University of Stavanger

**Faculty of Science and Technology**

# MASTER'S THESIS

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# ABSTRACT

Programmable sequence-specific nucleases (SSNs) produces double stranded breaks (DSBs) in the genome in a site-specific manner. The following repair through endogenous repair systems allows targeted genome editing, which among others can be used to genetically improve crops. The superior SSN is the RNA-guided engineered nucleases (RGENs) derived from the adaptive immune system - clustered regulatory interspaced short palindromic repeats (CRISPR) and its associated protein 9 (Cas9) of *Streptococcus pyogenes.* Only three components are required to generate site specific cleavage; Cas9 endonuclease, a single guide RNA (sgRNA) containing spacer, and a genomic target site upstream of a protospacer adjacent motif (PAM). Upon binding of spacer to genomic target site, it directs cleavage of the site by Cas9 endonuclease. DNA-free genome edited plants are more likely to be labelled as non-gene modified organisms (non-GMOs), which will have a great impact and value for agriculture, and resulting in a more efficient breeding of crops and production of food. The main object of this thesis is to investigate DNA-free genome editing in *Arabidopsis thaliana (A. thaliana)* using RGENs, and further on investigate the ability to produce a DNA-free gene modified whole plant. To achieve this, we shall directly deliver the RGEN components as both pre-assembled ribonucleoprotein (RNP) complex and in vitro transcribed transcripts, to protoplasts of *A. thaliana.*

The components to set up DNA-free RGENs genetic editing in protoplasts were successfully generated. Two sgRNA encoding gens, containing spacers targeting *Phytoene desaturase 3 (PDS3)* marker gene of *A. thaliana* was generated through sub cloning of spacers into a sgRNA expression vector, and success was confirmed by sequencing. Transcripts of the two sgRNA and Cas9 mRNA were generated by vitro transcription. To confirm transcripts functionality containing the predicted spacers, the sgRNAs and commercial Cas9 nuclease were combined to in vitro digest *PDS3* target sites. This proved the functionality for one of the designed sgRNA. To be able to do in vivo editing, we needed to optimize the isolation of healthy protoplasts, which proved to be a challenge. High numbers of healthy protoplasts were isolated, but during transformation, they died. However, after substantial trials, and by changing several parameters we were able to optimize the protocol and achieved high number of healthy protoplasts both before and after transformation. Subsequently, isolated protoplasts were transfected with both pre-assembled ribonucleoprotein (RNP) complex and in vitro transcribed transcripts of Cas9 and sgRNA. Genetic modifications were analysed using T7 Endonuclease I assay, but without success. In order to be able to experimentally optimize our plans, we also successfully expressed the Cas9 protein, which can be purified and used for the pre-assembled RNP complex. Successful in vivo studies would help us comparing the editing efficiency of the pre-assembled RNP complex to Cas9 transcripts.

# ABBREVIATIONS

| | |
|---|---|
| *A. thaliana* | *Arabidopsis thaliana* |
| β-ME | β-Mercaptoethanol |
| BSA | Bovine Serum Albumin |
| Cas | CRISPR associated |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| crRNA | CRISPR RNA |
| CTAB | Cetyltrimethyl ammonium bromide |
| DSB | Double stranded break |
| *E. coli* | *Escherichia Coli* |
| GMO | Gene modified organism |
| EU | European Union |
| HDR | Homology-directed repair |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| IVT | In vitro transcription |
| MS medium | Murashige and Skoog medium |
| NGS | Next generation sequencing |
| NHEJ | Non-homologous end joining |
| NLS | Nuclear localization signal |
| PAM | Protospacer adjacent motif |
| PDS3 | Phytoene desaturase 3 |
| PEG | Polyethylene glycol |
| pre-crRNA | precursor CRISPR RNA |
| RFLP | Restriction fragment length polymorphism |
| RGEN | RNA-guided engineered nucleases |
| RNase III | Endoribonuclease III |
| RNP | Ribonucleoprotein |
| sgRNA | Single guide RNA |
| SSN | Sequence-specific nuclease |
| T7E1 | T7 endonuclease I |
| TAE | Tris-Acetate-EDTA |
| TALEN | Transcription activator-like effectors nuclease |
| T-DNA | Transfer-DNA |

| | |
|---|---|
| TGS | Tris-Glycine-SDS |
| tracrRNA | trans-activating CRISPR RNA |
| US | United States |
| ZFN | Zinc-finger nuclease |

# 1 INTRODUCTION

## 1.1 Genome editing, improvement of crops and regulations

Programmable sequence-specific nucleases (SSNs) produces double stranded breaks (DSBs) in the genome in a site-specific manner, which in eukaryotes is followed by repair through endogenous repair system allowing targeted mutagenesis and genome editing. This has high value in research, medicine, and biotechnology (Kim and Kim 2014), including to be a tool to genetically improve crops. It can contribute to enhance agriculture to be able to produce enough food for the increasing population and to overcome possible agricultural challenges resulting from global climate changes (Kanchiswamy 2016).

In eukaryotes, the DSB induced by SSNs will either be repaired by Homology-directed repair (HDR) or by the error-prone non-homologous end joining (NHEJ). If the repair happens through NHEJ, small deletions or insertions (indels) is induced, which often result in a frameshift and knock out of gene. By knocking out a specific gene, the function of the gene can be studied. If the function is already known, unwanted genes can be knocked out to obtain phenotypes as desired. Alternatively, a gene can be knocked in at a pre-determined site if the repair happens through HDR. To obtain this, gene to be inserted needs to be flanked by arms with sequences that are identical to those near the break region, and be co-transformed together with the nuclease. Specific single nucleotide can also be inserted in the same matter. Two DSBs can also be induced. If the breaks are at the same chromosomes, it can lead to deletion or inversion of the region between the two breaks, while if the breaks are at two different chromosomes, it can lead to chromosomal translocation (Kim and Kim 2014). Thereby SSNs can be used to knock out genes, insert new genes and remove segments from the genome, all in a specific approach.

Traditional techniques for improvement of crops includes conventional breeding which takes advantage of naturally occurring mutations resulting in favourable traits, and the crossing of these to obtain new crop varieties. It also includes the induction of mutations by mutagenesis such as chemicals or irradiation followed by screening of mutants with desirable traits. A third approach is genome modification by randomly insertion of exogenous genes by different transfer methods (example by transfer-DNA (T-DNA) insertion) resulting in transgene plants with new traits that may not be achievable by crossing. These approaches act randomly on the genome, and can produce several unwanted mutations and rearrangement in the genome, which is time consuming to segregate away. Genome editing by SSNs can provide fast and precise plant breeding through specific mutations (Hartung and Schiemann 2014; Pacher and Puchta 2016).

It is also highly relevant if plants genetically edited using SSNs will be classified as gene modified organisms (GMOs). In the United States (US), they mainly have a product-based approach regarding regulation. This means that if the generated product is free of exogenous DNA, and therefore not a transgenic plant, the chances are high it will be considered as non-GMO and do not need to be regulated. Some genetically edited plants have already been approved as non-GMO in the US (Waltz 2016a, b). In the European Union (EU) it is more complicated. In Directive 2001/18/EC by the EU, a GMO is defined as "an organism […] in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". They have mainly a process-based approach regarding regulation, and the legislation is unclear regarding new methods including SSNs. Being considered a GMO by EU legislation can hamper the development of agriculture, because the approval procedure of new GMOs is time consuming and expensive, making it unfavourable using these techniques to get new crops varieties on the market (Hartung and Schiemann 2014; Pacher and Puchta 2016).

There are three different SSNs widely used, zinc-finger nucleases (ZFNs), Transcription activator-like effectors nucleases (TALENs) and the last in the line RNA-guided engineered nucleases (RGENs), which was developed from the type II clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) protein -system of the bacteria *Streptococcus pyogenes.* RGENs has been shown to replace ZFNs and TALENs, being simpler in design and therefore easier and faster to prepare, being more affordable and being more available for the general scientist (Kim and Kim 2014). One disadvantage is that off-targets has been seen, but the method is evolving and effort has been taken to reduce off-targets (Kanchiswamy 2016). In this project RGENs will be used to induce genome cleavage and knock out a desired gene.

## 1.2 CRISPR-Cas adaptive immune system

CRISPR-Cas system, is an adaptive immune system found in prokaryotes such as bacteria and archaea. The CRISPR-Cas loci consist of a CRISPR array of identical repeats interspersed by variable sequences called spacers, and an operon of Cas genes encoding the Cas proteins. The variable sequences in the CRISPR array originate from former invading DNA (Barrangou and Marraffini 2014; Doudna and Charpentier 2014).

The immune system operates in three phases. The adaptive phase, where fragments of invading foreign genome/DNA is inserted into the CRISPR array to form spacers (often 20 bp) and thereby creating memory. The expression phase (crRNA biogenesis), where the repeat-spacers elements are

transcribed into precursor CRISPR RNA (pre-crRNA), followed by enzymatic cleavage of pre-crRNA transcript to form short CRISPR RNA (crRNA) consisting of only one spacer and repeat. The last phase is the interference phase, where complementary binding of the spacers region of short crRNA to sequences of invading DNA, directs Cas nuclease cleavage of the invading DNA (Barrangou and Marraffini 2014; Doudna and Charpentier 2014; Jinek et al. 2012).

### 1.2.1 Type II CRISPR-Cas system of *Streptococcus pyogenes*.

CRISPR-Cas system has been classified into two classes containing five different types and several subtypes. The major differences between them are the Cas proteins and their contribution to the system, where type II system is one of the simplest of them regarding number of components (Makarova et al. 2015).

For type II system of *Streptococcus pyogenes* (Figure 1-1), it was shown by Deltcheva and co-authors (2011), that three components were required for the crRNA biogenesis; a trans-activating CRISPR RNA (tracrRNA), the hosts endoribonuclease III (RNase III) and Cas9 endonuclease. The binding of tracrRNA to to the repeat region of pre-crRNA, in the present of Cas9 endonuclease, triggers the cleavage of pre-crRNA by RNase III (Deltcheva et al. 2011). Further on, Jinek and co-authors (2012) showed that Cas9 protein is a dual-RNA-guided DNA endonuclease, and is responsible for the cleavage and silencing of foreign DNA during interference phase. It comprises two nuclease domains HNH and RuvC-like domain, where HNH domain cleaves DNA strand complementary to the spacer sequence, while RuvC-like domain cleaves the opposite strand. In addition to the tracrRNA:crRNA duplex and the following binding of spacer region of crRNA to the target site of the foreign DNA, to direct the Cas9 endonuclease cleavage, cleavage also requires the presence of a protospacer adjacent motif (PAM). PAM is a three-nucleotide short motif (5'-NGG-3` for *Streptococcus pyogenes*) on the invading DNA, directly downstream of the crRNA-binding sequence, on the opposite strand. Cas9 cleaves the target sequence, precisely three nucleotides upstream of PAM site, within the target region (Jinek et al. 2012). The complementary binding of spacer of crRNA to target site and the specific Cas9 cleavage of target site upstream of PAM site, makes the cleavage site-specific.

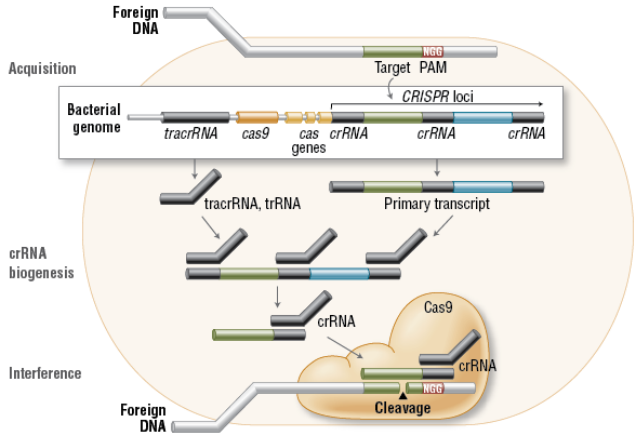


**Figure 1-1: CRISPR-Cas9 adaptive immune system**
In the adaptive phase (acquisition phase) fragments of invading foreign DNA are inserted into the CRISPR array as spacers to create memory. During the crRNA biogenesis, the CRISPR array is transcribed into pre-crRNA, followed by binding of tracrRNA to the repeat region, which in present of Cas9 induce cleavage of pre-crRNA into short crRNA by RNase III (Deltcheva et al. 2011). During interference, invading foreign DNA is cleaved by Cas9 due to complementary binding of spacer sequence of crRNA:tracrRNA duplex and the recognition of PAM sequence by Cas9 (Jinek et al. 2012). Illustration is collected from NEB webpage (Cas9 in vivo: Bacterial Adaptive Immunity 2014).

## 1.3 CRISPR-Cas9 technology

Type II CRISPR-Cas system from *Streptococcus pyogenes* was developed into a technology for targeted mutagenesis and precise editing of genes (Figure 1-2).

By joining the 3`end of the crRNA to the 5`end of the tracrRNA with a GAAA tetraloop, Jinek and co-authors engineered the tracrRNA:crRNA complex into a chimeric single guide RNA (sgRNA). The base-pairing interactions that occur between tracrRNA and crRNA is maintained, and the 5`end spacer of crRNA is free to base pair with target site (Jinek et al. 2012). Thereby, the system only needed three components; Cas9 endonuclease, a sgRNA, and a genomic target site directly upstream of a PAM motif (Ma et al. 2016). By changing the spacer sequence of the sgRNA, it can direct Cas9 to target any DNA sequence of interest as long as it is adjacent to a PAM (Jinek et al. 2012; Doudna and Charpentier 2014). ZFNs and TALENs require substantial protein engineering for each DNA target site to be modified, while CRISPR-Cas9 system (also called RGENs) only requires change of spacer sequence (Doudna and Charpentier 2014), thereby making it an easier and faster tool. In the start of the year 2013, three research articles demonstrated the CRISPR-Cas9 genetic editing mechanism in vivo in human cells and mouse cells (Cong et al. 2013; Jinek et al. 2013; Mali et al. 2013). Following this, thousands of research reports have been published, and the genome of a variety of animal and plants have been edited (Doudna and Charpentier 2014; Ma et al. 2016).

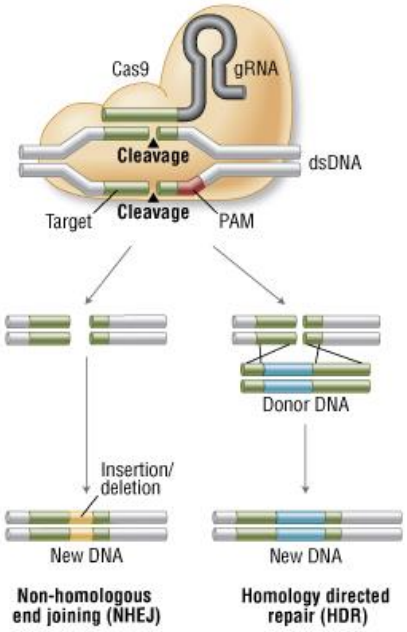


**Figure 1-2: CRISPR-Cas9 technology.**
The crRNA:tracrRNA duplex is engineered into a single gRNA. Upon binding of the sgRNAs 20 bp spacer (green) to the target site of a dsDNA, it directs Cas9 cleavage of both DNA strands. Cleavage occurs precisely three nucleotides upstream of PAM site (Jinek et al. 2012; Doudna and Charpentier 2014). Double stranded cleavages in the genome of eukaryotes is repaired by endogenous repair systems; NHEJ or HDR, which can be exploited to knock out or knock in genes in a specific approach(Kim and Kim 2014). Illustration is collected from NEB webpage (CRISPR/Cas9 System Applications 2014).

## 1.4 DNA free genome editing

The sgRNA and the Cas9 protein can be transferred to the plant by different means. Several sgRNA and Cas9 Expression Cassettes for editing of plants have been developed (Ma et al. 2016). Vector constructs carrying these cassettes are delivered into the plant cells typically by particle bombardment (biolistic) or by Agrobacterium-mediated transformation as for Arabidopsis by floral dipping (Ma et al. 2016). The Cas9 protein and sgRNA are expressed and transcribed inside the cells, which are followed by specific target site cleavage and modification of the hosts genome. This approach has successfully been performed by many research groups and for many plants (see list of plants and articles in Ma et al. 2016).

However, by plasmid-mediated delivery of Cas9 and sgRNA into the cells, the resulting genetic modified plant will continue to harbour foreign DNA; If using Agrobacterium and T-DNA or particle bombardment, the genes will be integrated into the hosts genome. If using non-integrating plasmid, the plasmid will eventually be degraded by endogenous nuclease and thereby disappear, but segments from the plasmid could be randomly integrated into the host genome (Woo et al. 2015; Kim et al. 2014). When continuing harbouring foreign DNA, the plants will be transgenic and considered a GMO

and covered by the GMO legislation both in the US and in the EU, and thereby must pass the expensive and time-consuming GMO approval procedure in the EU in order to be released to the market (Hartung and Schiemann 2014; Pacher and Puchta 2016). The unwanted inserted foreign DNA sequences could be removed through backcrossing, but only if the plants reproduce sexually. Crops like grape, potato and banana reproduces asexually, so this method will not be feasible (Woo et al. 2015). Also, the life cycle for the respective crop should be short (few month to one year) in order for it to be efficient (Kanchiswamy 2016).

A different DNA free based approach for delivery of Cas9 and sgRNA could possibly bypass the GMO labelling and regulations. In this approach preassembled Cas9 protein-sgRNA ribonucleoproteins (RNPs) or Cas9 mRNA and sgRNA are directly delivered to the cell. In cultured human cells, it was shown that RNPs cleaves chromosomal target site almost immediately after delivery and is thereafter rapidly degraded (Kim et al. 2014). Hence, this approach produces genetically edited plants, but with no trace of foreign genetic elements. Thereby it resembles conventional breeders use of mutagens such as chemicals or irradiation to induce mutations, which is not covered by the regulations in EU, only that CRISPR-Cas9 is specific and induce mutation only in the desired gene (Kanchiswamy et al. 2015; Hartung and Schiemann 2014).

In addition, genomic editing of target site is more efficient during direct delivery of RNPs than during plasmid-mediated delivery. As already noted, in cultured human cells it was shown that RNPs cleaves chromosomal target site almost immediately after delivery (Kim et al. 2014). It was also shown that the highest mutation frequency was achieved already one day after delivery, and when they compared to plasmid-mediated delivery, it took three days to achieve the same level (Kim et al. 2014). Other research groups, editing plants through same approach, also reported detection of mutation already 24 h after RNPs transfection, and suggested that target site is cleaved almost immediately after transfection (Subburaj et al. 2016; Woo et al. 2015).

Furthermore, using the DNA free delivery approach reduces the possibility for off-targets compared to plasmid-mediated delivery. The randomly integrated DNA sequences from the plasmids, could be integrated at off-target sites in the host genome and lead to knock out of other genes or enhance expression of genes. Cas9 can also create unwanted cleavage at off-target sites sequences that has high homology to on-target sites (Koo et al. 2015). If several DSBs is induces by off-target cleavage, severe chromosomal rearrangement can occur such as inversion or translocation (Kim and Kim 2014). It was shown in cultured human cells, that direct delivery of RNP complex lowered the amounts of off-targets when comparing to plasmid-mediated delivery. Cas9 protein was seen to be almost fully degraded 24 h post transfection, and suggested that continuous expression of Cas9 and sgRNA from

plasmids or from the hosts genome in case of integration may be the cause for the higher occurrence of off-targets during plasmid-mediated delivery (Kim et al. 2014). Also for plants, it has been shown lower amounts of off-targets when using direct delivery comparing to plasmid-mediated delivery (Liang et al. 2017; Svitashev et al. 2016).

The designing of a unique target site is nevertheless the most important step to reduce off-target sites. It should differ from any other sequence in the genome by at least two or three nucleotides (Koo et al. 2015). Several web based tools have been developed, to help synthesize spacer sequence of sgRNA with improved on-target specificity, and thereby reduce off-targets (Kanchiswamy 2016). It has also been developed other approaches to reduce off-targets furthermore, for example using truncated sgRNA or other Cas9 variants (Kanchiswamy 2016).

### 1.4.1 Delivery of RNP-complex or transcripts

In cultured human cells, vector-free direct delivery of RNP-complex has been achieved by using electroporation- and lipofection- mediated delivery (Kim et al. 2014; Liang et al. 2015; Lin et al. 2014; Zuris et al. 2015a). In difference from animal cells, plant cells have a cell wall making the delivery more difficult.

#### *1.4.1.1 Polyethylene glycol (PEG) -mediated transformation of protoplasts*

The issue of cell wall could be overcome by using protoplasts, which is plant cells lacking the cell wall due to enzyme digestion, this make them similar to mammalian cultured cells. PEG-mediated RNP transformation of protoplast have been successfully performed for plant species such as *Arabidopsis thaliana (A. thaliana),* tobacco, rice, lettuce (Woo et al. 2015), petunia (Subburaj et al. 2016), grapevine and apple (Malnoy et al. 2016). Transfection of lettuce protoplasts, was followed by regeneration into a whole plant, and the overall mutant frequency in lettuce calli (T0 generation) was measured to be 46 %.

#### *1.4.1.2 Particle bombardment of immature embryo cell*

For some plant species, especially monocots including maize and wheat, regeneration into whole gen modified plants from protoplasts is difficult (Eeckhaut et al. 2013; Svitashev et al. 2016). Therefore, another delivery method for RNP-complex has been explored, particle bombardment of embryo cells by using gold particles and gene gun, followed by regeneration of transformed embryotic cells into whole plants. This method has successfully been achieved for maize (Svitashev et al. 2016) and for bread wheat (Liang et al. 2017). For maize, when using markers and selective media, they obtained a mutation frequency at plant level at 47 %. Without using makers and selective media, mutant plants

in T0 generation were recovered at a frequency from 2.4 % to 9.7 %. For wheat, they recovered mutant plants in T0 generation at a frequency up to 4.4 %.

#### *1.4.1.3 Delivery of Cas9 transcript instead of protein*

As mention, instead of delivering Cas9 as protein, it could be delivered as a transcript. This could be favourable considering time and cost; generating Cas9 transcript is faster than producing Cas9 protein, and to commercially buy the Cas9 protein is expensive. The approach has successfully been performed in animal cells (Liang et al. 2015; Song et al. 2017), and when comparing to plasmid-mediated delivery, it has the same outcome as RNP-complex delivery, being superior (Liang et al. 2015). Very recently, in parallel to this project, genetic modified of wheat was achieved by this approach, and the delivery of Cas9 transcript and sgRNA were accomplished by particle bombardment of embryotic cells. Without using selective marker, mutant plants in T0 generation were recovered at a frequency of 1.1 % (Zhang et al. 2016). To the authors knowledge, no other plants has been modified delivering Cas9 as transcript.

