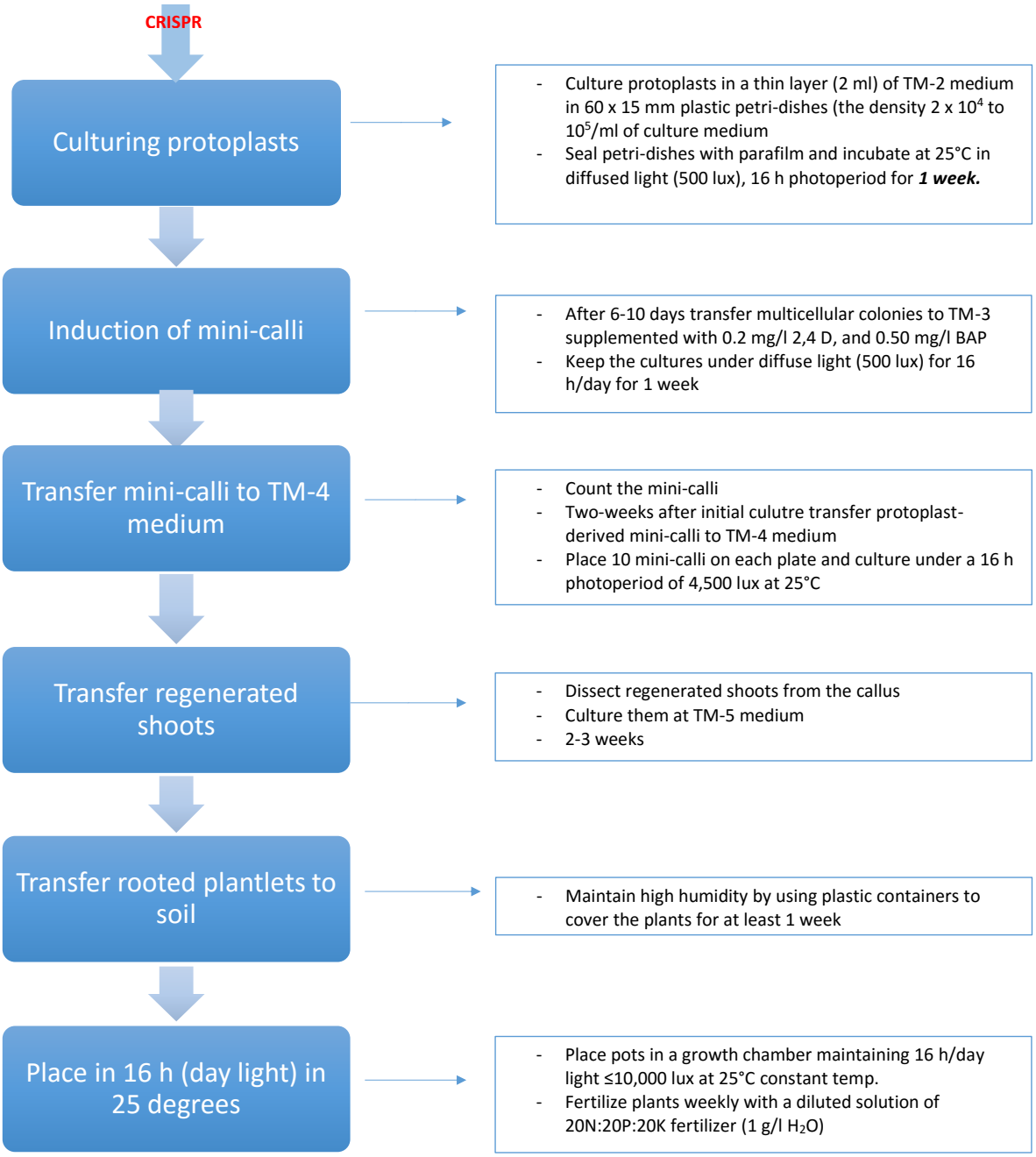

Figure 4 continues: Totipotency of tomato protoplasts. Shows the whole protocol (Shahin 1985) that was planned to follow during this study

Table 1: TM-1 solution. *g/ L except when notated. Solutions were made according to the original protocol (Shahin 1985)*