**Table 1-1: Summary of plants edited by CRISPR-Cas9 DNA-free method with direct delivery of the components as RNP-complex or transcripts.**

The table provide information of plant species edited, transformation method, amounts used of the different components and mutagenesis efficiency in the different cases. The mutagenesis efficiency are measured by different methods, such as next generation sequencing (NGS), T7 Endonuclease I (T7E1) assay and restriction fragment length polymorphism (RFLP) assay.

| Plants species | Transformation | Cas9 protein | sgRNA | Mutagenesis efficiency | Reference |
|---|---|---|---|---|---|
| *A. thaliana*<br>Tobacco<br>Rice<br>Lettuce | 1x $10^5$ (200 µl) protoplasts<br><br>210 µl PEG | 10-60 µg | 20-120 µg | NGS: 8.4 % to 44 % indels<br><br>T7E1 assay: 17 % to 23 %.<br><br>RFLP assay: average 46 % in calli | (Woo et al. 2015) |
| | | Molar ratio 1:2-10 Cas9:sgRNA<br><br>5-20 µl RNP complex | | | |
| *Petunia × hybrida* | Unclear amount Protoplasts<br><br>Unclear amount PEG | 90 µg | 50 µg | NGS: 11.5 % indels<br><br>T7E1 assay: 14.9 % | (Subburaj et al. 2016) |
| | | Unclear amount of RNP-complex | | | |
| Grapevine and apple | 2x $10^5$ (200 µl) protoplasts<br><br>Equal volume of PEG as protoplasts together with RNP complex | 90 µg | 30 µg | NGS: 0.1 % to 6.9 % indels<br><br>Mostly the 3:1 ration of Cas9:sgRNA gave highest mutation frequency. | (Malnoy et al. 2016) |
| | | 30 µg | 30 µg | | |
| | | 30 µg | 90 µg | | |
| | | Unclear amount of RNP-complex | | | |
| Maize | Particle bombardment of immature embryo cells. | 7 µg | 3 µg | Mutant production efficiency MPE in generated plants: 2.4 % to 9.7 % | (Svitashev et al. 2016) |
| | | 20 µl RNP complex | | | |
| Bread wheat | Particle bombardment of immature embryo cells. | 2 µg | 2 µg | Mutant production efficiency MPE in generated plants: Up to 4.4 % | (Liang et al. 2017) |
| | | 10 µl RNP complex | | | |
| **Plant species** | **Transformation** | **Cas9 mRNA** | **sgRNA** | **Mutagenesis efficiency** | **Reference** |
| Wheat | Particle bombardment of immature embryo cells. | 1 µg | 1 µg | Mutant production efficiency MPE in generated plants: 1.1 % | (Zhang et al. 2016) |

## 1.5 Phytoene desaturase 3 (PDS3)

The enzyme phytoene desaturase, encoded by *PDS3* in *A. thaliana*, catalyses the desaturation of phytoene into zeta-carotene, which is an important step of the carotenoids biosynthetic pathway (Ruiz-Sola and Rodríguez-Concepción 2012). Qin et al. analysed a T-DNA insertion mutant of *PDS3* for *A. thaliana*. The mutant showed albino and dwarf phenotype, and it was confirmed that the phenotypes resulted from the disruption of *PDS3* gene. (Qin et al. 2007). With this distinct phenotype, *PDS3* can serve as a model gene target for CRISPR-Cas9 induced mutation.

## 1.6 The objectives of this study

Investigated DNA-free genome editing in *A. thaliana* with pre-assembled CRISPR-Cas9 RNPs and transcripts, and further on investigate the ability to produce a DNA-free genome modified whole plant. Figure 1-3 provides a simple overview.

Methods: Molecular biology (cloning), protoplast production and transfection, in vitro transcription and mRNA purification, protein production and purification, and tissue culture methodology.

A. CRISPR-Cas9

    a. Cloning of sgRNAs containing spacer against marker gene *PDS3*.
    b. In vitro transcriptions for cloned sgRNAs and Cas9.
    c. Performing in-vitro CRISPR-Cas9 genetic modifications by Cas9 protein and transcribed sgRNAs against the marker gene *PDS3.*
    d. Performing in-vivo CRISPR-Cas9 genetic modifications through PEG transfection of isolated protoplasts by Cas9 protein and transcribed sgRNAs against the marker gene *PDS3.*
    e. Performing an T7E1 assay to detect in vivo genetic editing.

B. Expression and purification of Cas9 protein.
C. Establish tissue culture system to regenerate plants from isolated protoplasts.

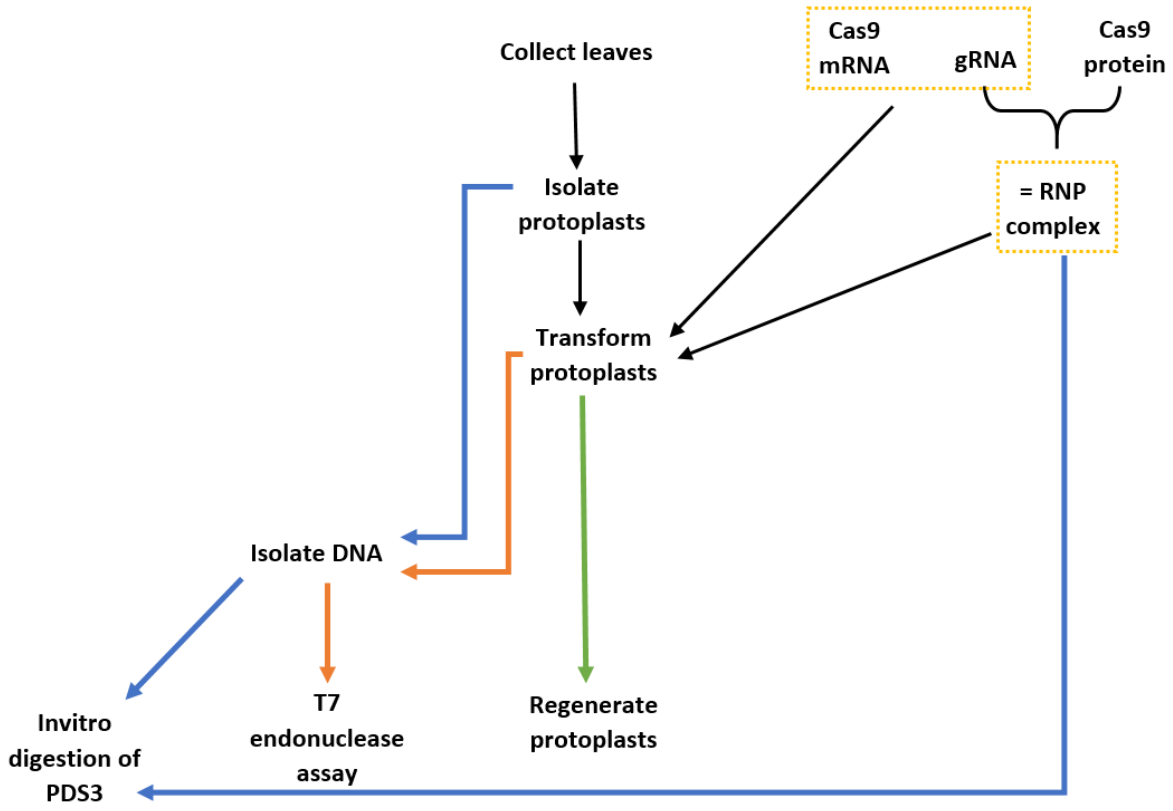


Figure 1-3: An overview of the objectives of this thesis.

# 2 MATERIAL AND METHODS

## 2.1 MATERIAL

### 2.1.1 Commercial kits

**Table 2-1: A list of the kits used and their purpose.**

| Commercial Kit | Function | Supplier | City, Country |
|---|---|---|---|
| MiniPrep NucleoSpin® Plasmid (NoLid) | Plasmid isolation | Machery Nagel | Düren, Germany |
| GenElute™ Plasmid Mini Prep Kit | Plasmid isolation | Sigma Aldrich | St. Louis, MO, USA |
| GenElute™ Gel Extraction Kit (NA1111) | Retrieval of DNA after gel electrophoresis | Sigma Aldrich | St. Louis, MO, USA |
| NucleoSpin® Gel and PCR Clean-up | Retrieval of DNA after gel electrophoresis | Machery Nagel | Düren, Germany |
| Monarch DNA Gel Extraction Kit (# T1020). | Retrieval of DNA after gel electrophoresis | NEB | Ipswitch, MA, USA |
| HiScribe™ T7 Quick High Yield RNA synthesis Kit (# E2050S). | In vitro transcription of sgRNA | NEB | Ipswitch, MA, USA |
| MEGAclear™ Kit Purification for Large Scale Transcription Reactions (AM1908) | Purification of sgRNA transcript | Ambion | Carlsbad, CA, USA |
| HiScribe™ T7 ARCA mRNA Kit (with tailing) (#E2060S). | In vitro transcription of Cas9 | NEB | Ipswitch, MA, USA |
| RNA Clean & Concentrator™ -25 (R1017). | Purification of Cas9 transcript | Zymo Research | Irvine, CA, USA |

### 2.1.2 Ladders

**Table 2-2: A list of the ladders used for gel electrophoresis.**

| Ladders | Supplier |
|---|---|
| Hyperladder™ 1 kb | Bioline |
| Quick-load® 100 bp DNA Ladder | NEB |
| GeneRuler 100 bp DNA Ladder | Thermo Scientific |
| GeneRuler 50 bp DNA Ladder | Thermo Scientific |
| GeneRuler 1 kb plus DNA Ladder | Thermo Scientific |
| 50 bp DNA Ladder | NEB |

### 2.1.3 Bacterial strains

1. **JM 109** *Escherichia coli (E. coli)* (Promega): This strain was used for cloning of plasmids, and is modified in such a way that makes it a good host for this purpose. The lack of the *E. Coli* K restriction system (genotype: *hsd*R17 ($r_k^-$, $m_k^+$)), stabilize the plasmid by preventing cleavage. Recombinase A– mutation (genotype: *Rec*A1), prevent recombination with host chromosomal DNA. Endonuclease A– mutation (genotype: *End*A1) prevents endonuclease activity and improves yield and quality of isolated plasmid.

2. **One Shot® BL21 Star™ (DE3)** Chemical Competent *E. coli* (Thermo Fisher Scientific): This strain was used as host for expression of NLS-Cas9-6xHis protein from pET-NLS-Cas9-6xHis vector, which is a T7 promoter based expression vector. The bacterium contains DE3 lysogen carrying the gene for T7 RNA polymerase which is needed for expression. This T7 RNA polymerase is itself under the control of the *lac*UV5 promoter and thereby isopropyl β-D-1-thiogalactopyranoside (IPTG) will induce expression. The bacterium is also modified to enhance mRNA stability and increase protein yield. Mutation in the RNaseE gene (*rne*131) reduces endogenous RNases, which reduces mRNA degradation, and the absent of the *lon* and outer membrane (*Omp*T) proteases reduces degradation of proteins.

### 2.1.4 Vectors

All the vectors used in this thesis are listed in Table 2-3 and are further on described in more detail in Section 2.2.3, 2.2.4 and 2.2.5.

**Table 2-3: A list of the vectors used in the thesis, and their purpose of use.**

| Vector/Plasmid | Function | Supplier |
|---|---|---|
| puC57-sgRNA expression vector | In vitro transcription of sgRNA | Addgene # 51132 |
| pST1374-NLS-flag-linker-Cas9 | In vitro transcription of Cas9 mRNA | Addgene # 44758 |
| pET-NLS-Cas9-6xHis | Expression of NLS-Cas9-6xHis protein | Addgene # 62934 |

### 2.1.5 Plant material

Seeds for *A. thaliana* Columbia (Col-0) wild-type (WT) were provided by NASC Nottingham, United Kingdom.

## 2.2 METHODS

### 2.2.1 Cloning

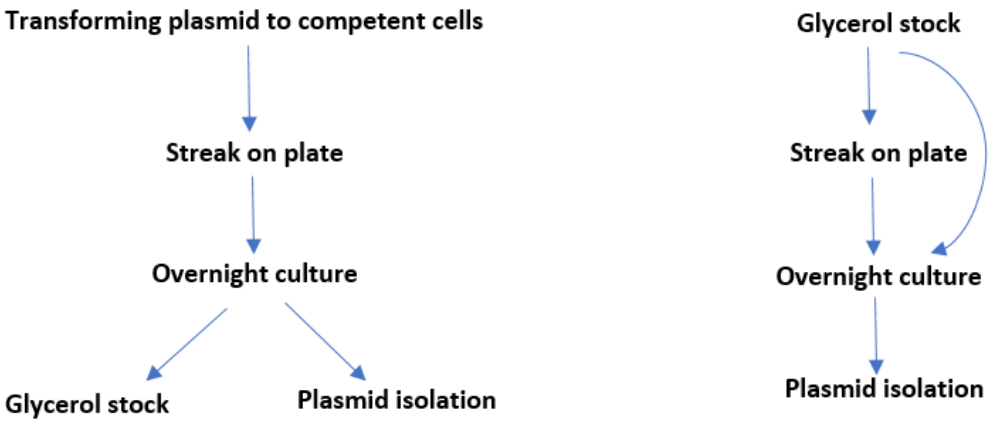


**Figure 2-1: An overview of different approaches to clone a plasmid.**

#### *2.2.1.1 Transformation of plasmid to competent cells by heat shock method:*

Plasmids were transformed to competent cells by heat shock method (Chung et al. 1989). Competent cells (~150 µl) were first thawed on ice for ~15 min. Then the plasmid (1 µl) was mix with the competent cells by slowly stirring. The mixture was left on ice for 20 - 30 min, before heat shock was performed by water bath at 42 ˚C for 50 seconds, followed by 2 min on ice. LB Broth (MILLER, Merck) (500 µl) were added, and the tubes were incubated at 37 ˚C and 200 rpm for 1-2 h. The transformed cells were plated on LB agar (MILLER, Merck) containing either 50 µg/ml kanamycin or 100 µg/ml ampicillin, depending on the antibiotic resistance of the plasmid transformed. The plates were incubated at 37 ˚C until next day.

#### *2.2.1.2 Overnight culture*

Overnight cultures were made by carefully touching a colony by a pipette tip, and dropping it into a tube with LB Broth (MILLER, Merck) (5 ml) containing 50 µg/ml kanamycin or 100 µg/ml ampicillin, depending on the antibiotic resistance of the plasmid. The colony could either come from transformed cells streaked on plates or a glycerol stock streaked on plates. Instead of a colony, a small piece of the glycerol stock could also be used. The tubes were then incubated on a shaker (250 rpm.) at 37 ˚C until next day.

#### *2.2.1.3 Glycerol stock*

Glycerol stocks were made by adding overnight culture (900 µl) to 70% glycerol (500 µl), followed by mixing well by inverting. The stocks were stored at -80 ˚C.

#### *2.2.1.4 Plasmid isolation*

Plasmids were isolated using kits (Table 2-1) and stored at -20 ˚C. The quality was checked using agarose gel and the concentration was measured using nanodrop. The plasmids were eluted with nuclease-free $dH_2O$ (60 µl), instead of using the elution buffers following the kits, otherwise the protocols from the kits were followed.

### 2.2.2 Agarose gels, and gel extraction

Both 1 % agarose and 2 % agarose gels were used together with 1x TAE (Tris/Acetate/EDTA) running buffer. Loading buffer DNA II (AppliChem) was used to make the samples sink nicely into the wells and for visual tracking of DNA during the migration thorough the gel. GelRed™ (Biotium) was used to stain to visualize the bands by UV-light. The gels were run at 85-90 V for 30-60 min, depending on percent agarose in the gels and the size of the DNA in the samples. The bands were compared to DNA ladders of known size (Table 2-2). Different ladders were used depending on the expected size of the DNA in the sample.

When the gels were used for gel extraction, the electrophoresis cell, gel caster and gel tray were rinsed with distilled water beforehand and new running buffer was used. This to avoid residues of old samples to contaminate the extracted DNA. UV-light was used to visualise the bands, and they were cut from the gel using a clean sharp scalpel. Different kits were used for gel extractions (Table 2-1), depending on availability at the lab. The extracted DNA was eluted in nuclease-free $dH_2O$ instead of the elution buffers following the kits, otherwise the kits protocols were followed. The concentration and purity of the extracted DNA was measured by nanodrop, and stored at -20 ˚C.

## 2.2.3 Generation of sgRNAs against *PDS3*.

### *2.2.3.1 Designing of spacers*

Spacers for sgRNAs production to edit *A. thaliana PDS3* (Table 2-4) were identified as described below and the oligos of the spacers were ordered from Thermo Fisher Scientific. In addition to the spacer target, adapters were added at the 5' end of each oligo (Table 2-4), which are needed for restriction ligation reaction and contain the nucleotides needed for T7 in vitro transcription.

**Table 2-4 Oligos for generation of two sgRNA to modify *A. thaliana PDS3* by CRISPR Cas9 method.**
The nucleotides coloured in red are adapters added to the oligos.

| Gene | Oligo name | Design Acr. | Sequence (5'--- 3') |
|---|---|---|---|
| At_*PDS3*_Class0 gRNA | CRISPR-5 | Forward | TAGGATAAGCCTGACCGCCGACCA |
| | CRISPR-6 | Revers | AAACTGGTCGGCGGTCAGGCTTAT |
| At_*PDS3*_Class1 gRNA | CRISPR-7 | Forward | TAGGCGCTTAAGACAAGAACAAGG |
| | CRISPR-8 | Revers | AAACCCTTGTTCTTGTCTTAAGCG |

To identify the spacers for sgRNAs production to edit *PDS3*, it is necessary to know where in the genome of *A. thaliana* the gene is located. This was found by searching for *PDS3* in the database at Arabidopsis Information Resource (TAIR) http://www.arabidopsis.org/. The gene locus is AT4G14210 and gene model number one is used (i.e. AT4G14210.1.), as the other gene models are splicer variants. It was found that *PDS3* is located on chromosome 4 from nucleotide number 8190212 to 8195265 (Appendix, Figure 7-1). This information was used as the query at CRISPR-PLANTS (Xie et al. 2014) (Appendix, Figure 7-2, A), which is a "…platform to help researches to design and construct specific sgRNA for CRIPSR-Cas9 mediated genome editing in plants" (Xie et al. 2014).The outcome of the search gives a list of recommended targets. These targets were compared to recommended targets from other prediction sites, such as CHOPCHOP (Labun et al. 2016) (Montague et al. 2014). At CHOPCHOP, the gene ID from TAIR (AT4G14210.1) together with species (*Arabidopsis thaliana*) and use (CRISPR/Cas9) were used as query (Appendix, Figure 7-3, A). CHOPCHOP also tells where in the gene, at which exon, the target is located. After comparing the targets from the two prediction sites, two sets of paired DNA oligos (spacer) were selected (Appendix Figure 7-2, B and C). The first set of paired DNA oligos, CRISPR -5 and -6 is located at exon 7, which is in the middle of the gene (Appendix Figure 7-3, B), while second set of paired DNA oligos, CRISPR -7 and -8 is located at exon 2, which is in the beginning of the gene (Appendix Figure 7-3, C).

### *2.2.3.2 Annealing of the complementary oligos*

Two protocols (A and B) for annealing of forward and revers oligos were performed for both CRISPR-5 and -6 (Reaction 1) and for CRISPR -7 and -8 (Reaction 2). The two protocols are modified previously described protocols (Ran et al. 2013) and (Liu et al. 2015), and the annealing buffer used in protocol B are from (Huang 2014). In protocol A the oligos were phosphorylated during the annealing by polynucleotide kinase (PNK), while in protocol B they were not. The annealing reactions was assembled as in Table 2-5 and the they were annealed in a thermocycler following the scheme in Table 2-6. After annealing, the oligos were diluted by nuclease-free $dH_2O$. In protocol A they were diluted 1/200 times, which gave a concentration of 0.05 µmol/L, while in protocol B they were diluted 1/100, which gave a concentration of 0.02 µmol/L.

**Table 2-5: Two protocols for annealing of complementary oligos.**
Protocol A is with T4 PNK, while protocol B is without.

| Protocol A | Volume | Protocol B | Volume | Reaction 1 | Reaction 2 |
|---|---|---|---|---|---|
| **Forward CRISPR, (100 µmol/L)** | 1 µl | **Forward CRISPR, (100 µmol/L)** | 1 µl | CRISPR 5 | CRISPR 7 |
| **Reverse CRISPR, (100 µmol/L)** | 1 µl | **Reverse CRISPR, (100 µmol/L)** | 1 µl | CRISPR 6 | CRISPR 8 |
| **10x T4 ligase buffer, Promega** | 1 µl | **Annealing buffer, 10x: 10 mM Tris, 50 mM NaCl, 1 mM EDTA** | 5 µl | | |
| **T4 PNK (10 000 u/ml), NEB** | 1 µl | | | | |
| **Nuclease-free $dH_2O$** | 6 µl | **Nuclease-free $dH_2O$** | 43 µl | | |
| **TOTAL** | 10 µl | **TOTAL** | 50 µl | | |

**Table 2-6: Thermocycler conditions for annealing of complementary oligos by protocol A and protocol B.**

| Protocol A | Protocol B |
|---|---|
| 37 ˚C for 30 min | 95 ˚C for 5 min |
| 95˚C for 5 min | A decrease of 3˚C for every 3. min until reaching 25˚C. |
| A decrease of 5˚C for every min until reaching 25˚C. | |
| 25 ˚C ∞ | 25 ˚C ∞ |

### *2.2.3.3 Restriction ligation reaction*

pUC57-sgRNA expression vector was a gift from Xingxu Huang (Addgene plasmid # 51132) (Shen et al. 2014). The vector was used for in vitro transcription of sgRNA by using the T7 promotor. Two restriction sites of BsaI were used for sub cloning of spacer into the vector, while three DraI restriction sites were used for linearization of the vector prior to in vitro transcription. It has resistance against the antibiotic Kanamycin, which was used as a selective marker. The vector sequence are attached in appendix.

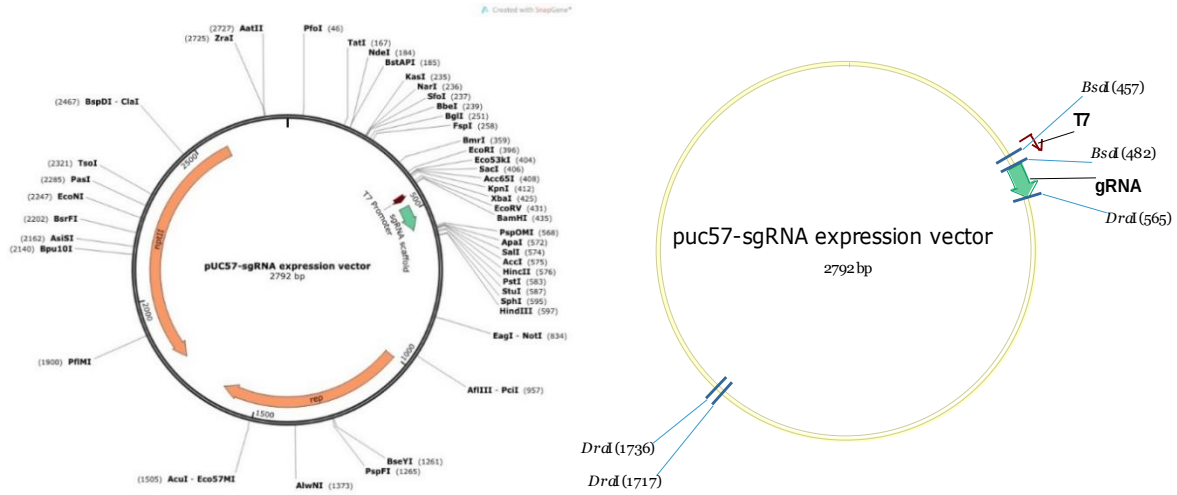


**Figure 2-2: Map of pUC57-sgRNA expression vector.**
The vector was used for T7 in vitro transcription of sgRNA. BsaI restriction sites was used during sub cloning of spacers into the vector, while DraI restriction sites were used during linearization of the vector prior to in vitro transcription. The first illustration was collected from Addgene webpage (Map Image for pUC57-sgRNA expression vector 2017), while the next was created by Dr. Amr Kataya for this thesis.

The annealed oligos were sub cloned into pUC57-sgRNA expression vector by restriction ligation reaction (golden gate) and the method used was modified from: (Xing et al. 2014). The assembling of the reactions and the reaction conditions are shown in Table 2-7. As a control, one sample with water was made instead of annealed oligos. During the reaction, the vector was digested by the restriction enzyme BsaI. The adapters at the 5' end of each oligo (Table 2-4), provides single stranded overhangs of the annealed complementary oligos, which are compatible with the overhangs from the vector after digestion by BsaI, thereby the spacers can be ligated into the vector.

**Table 2-7: Restriction ligation reaction for sub cloning of annealed oligos into pUC57-sgRNA expression vector.**
The concentration of oligos annealed with protocol A = 0.05 µmol/L and for oligos annealed with protocol B = 0.02 µmol/L.

| Component | Volume | Reaction conditions |
|---|---|---|
| Diluted annealed oligos | 2 µl | 3 h at 37 ˚C followed by 30 min at 70 ˚C. |
| pUC57, 100 ng/µl | 1 µl | |
| 10x T4 ligase buffer, Promega | 2 µl | |
| 10x BSA, NEB | 2 µl | |
| T4 ligase, Promega, 3u/µl | 0. 75 µl | |
| BsaI (HF), NEB, 20 000 u/ ml | 1 µl | |
| Nuclease-free $dH_2O$ | 11.25 µl | |
| TOTAL | 20 µl | |

#### *2.2.3.4 Cloning of the vector containing sgRNA and colony PCR*

The Restriction ligation mix "containing pUC57-sgRNA expression vectors with spacer insertion" was thereafter transformed to *E. coli* JM 109 (Section 2.2.1.1) for screening of successful reaction. From the successful colonies, overnight cultures were made followed by isolation of the vector from the bacteria (Section2.2.1.2 and Section 2.2.1.4).

During the transformation, half of the restriction ligation mix (10 µl, plasmid concentration = 5 ng/µl) was used, and all the transformed cells (500 µl) were plated on media. A colony PCR was accomplished for obtaining the correct transformed colonies (Table 2-8 and Table 2-9). This was done for three colonies of each sample-plate, one colony of the control plate, and as a second control it was done with plasmid without oligo insert (0.5 µl, 100ng/µl). The colonies were transferred to the reaction by touching the colonies with a pipette tip followed by swirling it in the tube with the other reagents. CRISPR-5 was used as forward primer for samples containing annealed oligo insert CRISPER-5 and -6 (reaction 1), while CRISPR-7 were used as forward primer for samples containing annealed oligo insert CRISPER-7 and -8 (reaction 2). M13R was used as reverse primer for all samples. The colonies from the control plate were divided in two, half used with reaction 1 and half with reaction 2. The colony PCR products were loaded on a 2 % agarose gel and analysed (Section 2.2.2).

**Table 2-8: Colony PCR.**
The assembly of a colony PCR reaction to screen for colonies containing vector with spacer insert. Reaction one was performed to screen for vectors with spacer consisting of annealed CRISPR-5 and -6, and reaction two was performed to screen for vectors with spacer consisting of annealed CRISPR-7 and -8.

| Components | Volume | Reaction 1 | Reaction 2 |
|---|---|---|---|
| 10x Dream Taq buffer, Thermo Scientific | 2 µl | | |
| dNTP mix, Bioline, 10 mM | 2 µl | | |
| Forward primer 10 µM | 2 µl | CRISPR-5 | CRISPR-7 |
| Reverse primer 10 µM | 2 µl | M13R | M13R |
| Dream Taq DNA polymerase, Thermo Scientific | 0.2 µl | | |
| Nuclease-free $dH_2O$ | 11.8 µl | | |
| TOTAL | 20 µl | | |

**Table 2-9: Colony PCR, thermocycler conditions.**

| Step | Temperature ˚C | Time | Number of cycles |
|---|---|---|---|
| Initial Denaturing | 95 | 5 min | 1 |
| Denaturing | 95 | 30 sec | 40 |
| Annealing | 60 | 30 sec | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 10 min | 1 |
| Hold | 4 | ∞ | |

#### *2.2.3.5 Sequencing*

To verify that pUC57-sgRNA expression vector contained the spacer insertion, they were sequenced at Seqlab Sequencing Laboratories (Seqlab.de) in Germany. The sequences were aligned by Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7) (Kumar et al. 2016), and the alignment was exported and generated with the help of BOXSHADE.

#### *2.2.3.6 Linearization of vector followed by invitro transcription of two sgRNA and purification of transcripts*

For in vitro transcription of sgRNA, pUC57-sgRNA expression vector containing CRISPR-5 and CRISPR-6 annealed by protocol B (without phosphorylation) and pUC57-sgRNA expression vector containing CRISPR-7 and CRISPR-8 annealed by protocol A (with phosphorylation), were used. To be able to perform in vitro transcription, the pUC57-sgRNA expression vectors containing spacer insert needs to be digested by DraI (Table 2-10). Ten units of the restriction enzyme (DraI) is used per microgram of DNA and the reaction is incubated over night at 37 ˚C.

**Table 2-10: Linearization of pUC57-sgRNA expression vector, containing spacer insert, by DraI.**
Vector A2 contains spacer CRISPR-7 and -8 annealed by protocol A, with phosphorylation. Vector B1 contains spacer CRISPR-5 and -6 annealed by protocol B, without phosphorylation.

| | **A2** | **B1** | **Incubation** |
|---|---|---|---|
| **DraI, NEB** | 50 units | 50 units | 37˚C overnight |
| **Vector with spacer insert** | 5 µg | 5 µg | |
| **10x NEB buffer** | 1x (5 µl) | 1x (5 µl) | |
| **Nuclease-free $dH_2O$** | Up to 50 µl | Up to 50 µl | |

The linearized vectors were run on a 1% agarose gel (2.2.2) and DNA from the correct size band was retrieved by gel extraction (section 2.2.2). To increase the DNA concentration before in vitro transcription, some of the water was evaporated from the samples by heating them at 60 ˚C on a heat block with the lid open. Thereafter the DNA concentration was again measured by nanodrop.

Linearized pUC57-sgRNA expression vector was in vitro transcribed using HiScribe™ T7 Quick High Yield RNA synthesis Kit (NEB #E2050S). The reactions were assembled as in Table 2-11 and incubated overnight at 37 ˚C.

**Table 2-11: In vitro transcription of linearized pUC57-sgRNA expression vector by HiScribe™ T7 Quick High Yield RNA synthesis Kit (NEB #E2050S).**
Linearized vector A2 contains spacer CRISPR-7 and -8 annealed by protocol A, with phosphorylation. Linearized vector B1 contains plasmid with spacer CRISPR-5 and -6 annealed by protocol B, without phosphorylation. The control, which is provided by the kit, is a linearized plasmid containing a gene under the transcriptional control of the T7 promotor.

| | **A2** | **B1** | **FLuc Control Template, NEB** | **Incubation** |
|---|---|---|---|---|
| **NTP buffer mix (20 mM)** | 10 µl | 10 µl | 10 µl | 37 ˚C overnight |
| **Template DNA** | 1 µg | 1 µg | 1 µg | |
| **T7 RNA polymerase mix** | 2 µl | 2 µl | 2 µl | |
| **Nuclease-free $dH_2O$** | Up to 30 µl | Up to 30 µl | Up to 30 µl | |

Thereafter the transcripts, two sgRNA, were purified by MEGAclear™ Kit Purification for Large Scale Transcription Reactions (AM1908, ambion). The optional step 7, precipitation with 5M Ammonium Acetate, in the purification protocol was not performed. After purification, the concentration and purity of the sgRNAs were measured by nanodrop. The sgRNAs were stored in -80 ˚C, in aliquots to avoid unnecessary thawing.

#### *2.2.3.7 Checking the integrity of sgRNAs with Urea-PAGE*

The integrity of the in vitro transcribed and purified sgRNAs were analysed by Urea PAGE, and the protocol used was from: (Summer et al. 2009).

The gel was premade: Mini-PROTEAN® TBE-Urea Precast Gels, Cat. #456-6033, 10% 10-well comb, 30 µl, BIO RAD and 1x TBE buffer was used as running buffer. A 0.5 L stock solution of 10x TBE running buffer (Table 2-12) was made by dissolving tris base (54. 50 g) and boric acid (27.82 g) in distilled water (300 ml). Then EDTA (20 ml of 0.5 M solution) was added and the volume of the buffer adjusted to 0.5 L with distilled water. To make a 1 L working solution of 1x TBE running buffer, distilled water (900 ml) was added to 10x TBE buffer (100 ml). Both buffers were stored at room temperature.

Samples were prepared by mixing sample buffer (2x, 5 µl) (Table 2-13) with sgRNAs diluted in nuclease-free $dH_2O$ (5-6 µg, 5 µl) followed by heating at 80 ˚C for four min. Thereafter GelRed™ (Biotium) ("1/50", 2 µl) was added to the samples, and the samples cooled on ice before loading into wells. The ladder (5 µl), GeneRuler 50 bp DNA Ladder Thermo Scientific, was only added GelRed™ (Biotium) ("1/50", 2 µl) since it is containing loading buffer when bought.

Before the samples were loaded onto the gel, the wells were rinsed to get rid of possible accumulated urea. This was done by pipetting running buffer several times up and down into the wells. The gel was pre-run at 90 V for 30 min to remove possible remaining urea and to warm up the gel. After the pre-

run and right before loading the ladder and the samples, the wells were rinsed again by pipetting as before.

**Table 2-12: 10x TBE buffer**
Amounts of chemicals to make a 0.5 L of a 10x stock solution TBE buffer.

| **10x TBE buffer** | **0.5 L** |
|---|---|
| 0.9 M Trizma® Sigma | 54. 50 g |
| 0.9 M Boric acid | 27.82 g |
| 20 mM EDTA, pH = 8 | 20 ml of 0.5 M solution |

**Table 2-13: 2x Sample buffer for Urea-PAGE**
Amounts of chemicals to make 1 ml of a 2x sample buffer to use for Urea-PAGE. In the protocol followed (Summer et al. 2009) formamide was used, but di-methyl formamide was used instead because of availability at the lab.

| **2x Sample buffer** | **1 ml** |
|---|---|
| 90 % di-methyl formamide | 900 µl |
| 0.5 % EDTA | 5 µl of 5M, pH = 8 |
| 0.1 % Xylene cyanol | 0.001 g |
| 0.1 % Bromphenol blue | 0.001 g |
| 9.3 % Glycerol | 93 µl |

### 2.2.4 Producing Cas9 mRNA

Plasmid pST1374-NLS-flag-linker-Cas9 was a gift from Xingxu Huang (Addgene # 44758) (Shen et al. 2013). It was used to in vitro transcribe Cas9 mRNA by using the T7 promotor. Open reading frame 3 contains the nucleotides (4265 bp) for Cas9. Its AgeI restriction site was used for linearization of the vector prior to in vitro transcription. It has resistance against the antibiotic Ampicillin, which was used as a selective marker. Nucleotides for a nuclear localization signal (NLS), a flag-tag and a 32-amino acid linker is located upstream of *Cas9*, and will be fused to the N-terminal of the protein after expression inside the plant cell. The NLS will direct Cas9 protein to the nucleus, where it will have access to the plants genome. The 32-amino acid linker prevent the NLS peptide to be too close to the Cas9 protein (Shen et al. 2013). The flag-tag can be used for protein detection after transfection to the plant cell.

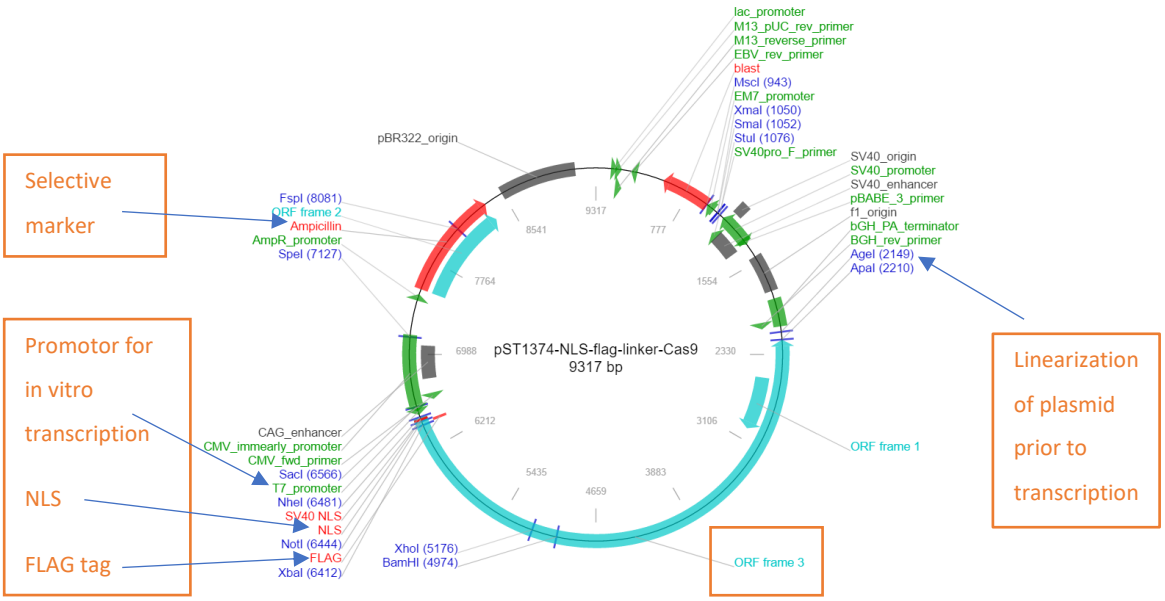


**Figure 2-3: Map of pST1274-NLS-flag-linker-Cas9.**
The vector was used for T7 in vitro transcription of Cas9 (open reading frame 3). The relevant functions of the vector for this purpose are highlighted by orange boxes and blue arrows. The illustration was collected from Addgene webpage (Full Sequence Map for pST1374-NLS-flag-linker-Cas9 2017).

### *2.2.4.1 Linearization of pST1374-NLS-flag-linker-Cas9 followed by in vitro transcription of Cas9 and purification of transcript.*

Before being able to transcribe Cas9, the plasmid needs to be linearized (Table 2-14). This was done by digesting the plasmid with the restriction enzyme AgeI (HF). Ten units of AgeI (HF) are normally used per microgram of DNA, but since there was a small amount of enzyme left in the tube, only eight units of AgeI (HF) were used per microgram of DNA. The reaction was incubated over night at 37 ˚C. It was not necessary with overnight incubation because this enzyme is high fidelity, and during pre-experiment it was seen that two hours were enough for digestion, but since these samples were made together with the sgRNA samples, which needed overnight incubation, these samples were also incubated overnight.

**Table 2-14: Linearization of PST1374-NLS-flag-linker-Cas9 by AgeI (HF).**

| | **Cas9** | **Incubation** |
|---|---|---|
| **AgeI (HF), NEB** | 40 units | 37˚C overnight |
| **Plasmid** | 5 µg | |
| **10x NEB buffer** | 1x (5 µl) | |
| **Nuclease-free $dH_2O$** | Up to 50 µl | |

The digested plasmids were run on a 1% agarose gel and DNA from the correct sized band was retrieved by gel extraction (2.2.2). To increase the DNA concentration before in vitro-transcription, some of the

water was evaporated from the samples by heating them at 60 ˚C on a heat block with the lid open. Thereafter the DNA concentration was measured again by nanodrop.

Linearized PST1374-NLS flag-linker-Cas9 plasmid was in vitro transcribed using HiScribe™ T7 ARCA mRNA Kit (with tailing) (NEB #E2060S). The linearization reaction was assembled and incubated as in Table 2-15, and the tailing reaction was assembled and incubated as in Table 2-16. The DNase treatment (step 3 in the kit protocol) was not performed.

**Table 2-15: In vitro transcription of linearized PST1374-NLS flag-linker-Cas9 by HiScribe™ T7 ARCA mRNA Kit (with tailing) (NEB #E2060S).**
The control, which is provided by the kit, is a linearized plasmid containing a gene under the transcriptional control of the T7 promotor.

| | Cas9 | CLuc Control Template, NEB | Incubation |
|---|---|---|---|
| **2x ARCA/NTP mix** | 10 µl | 10 µl | 37 ˚C for 1 h. |
| **Template DNA** | 1 µg | 1 µg | |
| **T7 RNA poly mix** | 2 µl | 2 µl | |
| **Nuclease-free $dH_2O$** | Up to 20 µl | Up to 20 µl | |

**Table 2-16: Tailing reaction in HiScribe™ T7 ARCA mRNA Kit (with tailing) (NEB #E2060S).**
IVT is the in vitro transcription reaction (Table 2-15). The control, which is provided by the kit, is a linearized plasmid containing a gene under the transcriptional control of the T7 promotor.

| | Cas9 | CLuc Control Template, NEB | Incubation |
|---|---|---|---|
| **IVT reaction** | 20 µl | 20 µl | 37 ˚C for 1 h. |
| **10x poly (A) Polymerase reaction buffer** | 5 µl | 5 µl | |
| **Poly (A) Polymerase** | 5 µl | 5 µl | |
| **Nuclease-free $dH_2O$** | Up to 50 µl | Up to 50 µl | |

The transcripts, Cas9 mRNA, were purified by RNA Clean & Concentrator™ -25 (R1017 ZymoResearch). The DNA was eluted with 50 µl nuclease-free $dH_2O$. After purification, the concentration and purity of Cas9 mRNA were measured by nanodrop. Cas9 mRNA was stored in -80 ˚C in aliquots to avoid unnecessary thawing.

#### *2.2.4.2 Checking integrity of Cas9 mRNA on 1 % denaturing agarose gel and 1 % native agarose gel.*

The integrity of the in vitro transcribed and purified Cas transcript was analysed by gel electrophoresis using both a 1 % denaturing agarose gel and a 1 % native agarose gel. The protocol for 1 % native agarose gel are described in Section 2.2.2, while the protocol for 1 % denaturing agarose gel from: (Kataya 2011) are described here.

To make a 50 ml 1 % denaturing agarose gel, agarose (0.5 g) and distilled water (36 ml) were mixed together and melted in a microwave and cooled down to ≈ 60 ˚C. MOPS buffer (10x, 5 ml) and

formaldehyde (37%, 9 ml) was mixed together and heated to 55 ˚C in a water bath. The two mixtures were thereafter mixed well, added GelRed™ (Biotium) (concentrated, 5µl) and poured immediately into the gel cassette.

1x MOPS buffer was used as running buffer. A 0.5 L stock solution of 10x MOPS (Table 2-17) buffer was made by dissolving MOPS (41.85 g) in distilled water (300 ml) and adjusting the pH to 7 by addition of NaOH. Then Sodium Acetate (4.10 g) and EDTA (10 ml of 0.5 M solution) were added and dissolved. The buffer volume was adjusted to 0.5 L by the addition of distilled water. To make a 1L working solution of 1x MOPS running buffer, distilled water (900 ml) was added to 10x MOPS buffer (100 ml). Both flask containing the buffers were wrapped in aluminium foil to avoid light, and stored at room temperature.

To prepare the samples, sample buffer 1 (10 µl) (Table 2-18) was mixed with Cas9 mRNA (2.5 µl) followed by heating at 55 ˚C for 15 min. Then sample buffer 2 (2.5 µl) (Table 2-18) and GelRed™ (Biotium) was added.

The samples and the ladder (5 µl Hyperladder™ 1 kb, (Bioline) added 2 µl "1/50" GelRed™ (Biotium)) were loaded into the wells. The gel run for 50 min at 90V. The bands were visualized by UV-light.

**Table 2-17: 10x MOPS running buffer.**
Amounts of chemicals to make a 0.5 L of a 10x stock solution MOPS buffer.

| **10x MOPS buffer** | **0.5 L** |
|---|---|
| 0.4 M MOPS, pH =7 | 41.85 g |
| 0.1 M Sodium Acetate | 4.10 g |
| 10 mM EDTA | 10 ml of 0.5 M solution |

**Table 2-18: Sample buffers for denaturing agarose gel.**
Amounts of chemicals to make 1 ml solution of Sample buffer 1 and Sample buffer 2, both used during preparation of samples for 1 % denaturing agarose gel electrophoresis.

| **Sample buffer 1** | **1ml** |
|---|---|
| 65 % Di-methyl formamide | 650 µl |
| 22 % Formaldehyde (37 %) | 220 µl |
| 13 % 10x MOPS | 130 µl |
| **Sample buffer 2** | **1 ml** |
| 50 % Glycerol | 500 µl |
| 1 mM EDTA | 2 µl of 0.5M solution |
| 0.3 % Bromphenol blue | 0.003 g |
| 0.3 % Xylene cyanol | 0.003 g |
| Nuclease-free $dH_2O$ | 492 µl |

## 2.2.5 Producing Cas9 protein

Plasmid pET-NLS-Cas9-6xHis was a gift from David Liu (Addgene plasmid # 62934) (Zuris et al. 2015b). It was used to induce expression of NLS-Cas9-6xHis protein by T7 promotor and IPTG. The plasmid has resistance against the antibiotic Ampicillin, which was used as a selective marker. Open reading frame 2 contains the nucleotides (4148 bp) for the protein. Downstream *Cas9*, the plasmid is coding for a 6xHis tag, which will be fused to the N-terminal of Cas9 after expression. This tag can be used to purify the protein during isolation. Upstream *Cas9*, the plasmid is coding for a NLS, which will be fused to the C terminal of the protein after expression. The NLS will direct the Cas9 protein to the nucleus of the plant cell, where it will have access to the plants genome.

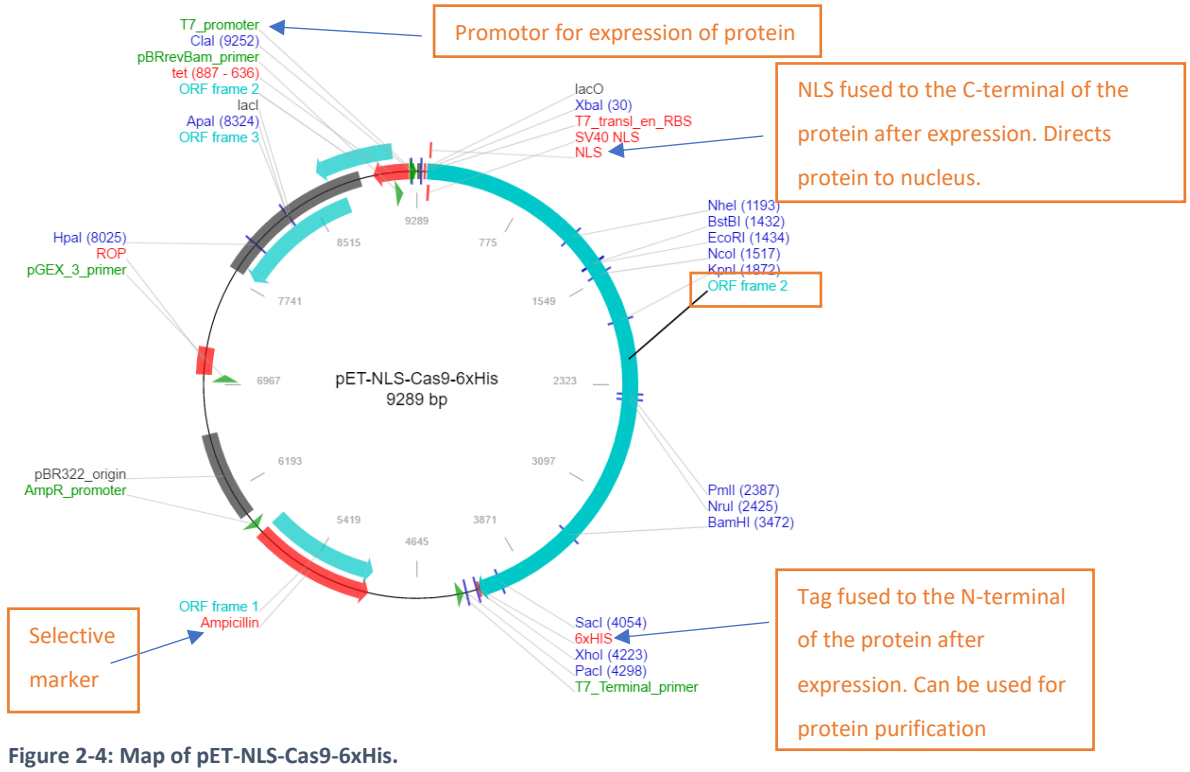


**Figure 2-4: Map of pET-NLS-Cas9-6xHis.**
The vector was used to express NLS-Cas9-6xHis protein (open reading frame 2). The relevant functions of the plasmid for this purpose are highlighted by orange boxes and blue arrows. The illustration are collected from Addgene webpage (Full Sequence Map for pET-NLS-Cas9-6xHis 2017).