TM-1					
	1 l (g/L)	**500 ml**(g/L)		**1 l** (g/L)	**500 ml**(g/L)
Macronutrients			***Vitamins***		
KH_2PO_4	-	-	Nicotinic acid	0.0025	0.00125
$CaCl_2$• 2 H_2O	0.15	0.075	Thiamine HCl	0.01	0.005
KNO_3	2.53	1.265	Pyridoxine HCl	0.001	500 µl
NH_5NO_3	0.32	0.16	Folic acid	0.0005*	250 µl
$NH_4H_2PO_4$	0.23	0.115	Biotin	0.00005*	25 µl
$(NH_4)2SO_4$	0.134	0.067	D-Ca-Pantothenate	0.0005*	250 µl
$MgSO_4$•$7H_2O$	0.25	0.125	Choline chloride	0.0001*	50 µl
			Glycine	0.0005*	250 µl
Micronutrients			Cacein hycrolysate	0.05	0.025
KI	0.00038*	190 µl	L-Cysteine	0.001	500 µl
H_3BO_3	0.0062	0.0031	Malic acid	0.01	0.005
$MnSO_4$• $4H_2O$**	0.0223	0.01115	Ascorbic acid	0.0005*	250 µl
$ZnSO_4$ • $7H_2O$	0.0086	0.0043	Adenine Sulfate	-	-
Na_2MoO4• $2H_2O$	0.00025*	125 µl	L-Glutamine	-	-
CuSO4 • 5H2O		12, 5 µl	Myo-inositol	0.1	0.05
$CoCl_2$• $6H_2O$	0.00025*	125 µl	Riboflavin	0.00025*	125 µl
$FeSO_4$• $7H_2O$	0,0139	0.00695			
Na_2• EDTA	0.0185	0.00925	***Others***		
			Sucrose	30	15
Hormones			Mannitol	-	-
NAA	-	-	Xylitol	-	-
Zeatin Riboside	-	-	Sorbitol	-	-
2,4-D	-	-	MES	-	-
BAP	-	-	Noble agar	6.0	3.0
GA_3^B	-	-			
IBA	-	-			
pH	5.8	5.8			

*Note *=stock solutions. 100 mg/100ml stock solution were made 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 $MnSO_4$ x H_2O was used instead.*

Table 2: 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
Cacein hycrolysate	0.05	0.0125	0.15	0.015	0.15	0.0125
L-Cysteine	0.001	250 µl	0.001	0.0001	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	0.025
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 x 10^{-5}*	0.05 M	2.5 ml
pH	5.80	5.80	5.60		5.8	
					*Filter sterilize	

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

Figure 5-9 shows different ladders used during this study.

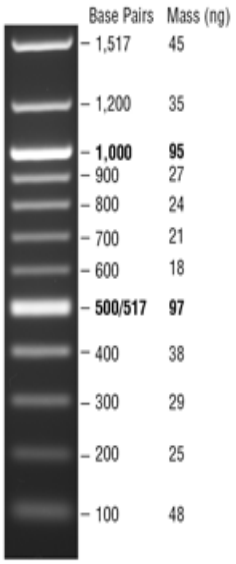

Figure 5: 100 bp DNA ladder from New England Biolabs

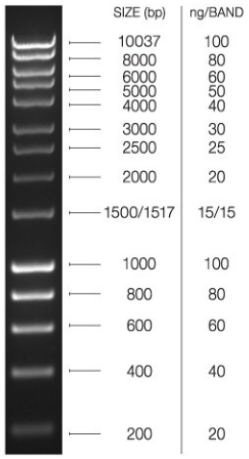

Figure 6: One kb Hyperladder from Bioline

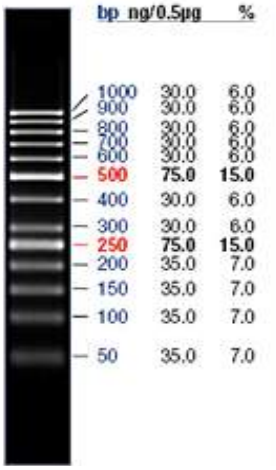

Figure 7: Gene ruler 50 bp from Thermo Fisher Scientific

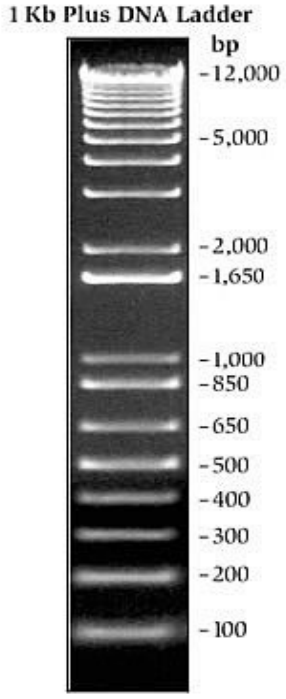

Figure 8: One kb plus DNA ladder from Thermo Fisher Scientific

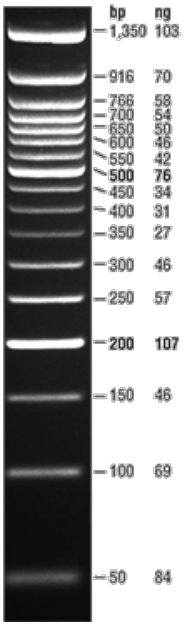

Figure 9: 50 bp DNA ladder from New England Biolabs