### *2.2.5.1 Isolation of pET-NLS-Cas9-6xHis from Mach1 followed by transformation to expression strain BL21 Star™ (DE3).*

The plasmid, pET-NLS-Cas9-6xHis arrived in Mach1 bacterial strain, and was isolated from overnight culture as described in section 2.2.1.2 and 2.2.1.4 using MiniPrep (Machery-nagel) NucleoSpin® Plasmid (NoLid). The plasmid (1 µl, 40 ng/µl) was then transformed to the expression strain, BL21 Star™

(DE3) (50 µl) using competent cells and heat shock method as described in section 2.2.1.1. Three plates were made, one with 100 µl of transformed bacteria, one with 200 µl and the last with 300 µl. From colonies on the plates, overnight cultures were made as described in section 2.2.1.2. From the overnight cultures, glycerol stocks were made as described in section 2.2.1.3, and new cultures were made from the glycerol stocks when needed for protein production.

#### *2.2.5.2 Expression of NLS-Cas9-6xHis.*

The protein was expressed using and optimizing a method previously described (Zuris et al. 2015a). Protocol for isolation and purification of the expressed protein exploiting the 6xHis-tag, can also be found at Zuris et al. (2015a).

1. **Overnight culture from glycerol stock of BL21 Star™ (DE3) containing pET-NLS-Cas9-6xHis.**
   Inoculated BL21 Star™ (DE3) containing pET-NLS-Cas9-6xHis in LB Broth (5ml) containing 100 µl/ml ampicillin with shaking (250/220 rpm) at 37 ˚C overnight (≈16 h).
   - When making overnight culture from glycerol stock 1 and 2, they were streaked on LB agar containing 100 µl/ml ampicillin, and the plates incubated at 37 ˚C until the next day. Then some of the colonies were used to make the overnight cultures as described in section 2.2.1.2.
   - When making overnight culture from glycerol stock 3, the glycerol stock (15 µl) was directly added to LB Broth (5ml) containing 100 µl/ml ampicillin, instead of making colonies beforehand.
2. **Dilute, grow and measure OD.**
   The overnight culture was diluted 1:100 into the same growth medium, and the cells were grown with shaking (230 rpm) at 37 ˚C until the $OD_{600}$ ≈ 0.6. Then the culture was incubated for another 30 min at approximately 20 ˚C (or room temperature and stirring by hand when the incubator did not work). Big autoclaved Erlenmeyer flask were used, to have enough space for air during growth.
   - Culture 1: Erlenmeyer flask (1000 ml) were filled with LB Broth (MILLER, Merck) (200 ml) containing 100 µl/ml ampicillin and overnight culture 1 (2 ml) were added.
   - Culture 2: Erlenmeyer flask (1000 ml) were filled with LB Broth (MILLER, Merck) (200 ml) containing 100 µl/ml ampicillin and overnight culture 2 (2 ml) were added.
   - Culture 3: Erlenmeyer flask (200 ml) were filled with LB Broth (MILLER, Merck) (50 ml) containing 100 µl/ml ampicillin and overnight culture 3 (500 µl) were added.

**3. IPTG induction**

Before adding IPTG to the culture, some of the culture was removed to use as control "non-induced" samples. The cells were collected by centrifugation and stored at -80 ˚C.

- Culture 1 (5 ml) centrifuged at 8000 xg for 1 min.
- Culture 2 (5 ml) centrifuged at 8000 xg for 1 min.
- Culture 3 (10 ml) centrifuged at 5000 xg for 4 min.

The rest of the culture were added IPTG to a concentration of 0.5 mM to induce Cas9 expression and were incubated approximately 16 h with shaking.

- Culture 1 and 2: Shaking = 230 rpm. Temperature = 24.3 ˚C for 45 min (because of non-working incubator), then 20 ˚C until next day.
- Culture 3: Shaking = 200 rpm. Temperature = 20 ˚C

After incubation, the cells were collected by centrifugation.

- Culture 1 (5 ml) centrifuged at 5000 xg for 2 min.
- Culture 2 (5 ml) centrifuged at 5000 xg for 2 min.
- Culture 3 (10 ml) centrifuged at 5000 xg for 4 min.

**4. Re-suspension of cells in lysis buffer**

Three different lysis buffers were used (Table 2-19), and some were also added lysozyme.

- Culture 1: Cells (from 5 ml culture) were re-suspended in lysis buffer (2.5 ml).
- Culture 2: Cells (from 5 ml culture) were re-suspended in PBS (2.25 ml) and lysozyme to a concentration at 1 mg/ml, followed by incubation at 30 min on ice.
- Culture 3: Cells (from 10 ml culture) were re-suspended in RB buffer (4.5 ml) and lysozyme to a concentration at 1 mg/ml, followed by incubation at 30 min on ice.

**5. Sonication**

The cells were thereafter lysed by sonicated, Branson Digital Sonifier® 250 (Branson Ultrasonic Corp., Danbury, CT). During sonication, the tubes were standing in ice to avoid overheating.

- Culture 1: 30 sec 30 % amp x 4-5 times for non-induced sample and x 6 times for induced sample. Between each sonication, the samples were cooled on ice.
- Culture 2: 30 sec pulse-on, 30 sec pulse-off at 20 % amp x 5 times
- Culture 3: 30 sec pulse-on, 30 sec pulse-off at 20 % amp x 5 times

6. **Centrifugation**

   The soluble lysate was obtained by centrifugation at 20 000 xg for 30 min. The pellets were again re-suspended in lysis buffer.

   - Culture 2: Cells were re-suspended in PBS (250 µl).
   - Culture 3: Cells were re-suspended in RB (250 µl).

**Table 2-19: Three different lysis buffers used during expression of the Cas9 protein.**

| Solution | Content concentration | Volume | Amount | Sterilization |
|---|---|---|---|---|
| Lysis buffer<br><br>(Zuris et al. 2015a) | 50 mM Tris (hydroxymethyl)-aminomethane (Tris)-HCl, ph=8<br><br>10 mM TCEP-HCl<br>1M NaCl<br>20 % glycerol | 100 ml | 10 ml 0.5M<br><br>0.28665 g<br>5.844 g<br>20 ml | Filter sterilized. Stored in room temperature. |
| Tris (hydroxymethyl)-aminomethane (Tris)-HCl, ph=8 | 0.5 M Trizma® Sigma<br><br>Adjusted pH = 8 with 37 % HCl | 500 ml | 30.285 g | Autoclaved |
| Re-suspension buffer<br><br>(Kataya 2011) | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br><br>Adjusted pH = 7.8 with NaOH | 100 ml | 0.7801 g<br>1.7532 | No |
| PBS<br><br>(Chazotte 2012) | 137 mM NaCl<br>2.7 mM KCl<br>10 mM $Na_2HPO_4$<br>1.8 mM $KH_2PO_4$<br><br>pH = 7.4 | 1L | 8 g<br>0.2 g<br>1.44 g<br>0.24 g | No |

### *2.2.5.3 SDS-PAGE*

Proteins from induced and non-induced cells were compared using SDS-PAGE, as well as proteins from soluble lysate and proteins from pellet.

The samples were re-suspended in Læmmli Sample buffer (BIO-RAD) containing β-Mercaptoethanol (β-ME) (Sigma) according to the description on the box. The samples were then heated at 95 ˚C for 1 min before cooling down on ice for 1 min followed by pulse-spin for some sec.

- Soluble lysate (75 µl) were added 4x Læmmli Sample buffer containing β-ME (25 µl).

- Pellets re-suspended in lysis buffer (75 µl) were added 4x Læmmli Sample buffer containing β-ME (25 µl).
- Pellets were added 2x Læmmli Sample buffer containing β-ME (20 µl) and nuclease-free $dH_2O$ (10 µl).

Proteins in samples were separated using SDS-PAGE with TGS (Tris/Glycine/SDS) buffer system. The gel was readymade Mini-PROTEAN® TGX™ Precast Gels (BIO-RAD) 12 %, 10 well comb, 30 µl /well Cat. # 456-1043. Ladder used was Precision Plus Protein™ WesternC™ standards (BIO-RAD). Samples (20 µl) and ladder (5µl) were slowly added to the wells, and the gel was run for 1-2 h at 100V.

The gel is inside two plastic covers, which was broken by hand to release it after the run. The gel was then rinsed by running tap water a few times before staining the proteins with Comassie Blue by completely covered the gel in the solution and incubate on a flipper for approximately 1 h. Thereafter the gel was de-stained by de-staining solution by completely covered the gel in the solution and incubate on a flipper for ½ h before replacing the solution with new one and incubate overnight.

**Table 2-20: Staining and de-staining solution for SDS-PAGE**
Chemicals and amounts to make 500 ml solutions.

| Solution | Content | Volume | Amounts |
|---|---|---|---|
| Coomassie Blue staining solution | Coomassie Blue R-250<br>Methanol<br>Acetic Acid | 500 ml | 0.5 g<br>200 ml<br>50 ml |
| De-staining solution | Ethanol<br>Acetic acid | 500 ml | 200 ml 95%<br>50 ml concentrated |

## 2.2.6 Protoplasts isolation and PEG transformation

### *2.2.6.1 Sowing seeds and growing plants*

*A. thaliana* Col-0 WT seeds were both sowed on ½ Murashige and Skoog (MS) medium containing 1 % sucrose and on soil.

Before sowing on media, the seeds were sterilized following a protocol previously described (Kataya 2011): Some seeds were put in an Eppendorf tube. 70 % ethanol with 0.01 % triton (1 ml) were added, and the tube incubated on a shaker for 15 min. The solution was then replaced by pure ethanol (1 ml) and incubated on a shaker for 10 min. The ethanol was removed and pure ethanol (1 ml) was added a second time and the tube inverted a few times, before removing the ethanol again. To dry the seed for remains of ethanol, the tube was left in the sterile hood with the lid open, before sealing the lid

with parafilm and placing it in 4˚C for storing. During the sterilization, the tube was only opened in the sterile hood.

½ MS medium containing 1 % sucrose without vitamins and solidified by Agar-agar (Merck) were made according to (Table 2-21). Before adding Agar-agar (Merck), the pH was adjusted to 5.8 by adding KOH solution. The media was autoclaved for 20 min at 110 ˚C.

Autoclaved magenta boxes were filled with 45 ml media, and sterile squared Petri dishes were filled with 50 ml media. Four seeds were sown in each magenta box and approximately 44 seeds in each Petri dish. They were seal with parafilm and put cold and dark for two days before putting them in growth chamber, some of them in 12-h light growth chamber and the others in 8-h light growth chamber.

**Table 2-21: MS-medium (Murashige and Skoog 1962).**
The first two columns contain chemicals and amount for stock solution needed to prepare MS-medium. Column number three contains amounts from stock solution, sucrose, and Agar-agar (Merck) to make 500 ml ½ MS solidified medium.

| **Stock solutions:** | | **500 ml ½ MS medium:** |
|---|---|---|
| $KNO_3$ for 1 l | 95 g | 5 ml |
| $NH_4NO_3$ for 1 l | 120 g | 3.25 ml |
| $MgSO_4$:$7H_2O$ for 1 l | 37 g | 2.5 ml |
| $KH_2PO_4$ for 1 l | 17 g | 5 ml |
| $CaCl_2$:$2H_2O$ for 1 l | 44 g | 2.5 ml |
| Fe-EDTA solution for 1 l: | | 12.5 ml |
| $Na_2$: EDTA | 0,373 g | |
| $FeSO_4$:$7H_2O$ | 0,278 g | |
| Minor I for 1 l: | | 2.5 ml |
| $ZnSO_4$:$7H_2O$ | 0,920 g | |
| $H_3BO_3$ | 0,620 g | |
| $MnSO_4$:$4H_2O$ | 2,230 g | |
| Minor II for 1 l: | | 2.5 ml |
| $Na_2MoO_4$:$2H_2O$ | 0,025 g | |
| $CuSO_4$:$5H_2O$ | 0,003 g | |
| $CoCl_2$:$6H_2O$ | 0,003 g | |
| KI | 0,083 g | |
| 1 % Sucrose | | 5 g |
| Agaragar | | 3.5 g |

Non-sterilized seeds were sown on soil containing vermiculite (3:1). In each box 5 -10 seeds were sown. After approximately 10 days, plants were removed to obtain maximum five plants in each box. The plants were grown in a 12-h growth chamber. The first week after sowing the plants were covered by plastic to increase the humidity. The plants were watered with water and 1x Hoagland solution when needed.

Sterilization of leaves before protoplast isolation was done by immersing 10-12 leaves in 70 % ethanol for up to 60 seconds in a falcon tube (size 50 ml), before transferring them to a petri dish onto 0.5 % sodium hypochlorite solution so that the surface was covered for 20 min. The leaves were then washed with autoclaved distilled water three times in the sterile hood. After sterilization, they were transferred to a petri dish containing 0.5 M Mannitol, ready for protoplast isolation.

#### *2.2.6.2 Mesophyll protoplast isolation*

Protoplasts were isolated from *A. thaliana* leaves following a protocol optimized from previously described protocols (Woo et al. 2015), (Yoo et al. 2007), and (Kataya 2011). Centrifuge used during isolation was JS, 7.5 Rotor, Beckman Coulter, Avanti J-26S XP. The solutions used during the optimization of the protocol and for the final protocol are listed in Table 2-22.

1. **Digestion of leaves:**

   One by one a leaf was transferred to a petri dish in a specific orientation (upper part down) onto 0.5 M mannitol. The lamina of the leaf was cut in narrow strips using a sharp razor blade. Afterwards the cut leaf was transferred to another petri dish in wrong orientation (upper part down) onto enzyme solution (10 ml), and then flipped to correct orientation (upper part up). Between 15-30 leaves were used (depending on the size of the leaves) until the petri dish was full. The petri dish was sealed with parafilm and incubated in dark at room temperature. Next day the petri dish was gently shaken to release protoplasts.
2. **Purification of protoplasts:**

   The enzyme solution containing the protoplasts were filtrated through a 75 µm nylon mesh into a 15-ml falcon tube using a 5-ml pipette (enlarged opening). W5 solution (4 ml) was added to the remains, the dish gently shaken to release the rest of the protoplasts and the solution filtrated into the same tube.
3. **To collect the protoplasts** in a pellet, the flow through from the filtration were centrifuged at 600 rpm for 1 min at 14 ˚C. The supernatant was removed by using a 5-ml pipette (enlarged opening).
4. **Distinguish between broken and intact protoplasts and washing of the intact once:**

   CPW 21S (10 ml) were carefully added to the pellet by a 5-ml pipette (enlarged opening), and the pellet was re-suspended by slowly inverting the tube. The solution was centrifuged at 600 rpm for 7 min at 14 ˚C to distinguish between broken and intact protoplasts. The protoplast floating on CPW 21S (upper layer/band) were transferred to a new 15-ml falcon tube by a 1000-µl pipette (enlarged opening).

   W5 solution (5 ml) were carefully added to the upper layer by a 5-ml pipette (enlarged opening) and mixed by slowly pipetting or inversing the tube. The solution was centrifuged at

700 rpm for 2 min at 14 ˚C. The supernatant was removed by using a 5-ml pipette (enlarge opening). Some of the lower part of the supernatant were still green and not removed. Washing was done two times. The second time, as much supernatant as possible were removed.

5. **Washing of protoplasts (only if step 4 was not performed):**

   W5 solution (5ml) was carefully added to the protoplasts by a 5-ml pipette (enlarged opening) and mixed by slowly inversing the tube. The protoplasts were collected in a pellet by centrifugation at 600 rpm for 1 min at 14 ˚C. The supernatant was removed by using a 5-ml pipette (enlarge opening), as much as possible without touching the pellet.

6. **Counting of protoplasts:**

   The protoplasts were re-suspended in W5 solution (5 ml) and counted under a light microscope using a haemocytometer.

7. **Dilution of protoplasts:**

   The protoplasts were incubated on ice for 30 min up to 1 h. During this time, the protoplasts are collected into a pellet due to gravity. The supernatant was removed by using a 5-ml pipette (enlarged opening), as much as possible without touching the pellet. The protoplasts were then re-suspended in MaMg solution (ice cold) to a concentration of 500 000 protoplasts pr. 300 µl of MaMg solution or 250 000 protoplasts pr. 300 µl of MaMg solution (both was done).

Table 2-22: Solutions used for protoplast isolation.

| Solution | Content concentration | Volume | Amounts | Sterilization |
|---|---|---|---|---|
| **Enzyme:** | 1.0% cellulase R10<br>0.5% macerozyme R10<br>0.45 M mannitol<br>20 mM MES [pH 5.7]<br><br>Before use, there were added 0.1 ml 1 M CaCl2 and 10 mg BSA to 12.5 ml enzyme solution and filter sterilized again. | 50 ml | 0.5 g<br>0.25 g<br>4.10 g<br>2 ml 500 mM | Filter sterilized. Stored at 4 ˚C if used the next couple of days, otherwise stored at -20 ˚C in 12.5 ml-aliquots. |
| **Enzyme number 2.** | Cellulase (SIGMA)<br>Pectinase (SIGMA)<br>0.4 M Mannitol<br>20 mM KCl<br>20 mM MES, pH = 5.7<br><br>Dissolved completely (for 30-60 min) at room temperature, and then it was added:<br>10 mM $CaCl_2$<br>Bovine Serum Albumin (BSA) | 50 ml | 75 mg<br>50 mg<br>40 ml 0.5 M<br>1 ml 1 M<br>2 ml 500 mM<br><br><br><br><br>0.5 ml 1M<br>50 mg | Filter sterilized. Stored at 4 ˚C if used the next couple of days, otherwise stored at -20 ˚C in 12 ml-aliquots. |
| **CPW** | 27.2 mg/L $KH_2PO_4$<br>101 mg/L $KNO_3$<br>1480 mg/L $CaCl_2$, $2H_2O$<br>246 mg/L $MgSO_4$, 7 $H_2O$<br>0.16 mg/L KI<br>0.025 mg/L $CuSO_4$, $5H_2O$<br><br>pH 5.8<br>CPW 21S = CPW + 21 % sucrose | 500 ml | 0.0135 g<br>0.0505 g<br>0.7500 g<br>0.1230 g<br>80 µl (1mg/ml)<br>12.5 µl (1mg/ml)<br><br><br>105 g | Autoclaved. Stored at 4 ◦C. After adding sucrose, filter sterilized or autoclaved. |
| **W5** | 2 mM MES [pH 5.7]<br>154 mM NaCl<br>125 mM $CaCl_2$<br>5 mM KCl | 500 ml | 0.1952 g<br>4.4999 g<br>6.9363 g<br>0.1864 g | Filter sterilized. Stored at 4˚C. |
| **W5 number 2.** | NaCl<br>$CaCl_2$<br>KCl<br>MES<br>Glucose | 250 ml | 7.7 ml 5 M<br>31.25 ml 1 M<br>1.25 ml 1 M<br>1 ml 500 mM<br>0.2252 g | Filter sterilized or autoclaved. Stored at 4˚C. |
| **MMG** | 0.4 M mannitol<br>15 mM $MgCl_2$<br>4 mM MES [pH 5.7] | 100 ml | 7.2869 g<br>0.1428 g<br>0.0781 g | Filter sterilized. Stored at 4˚C. |
| **MaMg** | 0.5 Mannitol<br>15 mM $MgCl_2$ x$6H_2O$<br>0.1 % MES<br><br>Adjusted the pH to 5.7 with KOH. | 50 ml | 4.555 g<br>0.152 g<br>0.100 g | Filter sterilized. Stored at 4˚C. |
| **Mannitol** | 0.5 M mannitol | 500 ml | 45.542 g | Autoclaved. Stored at 4˚C. |

#### *2.2.6.3 PEG Transformation of protoplasts*

For PEG transformation of isolated protoplasts a previously described protocol (Kataya 2011) was followed and optimized. Centrifuge used during transformation was JS, 7.5 Rotor, Beckman Coulter, Avanti J-26S XP. The solutions used during optimization of the protocol and for the final protocol are listed in Table 2-23. Transformation of fluorescence DNA was used to check the transformation ability, before transformation of CRISPR-Cas9 pre-assembled RNP complex and transcript was performed to obtain genetic modification.

1. **DNA/RNA/Protein** was added in the centre of a small petri-dish (30 µl). If sgRNA and Cas9 protein → mixed and incubation for 10 min at room temperature to assemble RNP complex.
2. **Protoplasts** re-suspended in MaMg solution (300 µl, 250 000 or 500 000 protoplasts) was added and mixed with the DNA/RNA/Protein by a 1000 µl pipette (enlarged opening).
3. **Transformation step:** PEG 4000 (500 µl) was dropwise added to the mixture with a 1000 µl pipette (not enlarged opening) at approximately 5 cm highs. Incubation for 30 min at room temperature with the lid on.
4. The mixture was then **diluted** stepwise (each 5 min) with W5 solution (0.5 ml + 1 ml + 2 ml + 3 ml → in total 6.5 ml). W5 was added dropwise in the same manner as PEG 4000.
5. The diluted mixture was then transferred to a 15-ml falcon tube using a 5-ml pipette (enlarged opening) and protoplasts collected by centrifuged at 500 rpm for 1 min (or 2 min) at 14˚C. After removing the supernatant, the protoplasts were re-suspended in 1x B5 solution (with addition of glucose and hormones) (3 ml) and transferred to a new small petri-dish and incubated at dark in room temperature until the next day.
6. Microscopy (Confocal, Nikon A1R): to check transformation ability and the survival of protoplasts.

Table 2-23: Solutions used for protoplast PEG transfection.

| Solution | Content concentration | Volume | Weights | Sterilization |
|---|---|---|---|---|
| **PEG 4000** | 40 % [w/v] PEG 4000 (Merck)<br>0.2 M mannitol<br>0.1 M $CaCl_2$. | 10 ml | 4 g<br><br>4 ml 0.5 M<br>1 ml 1M | Filter sterilized. Should be fresh or used within 5 days. |
| **PEG 6000 (Amr)** | 0.4 M mannitol<br>0.1 M $Ca(NO_3)_2$ x2$H_2O$<br>0.1 % MES<br>Adjusted the pH to 8 with NaOH.<br>PEG 6000 (Aldrich) | 50 ml | 3.65 g<br>1.181 g<br>0.05 g<br><br>20 g | Filter sterilized. Should be fresh or used within 5 days. |
| **W5** | 2 mM MES [pH 5.7]<br>154 mM NaCl<br>125 mM $CaCl_2$<br>5 mM KCl | 500 ml | 0.1952 g<br>4.4999 g<br>6.9363 g<br>0.1864 g | Filter sterilized. Stored at 4 ˚C. |
| **W5 number 2 (Amr)** | NaCl<br>$CaCl_2$<br>KCl<br>MES<br>Glucose | 250 ml | 7.7 ml 5 M<br>31.25 ml 1 M<br>1.25 ml 1M<br>1 ml 500 mM<br>0.2252 g | Filter sterilized or autoclaved. Stored at 4˚C. |
| **W1** | 0.5 M mannitol<br>20 mM KCl<br>4 mM MES (pH 5.7) | 100 ml | 4.5543 g<br>0.0746 g<br>0.0390 g | Filter sterilized. Stored at 4˚C. |
| **B5 containing glucose and hormones** | 1x B5<br><br><br><br>0.45M glucose<br>1 mg/L 2.4-D<br>0.15 mg/L BAP<br><br>Adjusted the pH to 5.7 with KOH. | 50 ml | 500 µl of all the 100x stock solutions (Table 2-24)<br><br><br><br>4.0036 g<br>50 µl 1mg/ml<br>7.5 µl 1mg/ml | Filter sterilized. Stored at 4˚C. |

## 2.2.7 Regeneration of protoplasts

Protoplasts were tried to be regenerated using and optimizing a previously described protocol (Woo et al. 2015). Only the first part of the protocol was performed. The rest of the protocol including shoot development from micro-calli, rooting and transfer to soil can be found at Woo. et. al (2015). Solutions used are listed in Table 2-24 and Table 2-25.

1. Re-suspended transfected protoplast in 0.5x B5 culture medium (containing supplement) to a concentration of 2.5x $10^5$ protoplast/ml.
2. Thereafter the protoplasts were embedded in a thin layer of agarose in a small Petri dish by mixing protoplast solution (1 ml) with 0.5x B5 containing 2.4 % agarose (1 ml).

3. After solidification of the agarose, it was overlaid with liquid 0.5x B5 culture medium (4 ml). The plate was then sealed with parafilm and incubated in dark at 25 ˚C.
4. After 7 days, the liquid medium was replaced with fresh liquid medium.
5. The culture was then transferred to light (16 h light and 8 h darkness) at 25 ˚C until micro-calli appeared.

**Table 2-24: Gamborg B5 Medium**

| | **Concentration in Gamborg medium (1x)** | | **Stock solutions (100x)** | **Volume of stocks to make 1L Gamborg (1x)** |
|---|---|---|---|---|
| **Macro elements** | **mM** | **Mg/L** | **g/100 ml** | |
| $CaCl_2x2H_2O$ | 1.02 | 150 | 1.5 g | 10 ml |
| $KNO_3$ | 24.73 | 2500 | 25 g | 10 ml |
| $MgSO_4x7H_2O$ | 1.01 | 250 | 2.5 g | 10 ml |
| $NaH_2PO_4$ $x2H_2O$ | 1.09 | 170 | 1.7 g | 10 ml |
| $(NH_4)2SO_4$ | 1.01 | 134 | 1.34 g | 10 ml |
| **FeNa EDTA** | 0.100 | 36.7 | 0.367 g | 10 ml |
| **Micro elements** | **µM** | **Mg/L** | **g/1000ml** | |
| $CoCl_2$ $x6H_2O$ | 0.11 | 0.025 | 0.0025 g | 10 ml |
| $CuSO_4$ $x5H_2O$ | 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 | |
| $MnSO_4$ $x1H_2O$ | 59.16 | 10.00 | 1.0 g | |
| $Na_2MoO_4$ $x2H_2O$ | 1.03 | 0.25 | 0.025 g | |
| $ZnSO_4$ $x7H_2O$ | 6.96 | 2.00 | 0.20 g | |

**Table 2-25: Solution used to regenerate protoplasts.**

| **Solution** | **Content concentration** | **Volume** | **Amounts** | **Sterilization** |
|---|---|---|---|---|
| **0.5x B5** | 0.5x B5 | 50 ml | 25 ml 1x B5 | Filter sterilized. Stored at 4˚C |
| **0.5x B5 with 2.4% sucrose** | 0.5x B5<br>2.4 % sucrose | 50 ml | 25 ml 1x B5<br>1.2 g | Filter sterilized before adding the sucrose. Stored at 4˚C |
| **5x B5 with supplement** | 0.5x B5<br>375 mg/L $CaCl_2$ $x2H_2O$<br>270 mg/L Sodium succinate<br>103 g/L Sucrose<br>18.35 mg/LNaFe EDTA<br>0.2 mg/L 2.4-D<br>0.3 mg/L BAP<br>0.1 g/L MES | 50 ml | 25 ml 1x B5<br>0.01875 g<br>0.01350 g<br>5.15 g<br>91.7 µl 10 mg/ml<br>10 µl 1 mg/ml<br>15 µl 1 mg/ml<br>51 µl 0.098g/ml | Filter sterilized. Stored at 4˚C |

### 2.2.8 Isolation of total genomic DNA from protoplasts

Total genomic DNA was isolated from both transfected protoplasts and non-transfected protoplasts. DNA from transfected protoplasts was further on used for T7 endonuclease I assay, while DNA from non-transfected protoplast was further on used for in vitro digestion by Cas9 nuclease. The isolation of DNA was performed by following a previously described protocol (Springer 2010) with some adjustments in the first three steps.

Un-transfected protoplasts re-suspended in MaMg solution (500 µl, 800 000 protoplasts) and transfected protoplasts re-suspended in 1x B5 solution (with addition of glucose and hormones) (3 ml, 250 000 protoplasts and 500 000 protoplasts) were carefully transferred to 1.5 ml Eppendorf tubes using 1000 ml pipette (enlarged opening). To collect the protoplasts, they were centrifuged at 40 xg for 2 min and the supernatant was discarded. Since protoplast easily break, there was no need to freeze-dry and grind them to be able to open the cells. CTAB plant extraction buffer (700 µl) (Table 2-26) was therefore directly added to the protoplasts and mixed by inversion, followed by incubation at 65 ˚C for 30 min. In case of a 3-ml protoplast solution, the solution was split in two 1.5 ml sized Eppendorf tubes, protoplast collected by centrifugation, half of the CTAB plant extraction buffer (350 µl) were added and then the two tubes combined before incubation to get the total volume of 700 µl. This was done to be able to collect all the protoplasts in the protoplast solution without excess centrifugation, which could break the protoplasts and lead to leakage of DNA into the supernatant to be discarded. To get a higher concentration of DNA at the end of the extraction, the DNA pellet was re-suspended in a volume of 100 µl instead of 250 µl. The rest of the steps were according to the protocol.

The DNA concentration was measured by nanodrop and the quality checked on 1% agarose gel according to section 2.2.2.

**Table 2-26: CTAB plant extraction buffer used during isolation of total genomic DNA from protoplasts (Springer 2010).**

| Solution | Content concentration | Volume | Amounts | Sterilization |
|---|---|---|---|---|
| Cetyltrimethyl ammonium bromide (CTAB) plant extraction buffer | 100 mM Tris, pH 7.5<br>700 mM NaCl<br>10 mM EDTA<br>1 % CTAB<br>1 % βME* | 1L | 100 ml 1 M<br>140 ml 5 M<br>20 ml 0.5 M<br>10 g | Autoclaved |
| * β-ME (100 µl) is added to CTAB plant extraction buffer (10 ml) directly before use and mixed well. | | | | |

### 2.2.9 Amplify regions of *PDS3* containing genomic target sites.

From isolated total genomic DNA, two regions of *PDS3* containing the gene specific part (target site) of the two sgRNA were amplified using PCR and site-specific primer.

Primes (Table 2-27) were design using the website CHOPCHOP. The gene ID from TAIR (AT4G14210.1) together with species (*Arabidopsis thaliana*) and use (CRISPR-Cas9) were used as query. Next a list of possible spacers is given, and the correct ones which were used to make sgRNAs were located. Then CHOPCHOP gives several options for primers to be used to amplify regions that includes target site. In addition, the gene location of the amplified PCR product is shown (Appendix, Figure 7-4).

Assembling for the two reactions are shown in Table 2-28 and the thermocycler conditions are shown in Table 2-29. After amplifying, a small amount of the PCR products was analysed by running a 2 % agarose gel and comparing the bands to a ladder to verify size and correct amplification. Thereafter the rest of the PCR reactions were run on a 2 % agarose gel, followed by gel extraction for retrieval of the DNA (Section 2.2.2).

**Table 2-27: Primers to amplify regions of *PDS3* containing genomic target site.**
The blue sequence of genomic target sites is the PAM sequence, while the black sequence is the gene specific part of sgRNA.

| Gene | PCR | Primer name | | Primer sequence 5`-3` | PCR amplification size | Genomic target site |
|---|---|---|---|---|---|---|
| ***PDS3*** | 1 | CRISPR-55 | F | GCTGAAATGTTCTGTGGTTGAA | 243 | ATAAGCCTGACCGCCGACCATGG |
| | | CRISPR-56 | R | ACTCAATAGCCTACTTGCCTGC | | |
| | 2 | CRISPR-57 | F | CACTTTCATCTGGAGGTTGTGA | 231 | CGCTTAAGACAAGAACAAGGAGG |
| | | CRISPR-58 | R | CTCTAGCTCTGGCCTTGGAATA | | |

**Table 2-28: PCR reactions to amplify regions of *PDS3* containing genomic target site.**

| Components | Reaction 1 | Reaction 2 |
|---|---|---|
| Template DNA | 0.09 µg - 0.1 µg | 0.09 µg - 0.1 µg |
| Forward primer | 1 µM CRISPR 55 | 1 µM CRISPR 57 |
| Reverse primer | 1 µM CRISPR 56 | 1 µM CRISPR 58 |
| 10x DreamTaq buffer | 5 µl | 5 µl |
| dNTP mix, 2 mM each | 5 µl | 5 µl |
| DreamTaq DNA polymerase (5u/µl) | 1.25 u (0.25 µl) | 1.25 u (0.25 µl) |
| Nuclease-free $dH_2O$ | To 50 µl | To 50 µl |

**Table 2-29: Thermocycle condition used when amplifying region of *PDS3* containing genomic target site.**

| Step | Temperature ˚C | Time | Number of cycles |
|---|---|---|---|
| Initial denaturing | 95 | 3 min | 1 |
| Denaturing | 95 | 30 sec | 38 |
| Annealing | 60 | 30 sec | |
| Extension | 72 | 1 min | |
| Final extension | 72 | 5 min | 1 |
| Hold | 12 | ∞ | |

### 2.2.10 In vitro digestion of DNA by Cas9 nuclease

PCR product amplified from genomic DNA isolated from non-transfected protoplasts were in vitro digested by Cas9 nuclease together with sgRNA. Protocol following enzyme NEB # M0386 from New England biolabs was followed with some modifications (In vitro digestion of DNA with Cas9 Nuclease, S. pyogenes (M0386) 2017).

1. During the digestion reaction, the molar ratio of Cas9 nuclease and sgRNA per target site should be at 10:10:1. Therefore, 1 µM stock-solutions for Cas9 nuclease and sgRNAs and 100 nM stock solutions for PCR-products, were prepared beforehand.
2. A digestion reaction was assembled as in Table 2-30.
    a. An RNP complex was first assembled by pre-incubation of Cas9 nuclease (NEB #M0641T) and sgRNA for 10 min at 25 ˚C, together with nuclease free water and Cas9 nuclease buffer.
    b. Then the substrate DNA was added to the reaction.
    c. The reaction was mixed thoroughly and pulse-centrifuged, before incubation for 1 h at 37 ˚C.
3. After the digestion reaction, the sgRNA of the reaction was degraded by adding 3 µl of a 10 mg/ml stock solution of RNase A (Thermo Scientific). The reaction was mixed by pipetting and incubated for 1 h at 37 ˚C.
4. The fragments were then analysed using a 2 % agarose gel (Section 2.2.2), and all the reaction (23 µl) was loaded onto the gel.

**Table 2-30: In vitro digestion of DNA by Cas9 nuclease**

| Components | Volume | Final concentration |
|---|---|---|
| Nuclease-free $dH_2O$ | 12 µl | |
| 10x Cas9 Nuclease buffer NEB | 2 µl | |
| 1 µM sgRNA | 2 µl | 100 nM |
| 1 µM Cas9 nuclease NEB #M0641T | 2 µl | 100 nM |
| | Pre-incubate for 10 min at 25 ˚C | |
| 100 nM substrate DNA | 2 µl | 10 nM |
| Total reaction volume | 20 µl | |

## 2.2.11 T7 Endonuclease I assay

PCR product amplified from genomic DNA isolated from CRIPSR Cas9 transfected protoplasts were analysed for mutations using T7 Endonuclease I assay. Protocol following enzyme NEB # M0302 from New England biolabs was followed with some modifications (Determining Genome Targeting Efficiency using T7 Endonuclease I (M0302) 2017).

1. First the PCR product were denatured and re-annealed. This was done by assembling the annealing reaction according to Table 2-31 and expose the reaction for the hybridization conditions in Table 2-32 using a thermocycler.
2. Next, the annealed hybrid PCR products were digested.
   This was done by adding T7 Endonuclease I (NEB #M0302) (1 µl) to the re-annealed PCR-products (19 µl), and incubated for 15 min at 37 ˚C.
3. The fragments were analysed using a 2 % agarose gel (Section 2.2.2).

**Table 2-31: Annealing reaction, T7 Endonuclease I assay.**

| Components | Amounts and volumes |
|---|---|
| DNA | 200 ng |
| 10x NEB Buffer 2 | 2 µl |
| Nuclease-free $dH_2O$ | Up to 19 µl |

**Table 2-32: Hybridization condition for thermocycler, T7 Endonuclease I assay.**

| Step | Temperature ˚C | Time or Ramp rate |
|---|---|---|
| Initial Denaturing | 95 | 5 min |
| Annealing | 95-85<br>85-25 | -2˚C/sec<br>-0.1˚C/sec |
| Hold | 4 | Hold |

# 3 RESULTS

## 3.1 Generation of sgRNAs transcript against *PDS3,* and generation of Cas9 transcript.

To be able to generate a site specific DSB in the genome of *A. thaliana* using CRISPR-Cas9, the different components in the system needed to be generated beforehand. The first goal was to generate sgRNAs containing spacer against *PDS3* in *A. thaliana*.

Two sgRNAs containing spacer against *PDS3* were made according to section 2.2.1. First, the complementary oligos for the spacers were designed (Section 2.2.3.1), the oligos annealed by two different methods (Section2.2.3.2) and sub cloned into pUC57-sgRNA expression vector by restriction ligation reaction (Section 2.2.3.3). The Restriction ligation mix "containing pUC57-sgRNA expression vectors with spacer insertion" was thereafter transformed to *E. coli* JM 109 (section 2.2.1.1) for screening of successful restriction ligation reaction.

As a control, there was one sample with water instead of annealed oligos during restriction ligation reaction. On the control-plate (Figure 3-1), after transformation of the restriction ligation mix to *E. coli* JM 109, some colonies were growing. This indicates that some plasmids were not digested or were closed again after digestion, and thereby the plasmid could be transformed into competent bacteria cells, which gives the bacteria the Kanamycin resistant and therefore it could grow colonies on the media containing kanamycin. Further on, this mean some colonies on the sample-plates could contain plasmid, but without spacer insert.

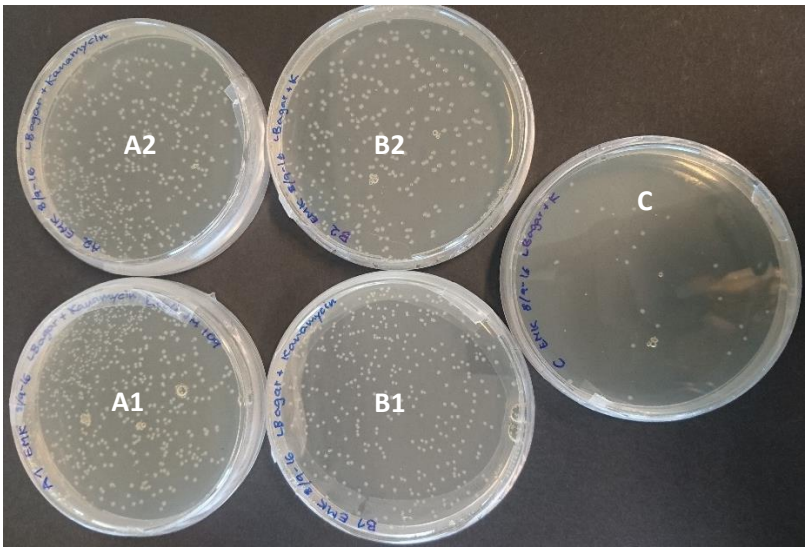


**Figure 3-1: *E. coli* JM 109 transformed with pUC57-sgRNA expression vector after restriction ligation reaction with annealed oligos.**
The bacteria are plated on LB agar with kanamycin (50 µl/ml). Sample A1 contains phosphorylated CRISPR – 5 and -6 spacer. Sample B1 contains non-phosphorylated CRISPR – 5 and -6 spacer. Sample A2 contains phosphorylated CRISPR – 7 and -8 spacer. Sample B2 contains non-phosphorylated CRISPR – 7 and -8 spacer. Sample C is a control sample, where water was added instead of oligos when performing restriction ligation reaction.

A colony PCR according to section 2.2.3.4, was accomplished for obtaining the correct transformed colonies. The spacer region was used as forward primer, and the reaction should only yield PCR product if the spacer was successfully ligated into the plasmid. The colony PCR products were loaded on a 2 % agarose gel (Figure 3-2). Most of the colonies gave a band around 200 bp, which was the expected size for the PCR product (Figure 3-3). Not all the colonies gave a band at this size, and probably contained bacteria with plasmid without spacer insertion. The control samples were positive and did not give a band at this size.

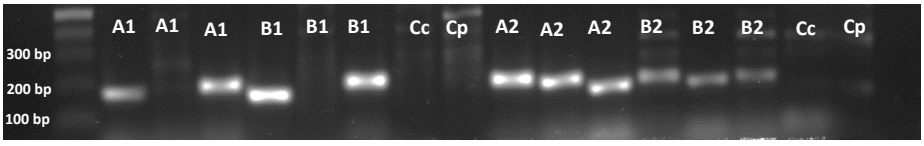


**Figure 3-2: Colony PCR products on a 2 % agarose gel.**
Bands are compared to Quick-load® 100 bp DNA Ladder, NEB. 1x TAE buffer is used as running buffer. GelRed™ (Biotium) is used to visualize the bands under UV-light. Sample 1 contains CRISPR -5 and -6 spacer. Sample 2 contains CRISPR -7 and -8 spacer. Sample Cc are colony from control plate, where water was used instead of spacers during restriction ligation. Sample Cp are controls containing plasmid without insert (0.5 µl, 100ng/µl). Sample A contains phosphorylated spacer. Sample B contains non-phosphorylated spacer.

/Taggtgagaccgagagaggtctca/gttttagagctagaaatagcaagttaaaataaggctagtccgttatca
acttgaaaaagtggcaccgagtcggtgctttttttaaagggcccgtcgactgcagaggcctgcatgcaagcttgg
cgtaatcatggtcatagctgtttcctg

➔ NNNNNNNNNNNNNNNNNNNNgttttagagctagaaatagcaagttaaaataaggctagtccgtta
tcaacttgaaaaagtggcaccgagtcggtgctttttttaaagggcccgtcgactgcagaggcctgcatg
caagcttggcgtaatcatggtcatagctgtttcctg

**Figure 3-3: Expected size of PCR product from colony PCR, 174 bp.**
The first sequence is a part of the pUC57 expression vector (5`→ 3`) containing BsaI restriction site (pink) and reverse complementary sequence of M13R primer (blue), The slashes ("/") indicates where the restriction enzyme cuts inside the restriction site. The next sequence is the pUC57 expression vector (5`→ 3`) after restriction ligation reaction and therefore containing the 20-nucleotide long spacer (yellow) plus the four nucleotides added to the 5` end of the oligo (red). The size of this sequence is 174 bp.

From the successful colonies, the plasmids were isolated (section 2.2.1.4), using MiniPrep (Machery-nagel) NucleoSpin® Plasmid (NoLid). To verify that the isolated plasmids contained the spacer inserts, they were sequenced (Section 2.2.3.5). Plasmid containing non-phosphorylated spacer 1, plasmid containing phosphorylated spacer 2, and plasmid containing phosphorylated spacer 1 were positive (Figure 3-4, the whole alignment can be found in appendix). The experiment was successful, both spacer 1 and spacer 2 were successfully sub cloned into the pUC57-sgRNA expression vector.

```
pUC57-sgRNA   454 CTATAGGT-GAGACCGAGAGAGGGTCTCA-GTTTTAGAGCTAGAAATAGCAAGTTAAAAT
gRNAb1       1016 CTATAGGATAAGCCTGACCGCCGACCA---GTTTTAGAGCTAGAAATAGCAAGTTAAAAT
gRNAa2       1019 CTATAGGC---GCTTAAGACAAGAACAAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
gRNAa1       1021 CTATAGGATAAGCCTGACCGCCGACCA---GTTTTAGAGCTAGAAATAGCAAGTTAAAAT
```

**Figure 3-4: Alignment of pUC57-sgRNA expression vector sequences after sub cloning of spacers.**
Sequence one, is the vector without any spacer insert. The next sequences are the vector containing spacer B1, A2 and A1, where the number one represents annealed oligos CRISPR -5 and -6, the number two represent annealed oligos CRISPR – 7 and -8, the letter A represent phosphorylated spacers, and the letter B represent non-phosphorylated spacers.

The next goal was to generate transcripts of the sgRNAs and transcript of Cas9, which further on directly could be used to produce DNA-free genetic edited plants where *PDS3* is knocked out. Then, in order to generate sgRNA transcript from the pUC57-sgRNA expression vector containing successfully sub cloned spacers, and Cas9 transcript from the PST1374-NLS flag-linker-Cas9 vector, the two plasmids needed to be linearized. This was first done with a small amount of plasmids, to test the restriction enzymes DraI and AgeI (HF), respectively. The digested samples were run on a 1% agarose gel (Figure 3-5). In addition to the digested samples, non-digested plasmids were run as a control. For an undigested plasmid, there could be more than one band, due to the coiling of plasmids, resulting in different shapes and therefore different migration ability. For a linearized plasmid, there should only be one band. The undigested PST1374-NLS flag-linker-Cas9 vector is showing three bands on the gel, while the digested sample are only showing one band on the gel, with a size between 8000 bp and 10

037 bp (Figure 3-5). This size is corresponding to the actual size of the linearized plasmid which is 9317 bp (Figure 2-3) which indicates that the digestion with AgeI (HF) worked. The undigested samples of pUC57-sgRNA expression vector are showing several bands on the gel (Figure 3-5), which is how undigested plasmids act. The pUC57-sgRNA expression vector contains three restriction sites for DraI (see Figure 2-2 for map). A fully digested plasmid will therefore show two bands, one around 1600 bp, one around 1100 bp and one too short to appear on the gel. For digested A1 sample, there are two bands showing, one band around 1500 bp and one band around 1000 bp. For digested A2 and B1 sample, there are one extra band between 2500 bp and 3000 bp. This is indicating that sample A1 is fully digested, while sample A2 and B1 still contained some plasmids only partly digested. Only the band containing T7 promotor and sgRNA near the end of the sequence can be used for in vitro transcription, which is the band around 1600 bp (see Figure 2-2 for map). To avoid partly digested plasmid, the incubation was extended to overnight instead of 2 h during the actual linearization (section 2.2.3.6 and 2.2.4.1).

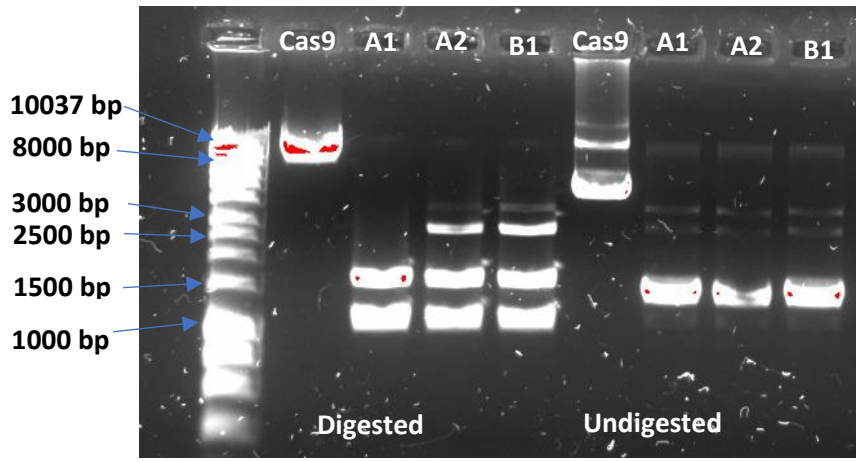


**Figure 3-5: 1 % agarose gel containing PST1374-NLS flag-linker-Cas9 digested by AgeI (HF) and pUC57-sgRNA expression vector digested by DraI.**
Bands are compared to Hyperladder™ 1 kb, (Bioline). 1x TAE buffer is used as running buffer. GelRed™ (Biotium) is used to visualize the bands under UV-light. The four first samples are digested while the four last samples are undigested samples used as a control. Sample A1 contains non-phosphorylated CRISPR -5 and -6 spacer. Sample A2 contains phosphorylated CRISPR -5 and -6 spacer. Sample B1 contains non-phosphorylated CRISPR -7 and -8 spacer.

After linearization of the vectors, the DNA from the correct sized band for sgRNA (1621 bp) and Cas9 (9317 bp) were retrieved by gel extraction by GenElute™ Gel Extraction Kit (NA1111), Sigma-Aldrich. The sgRNAs were in vitro transcribed from the linearized pUC57-sgRNA expression by HiScribe™ T7 Quick High Yield RNA synthesis Kit (NEB #E2050S), and the transcripts were purified by MEGAclear™ Kit Purification for Large Scale Transcription Reactions (AM1908, ambion). Cas9 was in vitro transcribed from linearized PST1374-NLS flag-linker-Cas9 plasmid by HiScribe™ T7 ARCA mRNA Kit (with tailing) (NEB #E2060S). The kit also provided the produced transcript with tailing and capping, which is needed

further on during the expression of the protein in vivo. The Cas9 mRNA transcript was purified by RNA Clean & Concentrator™ -25 (R1017 ZymoResearch). Transcription of both sgRNA and Cas9 were performed twice in order to have enough during CRISPR-Cas9 experiments.

The integrity of in vitro transcribed sgRNAs were analysed using a denaturing urea polyacrylamide gel (Section 2.2.3.7), without success. Difficulties raised when loading the samples and the ladder (50 bp Gene ruler Thermo Fisher Scientific Group), they would not sink nicely into the wells. Maybe accumulated urea was not completely removed when rinsing the wells, or maybe there was something wrong with the running buffer. What was supposed to be done was to load the samples and the ladder, then run the gel at 90 V for around 2 – 4 h. Thereafter UV-light should have visualized the band.

During another experiment (Section 3.5) the sgRNAs (the once transcribed the second time) were analysed using a 2 % agarose gel (Figure 3-14), with success. The two sgRNAs are showing sharp bands with no smear, which are indicating good quality RNA with no degradation. The size of the bands are around 100 bp, which is corresponding to the actual size which should be 105 bp (Figure 3-6).


**Figure 3-6: Expected size of the two sgRNA, 105 bp.**
pUC57 expression vector containing T7 promotor sequence (green and red), 20-nucleotide spacer sequence (yellow) with four nucleotides added at the 5`end (red) and DraI restriction site (blue). The slash ("/") indicates where the restriction enzyme cuts inside the restriction sites. T7 RNA polymerase starts transcription at the bolded g and transcribes until it reaches the end of the sequence, which is at the cutting site inside the DraI restriction site due to digestion with DraI restriction enzyme. The size of the sequence from "bolded g" to "slash" is 105 bp.

The integrity of in vitro transcribed Cas9 mRNA was analysed using a 1 % denaturing (Formaldehyde) agarose gel (Section 2.2.4.2) (Figure 3-7). In vitro transcriptions were done two times, and both transcripts were analysed. The amount of Cas9 mRNA loaded on the gel is different between the two samples. From the first transcribed Cas9 mRNA, only 0.61 µg was loaded, while from the second transcribed Cas9 mRNA, 1.89 µg was loaded. It is possible to see a weak white band between 4000 bp and 5000 bp for both the samples, Cas9 mRNA sample with highest amount of Cas9 mRNA being the least weak band. The Cas9 mRNAs were also analysed using a 1% native agarose gel (Figure 3-8). This time the same amount of Cas9 mRNA (1.88-1.89 µg) were loaded for both the transcripts. One sharp

band for both samples are shown, which indicated intact mRNAs. When comparing the bands to the ladder, they are bigger than 4000 - 5000 bp, which was the size shown on the denaturing gel. mRNAs in their secondary structure will migrate differently in the gel, compared to when denatured. The actual size of Cas9 mRNA should be 4265 bp (open reading frame 3 of PST1374-NLS flag-linker-Cas9 plasmid).

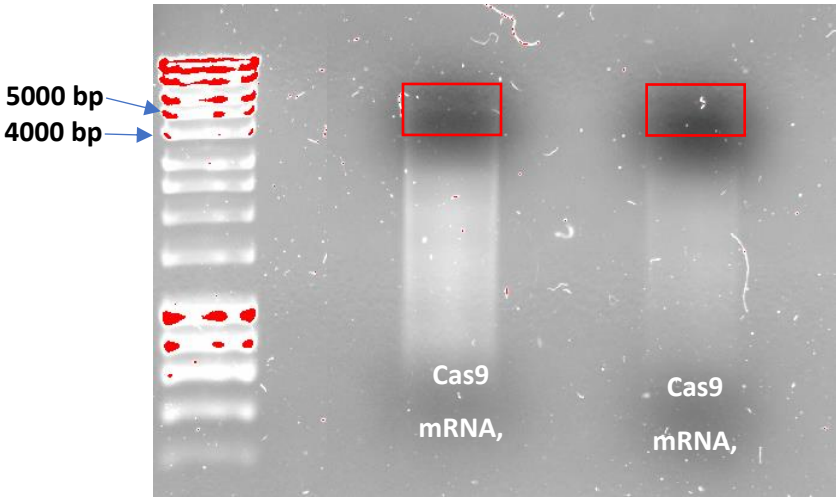


**Figure 3-7: Cas9 mRNA loaded on 1 % denaturing (formaldehyde) agarose gel.**
Bands are compared to Hyperladder™ 1 kb, (Bioline). 1x MOPS buffer is used as running buffer. GelRed™ (Biotium) is used to visualize the bands under UV-light. In vitro transcription were performed twice; the first well contains second transcript of Cas9 mRNA (1.89 µg), while the second well contains first transcript of Cas9 mRNA (0.61 µg). One very weak white band can be seen between 4000 bp and 5000 bp for both samples.

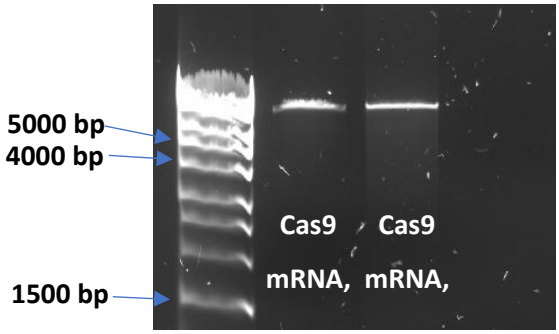


**Figure 3-8: Cas9 mRNA loaded on 1% native agarose gel.**
Bands are compared to Hyperladder™ 1 kb, (Bioline). 1x TAE buffer is used as running buffer. GelRed™ (Biotium) is used to visualize the bands under UV-light. In vitro transcription were performed twice; the first well contains second transcript of Cas9 mRNA (1.89 µg), while the second well contains first transcript of Cas9 mRNA (1.88 µg). One sharp band is showing for both samples.

## 3.2 Cas9 protein expression

The third goal, during the generation of the components for CRISPR-Cas9 genomic editing, was to express Cas9 and isolate and purify the protein, which in complex with sgRNA further on, directly could be used to produce DNA-free genetic edited plants where *PDS3* is knocked out.

Before being able to express the protein, the plasmid pET-NLS-Cas9-6xHis encoding the NLS-Cas9-6xHis protein was transformed to the expression strain BL21 Star™ (DE3) according to Section 2.2.5.1. To optimize the protocol for expression of the protein, several experiments were performed.

**Firstly,** expression of Cas9 protein was induced by IPTG and the protein content for pellet of induced samples were compared to protein content for pellet of non-induced samples by SDS-PAGE. Two overnight cultures of BL21 Star™ (DE3) containing pET-NLS-Cas9-6xHis were made according to section 2.2.1.2. The next day the cultures were diluted into same growth media to a final $OD_{600} \approx 0.8$. To allow the bacteria cells to grow and adapt to the new media, they were diluted a bit more and incubated on a shaker (250 rpm) at 37 ˚C for 30 min. "Non-induced" cells were collected (1.5 ml) by centrifugation (max speed, 2 min) and stored at -20 ˚C. To another 1.5 ml of the cultures, IPTG (4.5 µl, 1M) were added to make "Induced samples". They were incubated on a shaker (250 rpm) at 23 ˚C overnight before collecting the cells by centrifugation (max speed for 2 min). The protein content of the pellets from all samples, both induced and non-induced samples, were analysed using SDS-PAGE (method section 2.2.5.3). No expression was seen from the gel. When making overnight culture, the bacteria cells could have passed the lag phase and entered death phase. After diluting the cultures next day, they were only incubated for 30 min and this could have been too short time to get a good growing culture. Another reason could be that the protein may have been in the soluble lysate and not in the pellet.

**Secondly,** the protocol for protein expression section 2.2.5.2 was followed. From culture 1, the soluble lysate from non-induced and induced cells, as well as the cells (pellet) were analysed for protein content by SDS-PAGE (section 2.2.5.3). The marker worked well. The soluble lysate showed no bands, no proteins. The induced pellet sample was bigger than the non-induced pellet sample, and during the SDS-PAGE, the induced pellet sample gave a sharp smear (a lot of colour) and thereby no specific bands could be seen. The non-induced pellet sample gave some clear bands. The proteins in the supernatant could be not showing because of too high dilution. Otherwise, it could be no proteins in the sample because of incomplete lysis of cells.

**Third,** two different lysis buffers were used and compared. In addition to lysis buffer lysozyme used. The protocol for protein expression section 2.2.5.2 was followed, culture 2 and 3. The soluble lysate

from non-induced and induced cells, as well as the cells (pellets) re-suspended in lysis buffer from both cultures, were analysed for protein content by SDS-PAGE (section 2.2.5.3). The gel shows a clear and sharp band around the size of 150 kDa from the pellet for all the induced cultures, as well as from the soluble lysate for the induced culture 3 (Figure 3-9). For the non-induced cultures, this band is not seen or not as sharp.

Two approaches were used for theoretical size prediction of the NLS-Cas9-6xHis protein. Open reading frame 2 of the plasmid was translated to protein by addgene. The protein consisted of 1383 amino acids.

- The average weight of an amino acid residual is 110 Da. By multiplying number of amino acid residuals with the average weight, the protein weight is 152,130 Da, which is the same as 152 kDa.
- From the amino acid sequence, the theoretical protein weight was also calculated using http://web.expasy.org/compute_pi/. The tool calculated the protein weight to be 160 kDa.

The size of the protein bands "around 150 kDa" seen on the gel for the induced cultures correlates with the size of the theoretical protein size of NLS-Cas9-6xHis "152 kDa or 160 kDa", which indicates that the protein was successfully expressed. The isolation and purification of the expressed protein was not performed.

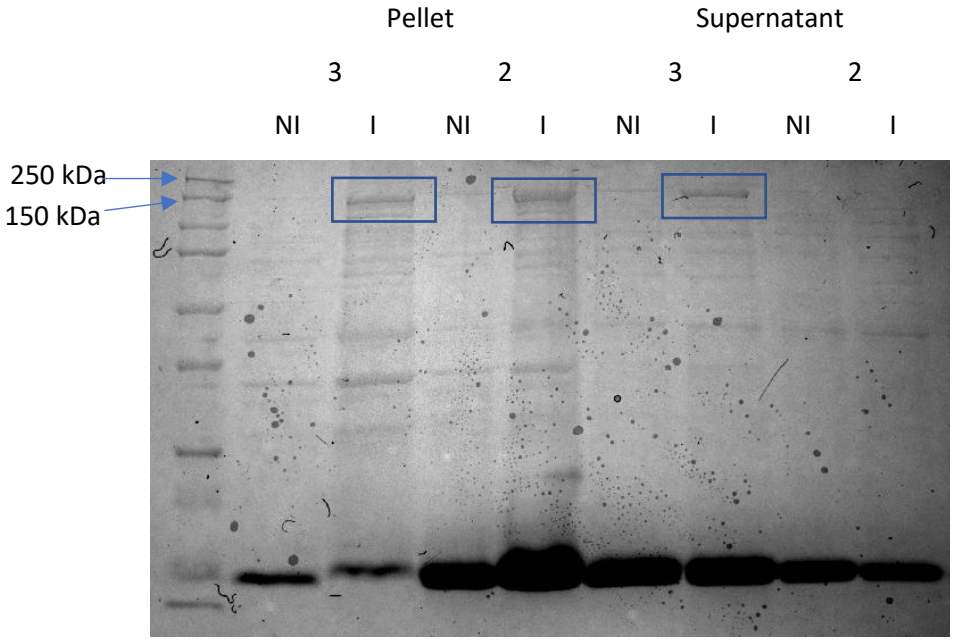


**Figure 3-9: SDS PAGE**
Protein expression from IPTG induced (I) and non-induced (NI) cultures of expression strain BL21 Star™ (DE3) transformed with pET-NLS-Cas9-6xHis. Two different lysis buffers were used beforhand, PBS for culture 2 and RB buffer for culture 3. Both pellet and supernatant are analysed. Ladder used was Precision Plus Protein™ WesternC™ Standards, BIO RAD.

## 3.3 Isolation and transfection of protoplasts.

To be able to directly deliver the components for CRISPR-Cas experiments to *A. thaliana* plant cells, protoplasts and PEG-mediated transfection were used.

Isolation of protoplasts was performed several times to optimize the protocol (section 2.2.6.2). Because of the need for bigger leave size, only the plants grown on soil were used and not the plants grown on ½ MS-medium. During the first isolation (8 weeks old plants), the digested leaves were filtrated into a big falcon tube (50 ml). After addition of CPW 21S and centrifugation (step 4), the top-layer consisting of floating protoplasts was very thin, and difficult to collect. Therefore, the top-layer and pellet were re-suspended and transferred to a smaller falcon tube (15 ml), and the centrifugation repeated. The top-layer then became more concentrated and easier to collect. During the following isolations, the digested leaves were filtrated into this smaller falcon tube (15 ml).

The top-layer and pellet (during the first isolation) were analysed using light microscope. It was seen that the top-layer consisted of intact protoplasts, only some of them were broken, while most of the protoplasts in the pellet were broken. However, during another experiment, the isolation of protoplasts (7 weeks old plants) was done in two parallels; one sample where it was distinguished between intact and broken protoplasts using CPW 21S treatment (step 4), and one sample where this step was skipped and they were washed directly after purification and collection (step 3 → step 5). When analysing the protoplasts under microscope, there was seen a lot of protoplasts from the sample without CPW 21S treatment (step 4), and there were surprisingly many intact protoplasts. It was counted to be approximately 250 000 intact protoplasts/ml in the sample treated with CPW 21S (Figure 3-10) and approximately 500 000 intact once in the sample without CPW 21S treatment. This was done during another isolation (4 weeks old plants) as well, and the same was seen; it was counted to be approximately 300 000 intact protoplasts/ml in the sample treated with CPW 21S (Figure 3-10) and approximately 550 000 intact protoplasts/ml in the sample without CPW 21S treatment.

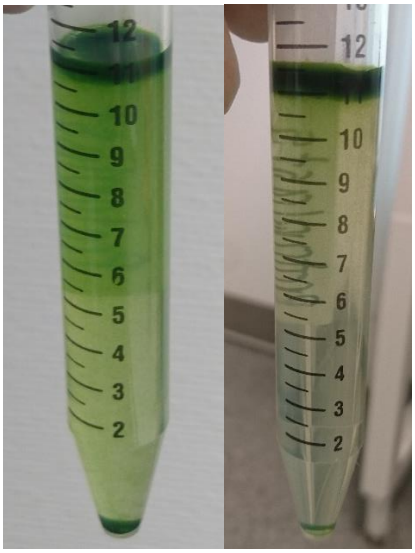

**Figure 3-10: Isolated protoplasts during CPW 21S treatment.**
On the first image, isolated mesophyll protoplasts from seven weeks old plants are shown, while on the second image isolated mesophyll protoplasts from four weeks old plants are shown. By the addition of CPW 21S solution, the healthy protoplasts are floating to the top, while the broken protoplasts remain in the pellet. However, during comparison in parallel to samples without CPW 21S treatment, higher numbers of intact protoplasts was seen for the samples without CPW 21S treatment.

PEG transformation of isolated protoplasts (Section 2.2.6.3) was done several times without success, so the protocol needed to be optimized. First protocol from Woo et al. (2015) was used. After overnight incubation, the protoplasts were analysed under a microscope (Confocal, Nikon), but almost all of them were dead. The transfection was performed the day after isolation, and not at the same day. Therefore, it was tried to do it again with the same protocol, but this time at the same day of isolation. The protoplast gave a nice band after addition of CPW 21S and it was counted to be approximately 300 000 intact protoplasts/ml. After transformation and overnight incubation, the protoplasts were analysed under microscope (Confocal, Nikon), but again almost all of them were dead.

It was decided to try another protocol (Kataya 2011), but with the solutions from the first protocol (Woo et al. 2015). The protoplasts kept on dying during transformation, even though they looked nice when counting them before transformation. It was believed that PEG could be harming the protoplasts, and make them break. Therefore, parallel experiments were run with and without PEG treatment, but there was no difference. This was done for both PEG 4000 and PEG 6000. It was believed there could be a problem with the solutions, and many of the solutions were made again or shifted out. It was tried with the solutions from both protocols (Woo et al. 2015; Kataya 2011). MaMg solution were used instead of MMG solution. Different solutions for the incubation overnight was tried; W1, 0.5xB5 and 1xB5. Two different enzyme solutions were tried, with enzymes from different companies. Two different W5 solutions were tried. It was also tried to not sterilize the leaves beforehand, comparing old and new leaves, and comparing CPW 21S treatment with no CPW 21S treatment. During two of several experiments, some protoplasts survived:

- Experiment one: seven weeks old plants, sterilized leaves, CPW 21S were both used and not used in parallel, MaMg solution, PEG 6000 solution, and both W1 and 1xB5 were used in

parallel. This gave approximately 5 % survival, and for the survival once it was seen that the transformation worked (Figure 3-11). There was seen no differences between the solution used in parallel.

- Experiment two: four weeks old plants, not sterilized leaves, CPW 21S were both used and not used in parallel, enzyme solution number 2, W5 solution number 2, MaMg solution, both PEG 4000 and no PEG was used in parallel, and W1 solution was used. During this experiment, most of them were dead, but there were found some surviving, which also were transformed. It was thought that it might be better without CPW 21S solution.

Further on, there was a suspicion that centrifugation could be an issue, therefore the centrifugation speed and time were shortened during both the isolation and transformation. Addition of CPW 21S and the following centrifugation at 600 rpm for 7 min, and the double washing, were skipped to avoid excessive centrifugation. It was also made a new solution for overnight incubation for the transformed protoplasts, 1x B5 containing glucose and hormones. The leaves were not sterilized beforehand and W5 solution 2, enzyme solution 2 and MaMg solution were used. Both young leaves (4 weeks old) and old leaves were used. When analysing the transformed protoplasts after incubation under the microscope (confocal laser-scanning microscope, Nikon A1R) almost all of them were alive, both from the sample containing young leaves and the sample containing old leaves. The experiment was repeated with the same success, and pictures were taken both before and after transformation (Figure 3-12 and Figure 3-13) using light microscope.

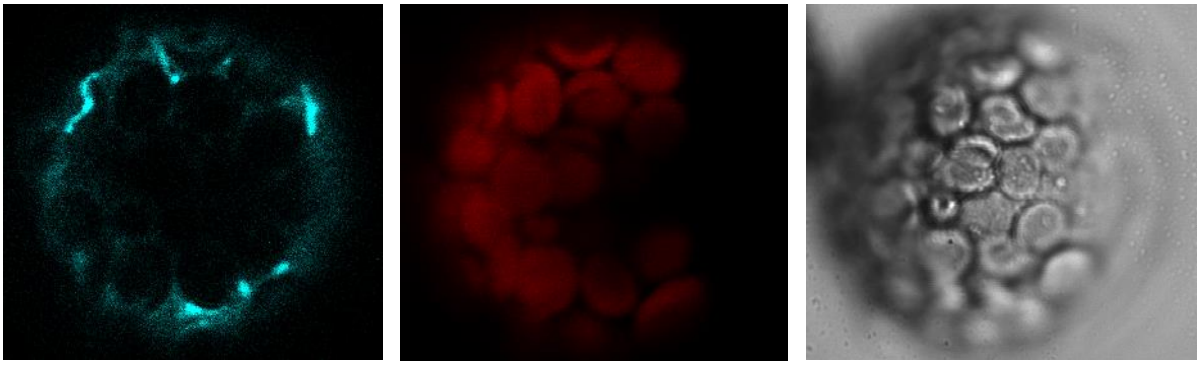

**Figure 3-11: Isolated protoplast from *A. thaliana* leaves undergone PEG-mediated transformation and visualised by a confocal laser-scanning microscope (Nikon A1R).**
On the first image, the protoplast is transiently expressing cyan fluorescent protein (CFP) confirming transformation ability. On the second image, the chloroplasts auto fluorescence of the protoplast is visualized. On the third image, white light is used to visualise the protoplasts.

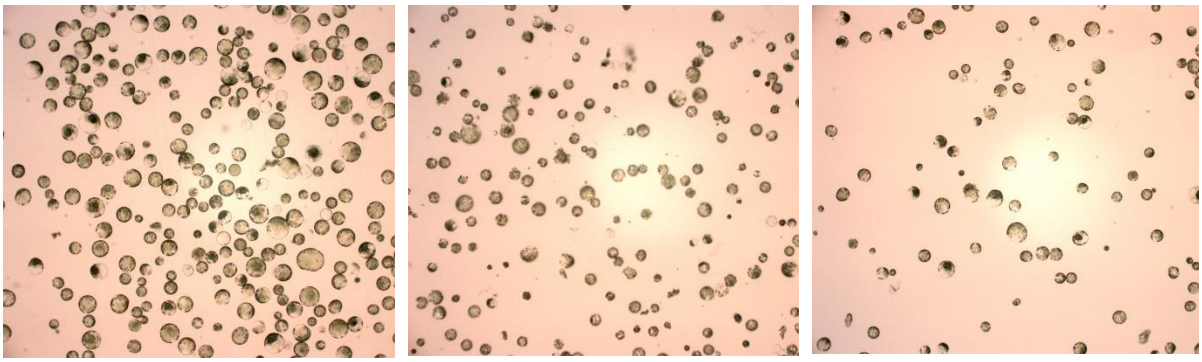

**Figure 3-12: Isolated protoplasts from *A. thaliana* leaves visualised by a light microscope.**

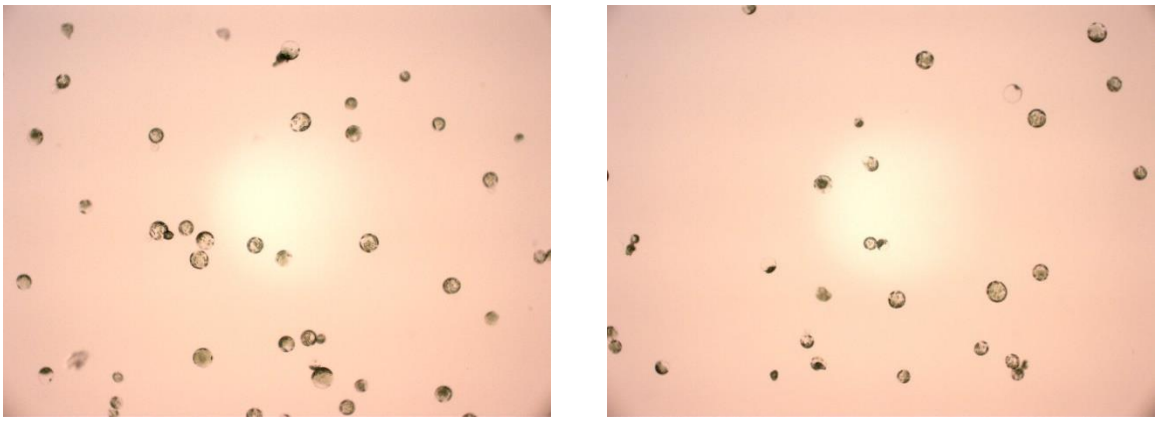

**Figure 3-13: Isolated protoplasts from *A. thaliana* leaves after undergone PEG-transformation and visualised by a light microscope.**

## 3.4 Protoplast regeneration

To be able to obtain DNA- free genetic modified plants, a tissue culture system to regenerate the protoplasts is needed. Protoplast regeneration was tried two times (Section 2.2.7). The first time, isolated protoplast which had not undergone transformation was used. The first-time mixing protoplasts re-suspended in 0.5x B5 (with supplement) with 0.5x B5 containing 2.4 % agarose, it was done in a falcon tube (15 ml). The agarose solidified quickly, and got stuck in the tube. Therefore, it was tried to mix the two solutions directly in a 6-wells plate. After solidification of the agarose, 0.5x B5 was added on top of the layer. It was then seen that not all the protoplasts were embedded in the agarose, some were floating on top. The plate was sealed with parafilm and incubated in dark at 25 ˚C. After one week, the liquid media was replaced by new fresh liquid media, and the plate transferred to light (16-h light and 8-h dark growth chamber). After three weeks in light, the plate was checked for callus formation, but all the protoplasts were gone. The second time, isolated protoplast that had undergone transformation were used. This time, the protoplast re-suspended in 0.5x B5 (with

supplement) were mixed with 0.5x B5 containing 2.4 % agarose in a small petri-dish. After one week incubation in dark, it was seen that the plate had been contaminated. Further trial and optimization of the protocol was postponed after checking the success of transient CRISPR digestion, but time was too limited to complete.

## 3.5 In vitro digestion of *PDS3* by Cas9 nuclease

To check the functionality of the CRISPR-Cas9 components i.e. sgRNAs and Cas9 protein, an in vitro digestion of the genomic target site was performed. Total genomic DNA were isolated from non-transfected protoplasts (Section 2.2.8) and two regions of *PDS3* containing the gene specific parts of sgRNAs were amplified using PCR and site-specific primer. The two PCR products were thereafter in vitro digested by Cas9 nuclease (NEB, #M0641T) after binding of sgRNA to its gene specific part (Section 2.2.10).

The in vitro digested PCR products were run on a 2 % agarose gel (Figure 3-14, A). Both in vitro digested PCR products show a band between 200 bp and 250 bp. The size correlates to the size of undigested PCR products (Figure 3-16), which for PCR product 1 containing gene specific part of sgRNA B1, should be 243 bp and which for PCR product 2 containing gene specific part of sgRNA A2, should be 231 bp. Both samples also show a band around 100 bp. This band was suspected to be sgRNA instead of digested PCR product. Therefore, another gel was run loading the undigested PCR products and the sgRNAs separately (Figure 3-14). This gel shows that the sgRNAs are visible on the gel around the size of 100 bp, which supports the suspicion. For PCR product 2 containing gene specific part of sgRNA A2 digested in vitro with Cas9 nuclease, a third band appears between 100 bp and 150 bp. This band is believed to be one of the two fragments from digested PCR product. Digested PCR product should give one fragment at the size of 154 bp and one at the size of 77 bp. This sample was therefore believed to be positive and sgRNA A2 believed to be working together with Cas9 nuclease and give successful in vitro digestion of DNA.

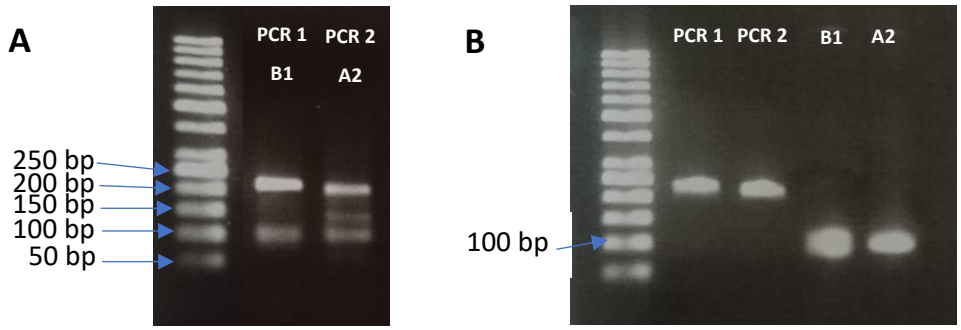


**Figure 3-14: In vitro digested *PDS3* by Cas9 nuclease, undigested *PDS3* and sgRNAs loaded on 2 % agarose gels.**
Bands are compared to GeneRuler 50 bp DNA Ladder, Thermo Scientific. 1x TAE buffer is used as running buffer. GelRed™ is used to visualize the bands under UV-light. **A**: Amplified regions containing genomic target site and respective gRNA together with Cas9 nuclease (NEB, #M0641T). For the reaction with gRNA A2, a band between 100 and 150 bp are showing. It is believed that this band results from successfully digested PCR product. **B**: Undigested PCR products are showing bands between 200 and 250 bp. gRNAs are showing bands at around 100 bp.

Since the sgRNAs most likely gave a band on the previous gel, the in vitro digestion of the PCR products with Cas9 nuclease was performed one more time, this time including an RNase A (Thermo Scientific) step before loading the gel (Figure 3-15). In addition to this, it was performed three reactions for each PCR product, one without adding the Cas9 nuclease and the sgRNA, the second without adding the sgRNA and the last containing all the reactants.

All the reactions containing PCR product 1, only give one band on the gel. The band is between 200 bp and 300 bp, which is corresponding to the size of the undigested PCR product (Figure 3-16, A). There is no extra band(s) for the reaction containing PCR product 1 together with both Cas9 nuclease and sgRNA B1. The reaction containing PCR product 2 and the reaction containing PCR product 2 together with Cas9 nuclease, only gave one band. The band is between 200 bp and 300 bp, which is corresponding to the size of the undigested PCR product (Figure 3-16). For the reaction containing PCR product 2 together with both Cas9 nuclease and sgRNA A2, there is two bands on the gel. One band is at the size of undigested PCR product, between 200 bp and 300 bp (Figure 3-16, B), while the other band is shorter than 200 bp. This band is believed to be a fragment of digested PCR product. Again, sgRNA A2 is believed to be working together with Cas9 nuclease and successfully cleave target site in vitro.

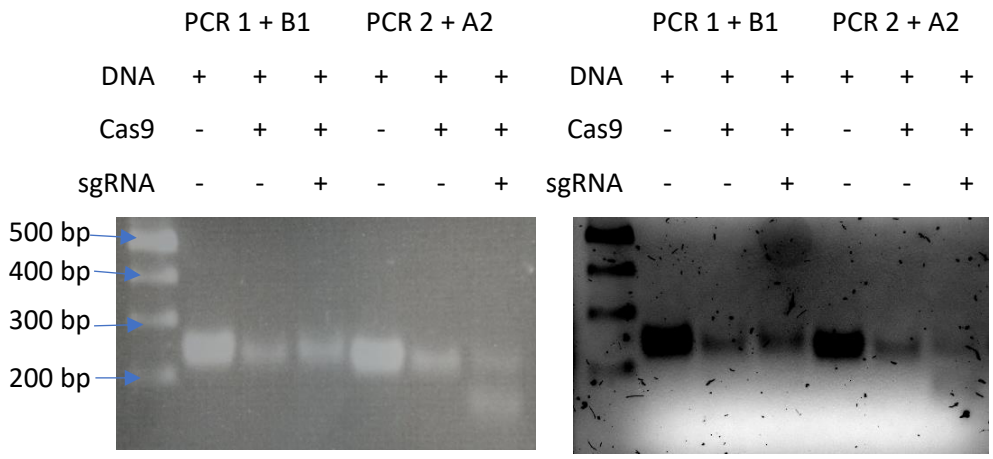


**Figure 3-15: In vitro digested *PDS3* loaded on 2 % agarose gel.**
Bands are compared to GeneRuler 1 kb Plus DNA Ladder, Thermo Scientific. 1x TAE buffer is used as running buffer. GelRed™ is used to visualize the bands under UV-light. All the reactions without gRNAs, are only showing one band on the gel. All the reactions without gRNA are only showing one band. For the reaction with gRNA A2 together with Cas9 nuclease (NEB, #M0641T), one extra band is showing at a size between 100 and 150 bp. It is believed that this band results from successfully digested PCR product.

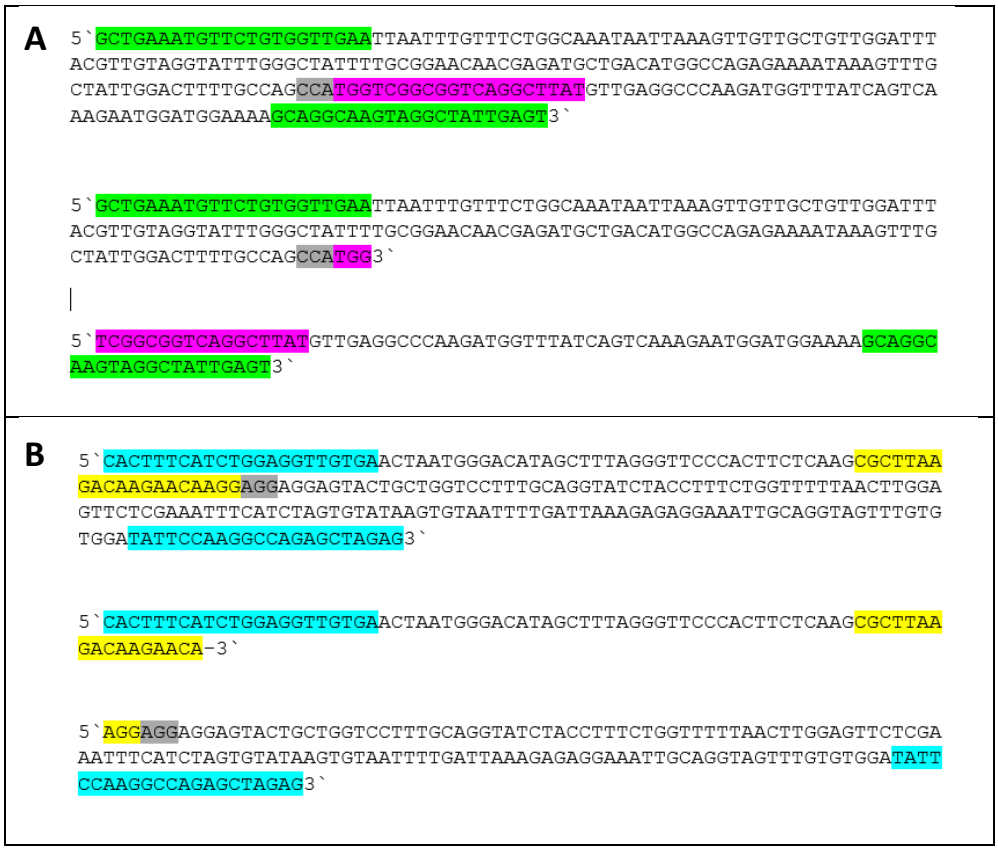


**Figure 3-16: Size of PCR amplified sequences of *PDS3* containing target site.**
A: PCR product (243 bp) from PCR 1 containing gene specific part of sgRNA containing B1 spacer (pink). Digestion of PCR product in vitro with Cas9 nuclease will give two fragments, one consisting of 160 bp and the other consisting of 83 bp. B: PCR product (231 bp) from PCR 2 containing gene specific part of sgRNA containing A2 spacer (yellow). Digestion of PCR product in vitro with Cas9 nuclease will give two fragments, one consisting of 77 bp and the other consisting of 554 bp. The PAM site is coloured in grey.

## 3.6 In vivo cleavage of *PDS3* and T7 endonuclease I assay

In vivo editing by CRISPR-Cas9 system was performed two times, and to identify mutations at *PDS3* genomic target site T7 endonuclease I was performed.

Isolated protoplasts (500 000) were transfected with Cas9 mRNA (10,61 µg) together with sgRNAs (A2: 24 µg and B1: 27.36 µg) and RNP complex; Cas9 nuclease (40 µg) together with sgRNAs (20 µg of both). Total genomic DNA from protoplasts from both transfections was thereafter isolated (Section 2.2.8), and the two segments of the *PDS3* gene was amplified using PCR and site specific primers (Section 2.2.9). The four PCR products, two from each transfection, were thereafter subject for T7 endonuclease I assay (Section 2.2.11). As a control, the same two segments of *PDS3* gene amplified from DNA isolated from non-transfected protoplasts were used. After T7 endonuclease I assay, the samples were run on a 2% agarose gel to analyse the fragments (Figure 3-17). For a mixture of PCR products with mutations at genomic target site and not mutation at genomic target site, there will be produced hybrid PCR products after denaturing and re-annealing (Section 2.2.11). Hybrid DNA is recognized by T7 endonuclease and cleaved. For all the samples, there is only one band showing on the gel, indicating no hybrid PCR products and no mutation at genomic target site.

T7 endonuclease I assay was done one more time, using protoplasts (250 000) transfected with Cas9 mRNA (28.99 µg) together with sgRNA A2 (76.31 µg). This time there were more Cas9 mRNA and sgRNA per protoplasts compared to the first time. Only sgRNA A2 were used, since this one gave positive results during in vitro digestion of the target site. Total genomic DNA was isolated from the transfected protoplasts (Section 2.2.8), and the region *PDS3* containing the gene specific site of sgRNA A2 was amplified using PCR and site specific primers (Section 2.2.9). The fragments from the T7 endonuclease I assay, was analysed on a 2% agarose gel (Figure 3-18). Only one band is showing on the gel, again indicating no hybrid PCR product and no mutation at genomic target site.

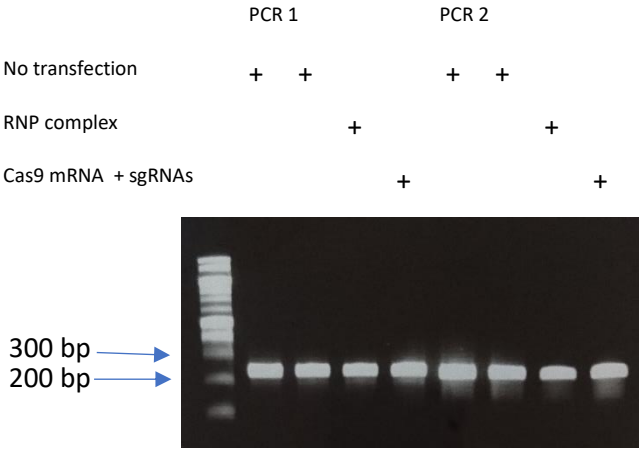


**Figure 3-17: In vivo cleavage of target site and T7 endonuclease I assay. 2% agarose gel.**
Bands are compared to GeneRuler 100 bp DNA Ladder, Thermo Scientific. 1x TAE buffer is used as running buffer. GelRed™ is used to visualize the bands under UV-light. The four first wells contain PCR 1 product containing gene specific sequence of sgRNA B1, while the four last wells contains PCR 2 product containing gene specific sequence of sgRNA A2. Well 1, 2, 5 and 6 contains PCR products from DNA isolated from non-transfected protoplasts. Well 3 and 7 contains PCR products from DNA isolated from protoplasts transfected with RNP complex. Well 4 and 8 contains PCR product from DNA isolated from protoplasts transfected with Cas9 mRNA and sgRNAs.

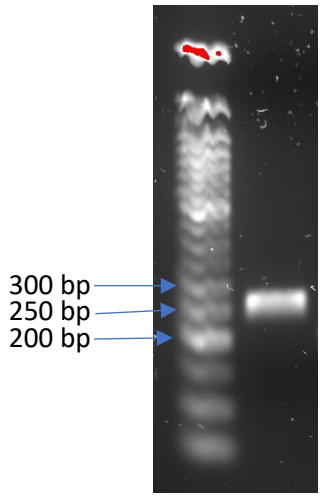


**Figure 3-18: In-vivo cleavage of target site and T7 endonuclease I assay. 2% agarose gel.**
Bands are compared to 50 bp DNA ladder, NEB. 1x TAE buffer is used as running buffer. GelRed™ is used to visualize the bands under UV-light. PCR 2 product containing gene specific sequence of sgRNA A2 amplified from DNA isolated from protoplasts transfected with Cas9 mRNA and sgRNAs.

# 4 DISCUSSION

The CRISPR-Cas9 system is exciting to work with. It can generate site specific DSB at large ranges of sites in the genome. It supersedes traditional mutagenesis methods and transgenic methods, in being specific and fast, and it supersedes other programmable nucleases such as ZFNs and TALENs in being simple, fast, and more affordable. It only needs three components; Cas9 nuclease, gRNA, and a genome target site upstream to a PAM site. The only change needed to direct the system at another genomic target site, is to change the spacer sequence of the gRNA. Through endogenous repair systems DSB repair can result in knock out and knock in of specific genes. This opens the possibility to genetically edit crops in a much faster and straight forward approach than earlier (Pacher and Puchta 2016; Hartung and Schiemann 2014; Kim and Kim 2014; Ma et al. 2016; Jinek et al. 2012; Doudna and Charpentier 2014). The purpose of this project was to investigate CRISPR-Cas9 genome editing in *A. thaliana* through a DNA-free approach, by directly deliver the components as both pre-assembled RNP complex and transcripts to protoplast using PEG mediated transfection. Further on, investigate the ability to regenerate transfected protoplasts into a DNA-free modified whole plant.

To be able to generate CRISPR-Cas9 genome editing through this DNA-free method is important because the generated modified plants will be free of foreign DNA, making it more likely that the plant will bypass the GMO legislation both in the US and EU. This means avoiding the expensive and time-consuming regulations of EU, making it more favourable to use CRISPR-Cas9 for crops improvement which allows the production of more food for the growing population during climate change (Hartung and Schiemann 2014; Pacher and Puchta 2016; Kanchiswamy 2016). In the last years, direct delivery of RNP-complex, to generate genetic modification, has been achieved for several plants and crops; *A. thaliana*, tobacco, rice, lettuce (Woo et al. 2015), petunia x hybrid (Subburaj et al. 2016), grapevine and apple (Malnoy et al. 2016), maize (Svitashev et al. 2016) and for bread wheat (Liang et al. 2017). In addition to being transgene free, plants checked for RGEGN RNPs off-targets cleavage showed no cleavage at homologous sites that differed by 1-5 or 2-5 nucleotides from on-target site (Woo et al. 2015; Svitashev et al. 2016; Liang et al. 2017), which could contribute to the likelihood of passing as non-GMOs.

In this project, direct delivery of Cas9 as transcript was investigated in addition to the direct delivery as protein (as pre-assembled RNP complex). This was of interest, because it could make the system of CRISPR-Cas9 genetic editing to likely be simpler. The protein is expensive to buy, and the setup for in vitro transcription of Cas9 is easier and faster than the setup for Cas9 protein expression and isolation. In parallel to our work, Zhang and his group were attempting to achieve the same target; obtaining genetic modification through delivery of Cas9 as a transcript. They chose a different delivery method;

particle bombardment to immature embryotic cells instead of PEG-mediated protoplast transfection. They succeeded, and obtained genetic modified wheat (Zhang et al. 2016). To the authors knowledge, PEG mediated protoplasts transfection of Cas9 as transcript, has not yet been performed.

Two sgRNAs transcript containing spacers against *PDS3* for *A. thaliana* were generated. The sub cloning of annealed oligos into pUC57-sgRNA expression vector were successful and confirmed by sequencing. Both two approaches for the annealing of the complementary oligos were also confirmed to work. The sgRNAs were in vitro transcribed from pUC57-sgRNA expression vector, and the integrity of the transcripts were analysed. The gel showed clear bands, which indicated intact and good quality sgRNAs. Cas9 mRNA was invitro transcribed from pST1374-NLS-flag-linker-Cas9, and the integrity of the transcript was analysed using both a 1 % denaturing agarose gel and a 1 % native agarose gel. Both gels showed clear bands, which indicated intact transcript of good quality. To further confirm transcripts functionality containing the predicted spacers, we performed in vitro digestion of genomic target site of *A. thaliana* by sgRNA preassembled with commercial Cas9 protein. Cas9 protein together with one of the two sgRNA (A2) successfully cleaved the target site, and proved its functionality. Hence, it was a successful setup of CRISPR-Cas9 digestion, all the components were ready.

To generate in vivo genetic modification with this setup, healthy protoplasts were needed. Protoplast were successfully isolated, but to still obtain healthy protoplasts following PEG-mediated transfection was found to be a challenge to achieve. Substantial trials were performed, changing many parameters to figure out the issue. During two of the experiments, some of the protoplasts survived (around 5 %). For this small number of surviving protoplasts, it was seen that transfection worked with fluorescence DNA. However, higher number of surviving protoplasts was needed, and the important parameter to achieve this, was found to be centrifugation. By lowering the centrifugation time and speed during both the isolation and transformation, high numbers of protoplasts survived the transformation. Hence, the protocol for protoplast isolation and transformation was successfully optimized to get a good number of surviving protoplasts following transfection. Subsequently, isolated protoplasts were transfected with both pre-assembled RNP complex and in vitro transcribed transcripts of Cas9 and sgRNA. Total genomic DNA was isolated from the transformed protoplasts, and target sites amplified using PCR. T7E1 assay was performed to identify mutations, but without success. Successful in vivo experiments, would have allowed comparison between genomic modifications through delivery of RNP complex and transcripts.

In order to optimize the protocol for in vivo editing, the amounts of the different components could have been experimentally optimized; the ratio of Cas9 protein to sgRNA, the ratio of Cas9 mRNA to sgRNA, and the number of protoplasts to be transfected. This was investigated theoretically

beforehand (see introduction, Table 1-1). When comparing amounts of the components used in our experiment to other groups work, it is seen that they used a smaller amount of protoplasts and a higher amount of Cas9 protein and sgRNA during transformation (Malnoy et al. 2016; Woo et al. 2015). Therefore, in our experiment, the non-successful T7 endonuclease I assay could be due to a possible low amount of transfected protoplasts compared to non-transfected protoplast, making the majority genome isolated from the total amount of protoplast unmodified.

In addition, it was tried to produce Cas9 protein. This would have allowed more experiments during optimization of the transient CRISPR digestion, due to lower cost than commercial protein. Cas9 protein expression was induced by IPTG from BL21 Star™ (DE3) expression strain containing pET-NLS-Cas9-6xHis plasmid. Protein expression from induced and non-induced cultures were compared by SDS-PAGE. The gel showed a clear protein band around 150 kDa, which was not seen for the non-induced samples. The band size correlated to the theoretically predicted size of Cas9 protein, which indicated that the expression of the protein was successful. Further on, the protein could have been isolated and purified and used for the pre-assembled RNP complex.

To confirm that the Cas9 mRNA is expressed in vivo, the level of Cas9 protein after protoplast transfection with Cas9 transcript, could have been investigated. The Cas9 vector, pST1374-NLS-flag-linker-Cas9, has been set up to include a flag-tag fused with the expressed Cas9 protein. A western blot against tagged Cas9 protein could have revealed expression level.

# 5 CONCLUSION AND FUTURE PERSPECTIVE

To summarise, all the components to generate genetic modifications through CRISPR/Cas9 were successfully generated; a *PDS3* target site in the genome of *A. thaliana* directly upstream to a PAM site, sgRNA transcript against the genomic target site, and Cas9 transcript. Commercial Cas9 protein together with one of the sgRNAs was shown to produce cleavage of target site in vitro, confirming the sgRNAs functionality. Cas9 protein was shown to be expressed, and can further be purified and used for pre-assembled RNP complex. Protocol for isolation and transfection of protoplasts was successfully optimized. Genetic modification in vivo both by using pre-assembled RNP complex and using in vitro transcribed Cas9 together with sgRNA was attempted, but without success.

Further on, to achieve in vivo genetic modification, the protocol needs to be experimental optimized. One aspect is to investigate the different amounts of the components to be used in order to obtain cleavage; the ratio of Cas9 protein to sgRNA, the ratio of Cas9 mRNA to sgRNA, and the number of

protoplasts to be transfected. Then level of different components that results in highest genomic cleavage efficiency needs to be defined. Purification of Cas9 protein to be used in pre-assembled RNP complex, would allow more experiments at a lower cost during optimization. Western blot against flag-tagged Cas9 protein can be used to confirm in vivo expression of Cas9 after transfection of protoplasts by Cas9 transcript. Successful in vivo editing using both pre-assembled RNP complex and in vitro transcribed Cas9 and sgRNA, would allow comparison between the methods, regarding the editing efficiency. Other successful studies using RNP-complex could also have help during comparison. The ability to regenerate transfected protoplast would further allowed generation of DNA-free modified whole plants.

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# 7 APPENDIX

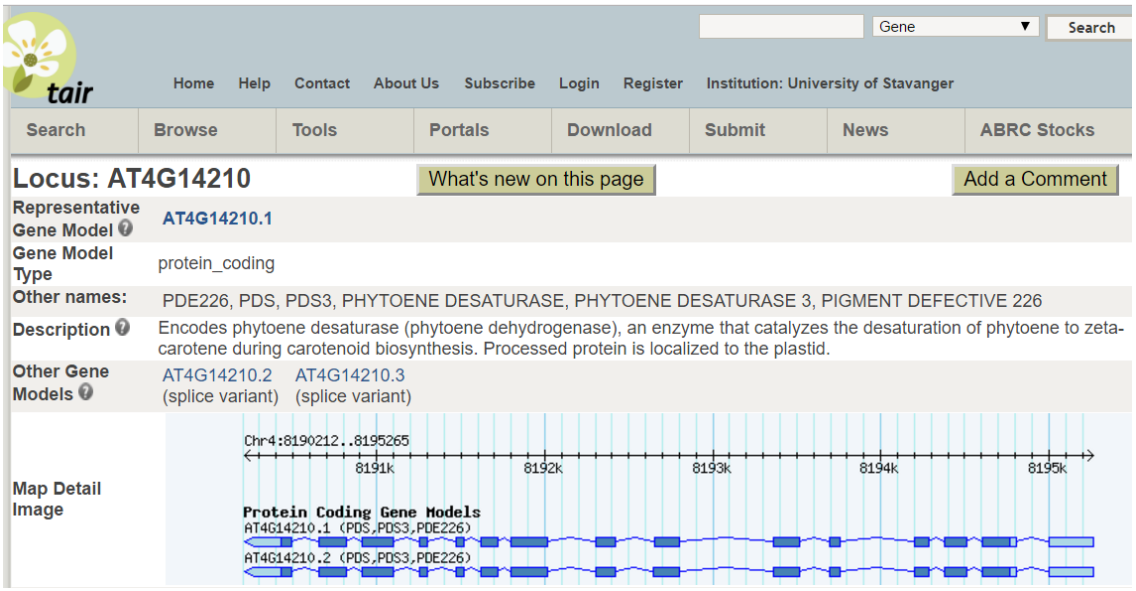
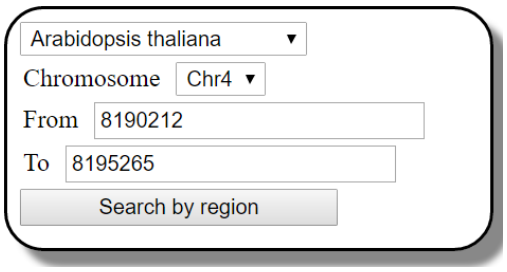



**Figure 7-1: *A. thaliana PDS3* at TAIR**
The gene locus number is AT4G14210 and gene model one is shown. The gene is located at chromosome 4 from nucleotide number 8190212 to number 8195265. This information was used during the design of spacers for sgRNAs targeting *PDS3*.

**A**




**B**

| SeqID | minMM_GG | minMM_AG | Spacer seq (5'->3') | PAM (5'->3') | strand | location |
|---|---|---|---|---|---|---|
| Chr4:8192698-8192718 | NA | NA | ATAAGCCTGACCGCCGACCA | TGGCTGGCAA | + | exon |

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

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

GC content of Spacer sequence: 0.6

Potential Pol III terminator (TTTTT): null

4 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
NlaIII cut CATG
CviAII cut CATG
FatI cut CATG
NcoI cut CCATGG

Total of 1 class0.0 gRNA seeds were found in this region

**C**

| | | | | | | |
|---|---|---|---|---|---|---|
| Chr4:8194628-8194648:c | 3 | 4 | CGCTTAAGACAAGAACAAGG | AGGAGGAGTA | - | exon |

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

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

GC content of Spacer sequence: 0.45

Potential Pol III terminator (TTTTT): null

1 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BseRI cut GAGGAG

**Figure 7-2: CRISPR-PLANTS, target prediction site for spacer designing of sgRNA (Xie et al. 2014).**
A: The query at CRISPR-PLANTS during the search for recommended targets of *PDS3* to design spacer of sgRNAs against.

B: The first pair of oligos chosen for spacer design against *PDS3*, CRISPR -5 and -6.

C: The second pair of oligos chosen for spacer design against *PDS3*, CRISPR -7 and -8.

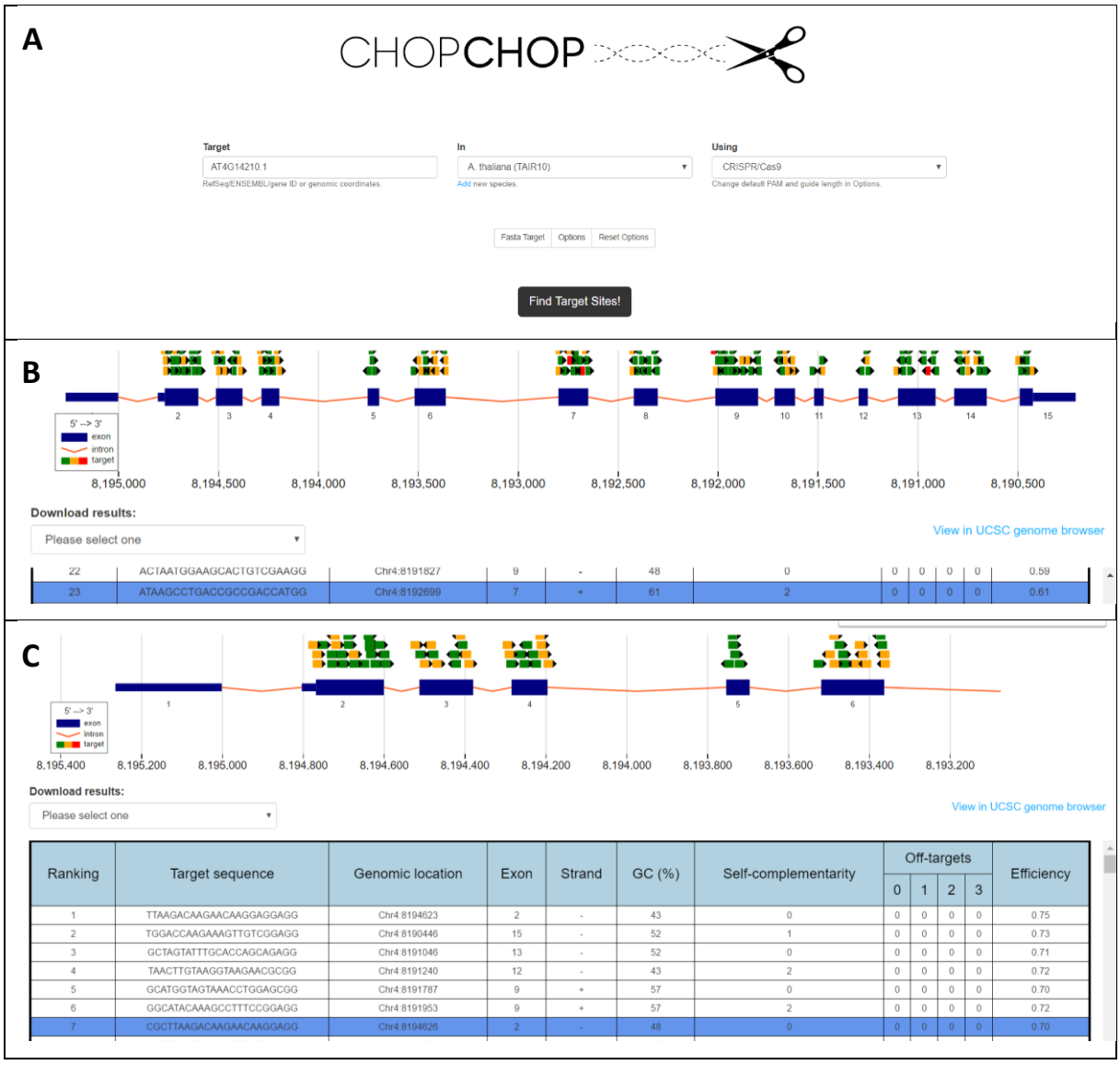
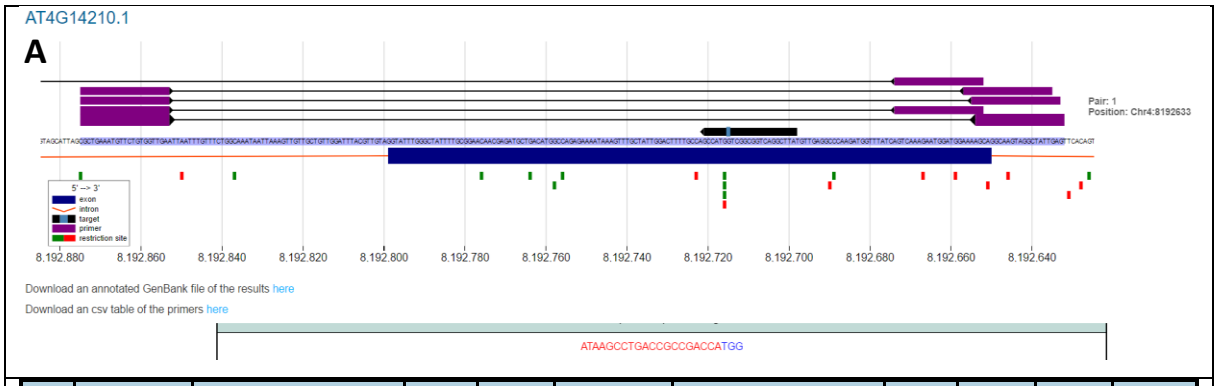


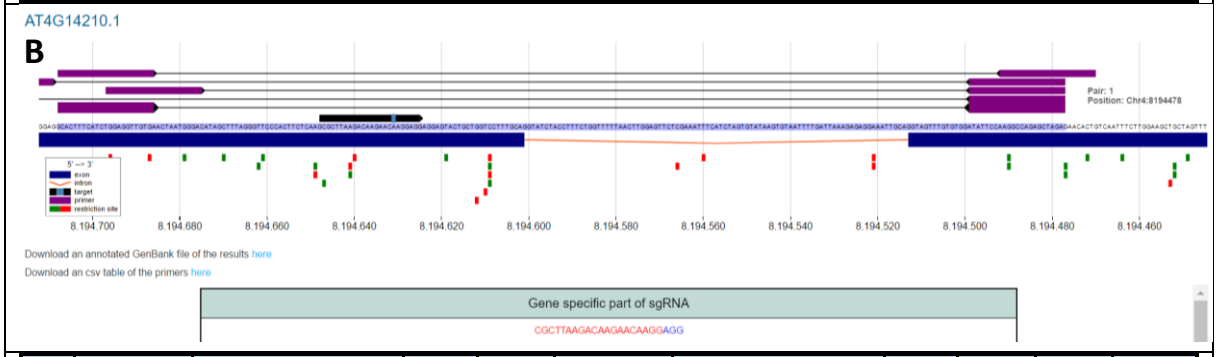

**Figure 7-3: CHOPCHOP, target prediction site for spacer designing of sgRNA (Labun et al. 2016; Montague et al. 2014).**
A: The query at CHOPCHOP during the search for recommended targets of *PDS3* to design spacer of sgRNAs against.

B: The first pair of oligos chosen for spacer design against *PDS3*, CRISPR -5 and -6. The target site is located at exon 7.

C: The second pair of oligos chosen for spacer design against *PDS3*, CRISPR -7 and -8. The target site is located at exon 7.




| Pair | Left primer coordinates | Left primer | Left primer Tm | Left primer off-targets | Right primer coordinates | Right primer | Right primer Tm | Right primer off-targets | Pair off-targets | Product size |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Chr4:8192854-8192876 | GCTGAAATGTTCTGTGGTTGAA | 60.2 | 0 | Chr4:8192633-8192655 | ACTCAATAGCCTACTTGCCTGC | 59.9 | 0 | 0 | 243 |




| Pair | Left primer coordinates | Left primer | Left primer Tm | Left primer off-targets | Right primer coordinates | Right primer | Right primer Tm | Right primer off-targets | Pair off-targets | Product size |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Chr4:8194687-8194709 | CACTTTCATCTGGAGGTTGTGA | 60.1 | 0 | Chr4:8194478-8194500 | CTCTAGCTCTGGCCTTGGAATA | 60.0 | 0 | 0 | 231 |

**Figure 7-4: Designing of primers at CHOPCHOP, to amplify regions of *PDS3* that includes genomic target site (Labun et al. 2016; Montague et al. 2014).**

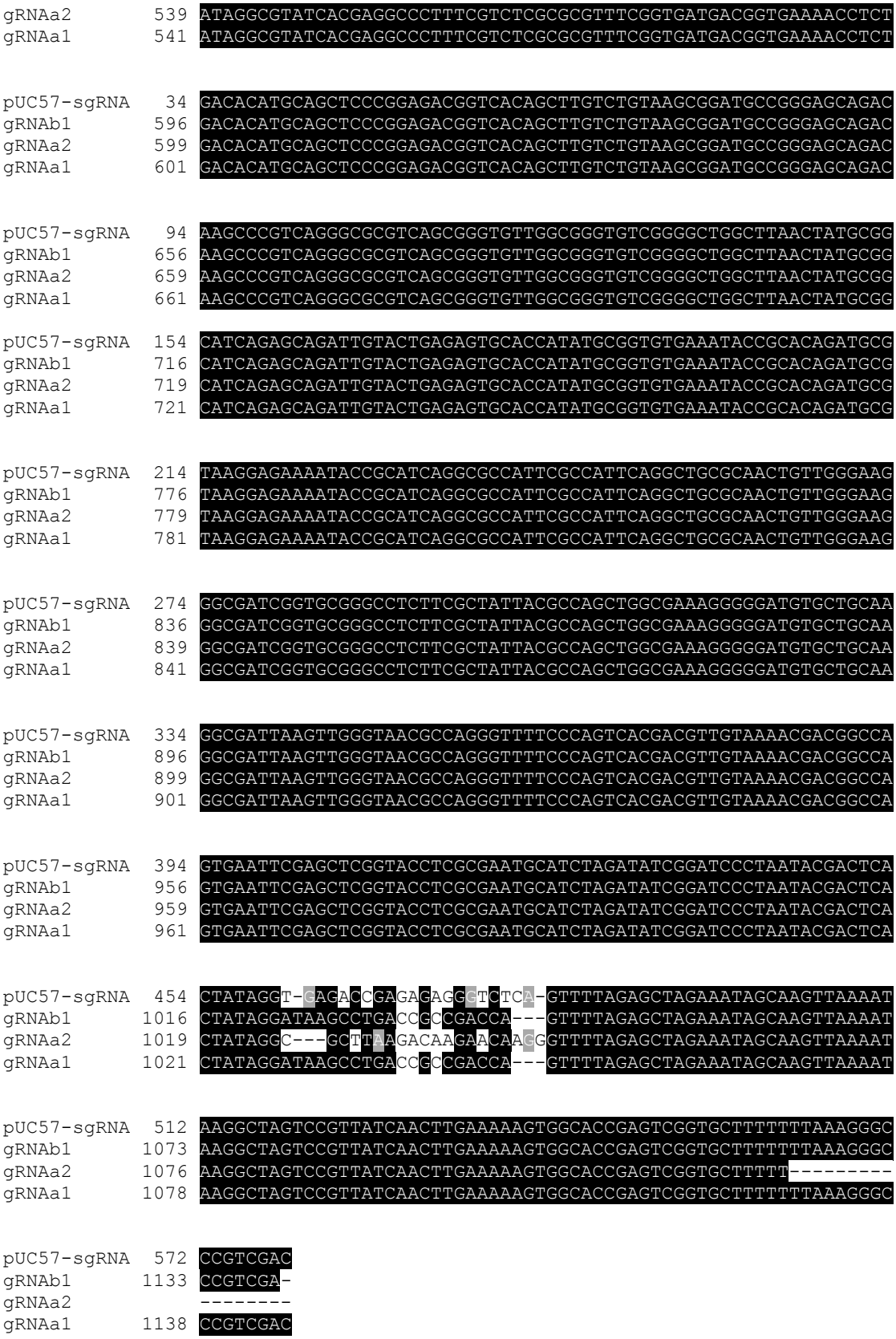
A: Primers to be used to amplify region of PDS3 that includes target site of sgRNA with spacer 1 (CRISPR -5 and -6.)

B: Primers to be used to amplify region of PDS3 that includes target site of sgRNA with spacer 2 (CRISPR -7 and -8.)

In addition, the gene location of the amplified PCR products is shown.

**Alignment of pUC57-sgRNA expression vector sequences after sub cloning of spacers.** The first sequence is the vector without any spacer insert. The next sequences are the vector containing spacer B1, A2 and A1, where the number one represents annealed oligos CRISPR -5 and -6, the number two represent annealed oligos CRISPR – 7 and -8, the letter A represent phosphorylated spacers, and the letter B represent non-phosphorylated spacers.

```
pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1          1 --TCAACAAT--TTTCACCTGAATCAGGAT-TTCTTCTAATACCTGGAATGCTGTTTTCC
gRNAa2          1 CATCAACAAT-TTTTCACCTGAATCNNGAT-TTCTTCTAATACCTGGAATGCTGTTTTCC
gRNAa1          1 CATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCC

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1         56 CAGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGG
gRNAa2         59 CAGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGG
gRNAa1         61 CAGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGG

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        116 TCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCAT
gRNAa2        119 TCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCAT
gRNAa1        121 TCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCAT

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        176 TGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACA
gRNAa2        179 TGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACA
gRNAa1        181 TGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACA

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        236 ATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATA
gRNAa2        239 ATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATA
gRNAa1        241 ATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATA

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        296 AATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATAT
gRNAa2        299 AATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATAT
gRNAa1        301 AATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATAT

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        356 GGCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA
gRNAa2        359 GGCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA
gRNAa1        361 GGCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        416 GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTC
gRNAa2        419 GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTC
gRNAa1        421 GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTC

pUC57-sgRNA     1 ------------------------------------------------------------
gRNAb1        476 CCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA
gRNAa2        479 CCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA
gRNAa1        481 CCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA

pUC57-sgRNA     1 ---------------------------TCGCGCGTTTCGGTGATGACGGTGAAAACCTCT
gRNAb1        536 ATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT
```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gRNAa1 541 ATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCT
pUC57-sgRNA 34 GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC
gRNAb1 596 GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC
gRNAa2 599 GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC
gRNAa1 601 GACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC
pUC57-sgRNA 94 AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGG
gRNAb1 656 AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGG
gRNAa2 659 AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGG
gRNAa1 661 AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGG
pUC57-sgRNA 154 CATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG
gRNAb1 716 CATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG
gRNAa2 719 CATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG
gRNAa1 721 CATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG
pUC57-sgRNA 214 TAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAG
gRNAb1 776 TAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAG
gRNAa2 779 TAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAG
gRNAa1 781 TAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAG
pUC57-sgRNA 274 GGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAA
gRNAb1 836 GGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAA
gRNAa2 839 GGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAA
gRNAa1 841 GGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAA
pUC57-sgRNA 334 GGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
gRNAb1 896 GGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
gRNAa2 899 GGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
gRNAa1 901 GGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
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pUC57-sgRNA 512 AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTAAAGGGC
gRNAb1 1073 AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTAAAGGGC
gRNAa2 1076 AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT---------
gRNAa1 1078 AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTAAAGGGC
pUC57-sgRNA 572 CCGTCGAC
gRNAb1 1133 CCGTCGA-
gRNAa2 --------
gRNAa1 1138 CCGTCGAC

**The nucleotide sequence of *A. thaliana PDS3.***

Genomic target sites are highlighted in yellow and pink: (A2 gRNA, Crispr 6: 5`CGCTTAAGACAAGAACAAGG3` B1 gRNA, Crispr 5 → 5`-TGGTCGGCGGTCAGGCTTAT-3`).

Primers used during amplification of regions containing genomic target site are highlighted in green and blue: (Crispr 55: 5`GCTGAAATGTTCTGTGGTTGAA3` Crispr 56 reverse complement 5`GCAGGCAAGTAGGCTATTGAGT3`, Crispr 57: 5`CACTTTCATCTGGAGGTTGTGA3`Crispr 58, reverse complement: 5`-TATTCCAAGGCCAGAGCTAGAG3`

PAM sites are highlighted in grey.

>NC_003075.7:c8195320-8190190 Arabidopsis thaliana chromosome 4 sequence
CTTTGGTGGGCAAAAACATATTAGCTGAGAGGTCAATTTCTTTTTCCCCTAAACCAAATTACGTTGAGATGCATG
GTCTCTCTCTACTCAATTAACCAAATAAGGAAAAGAATCATATGGTCATCAATTCGTAAATCAAAATTTTAATTT
GTGTGGTATTTAATCCATCTACATGTTTCGTAAGCAACAAAAGAGCTTGGTCTGAAAACCAAACAAGACCATATG
GGCACTCGAATACTCCATTTTGTTATCGGCTACTTCCACTAGCCTCCTCCTTCGCTGCGTCTCCTGTTTCTCTAC
TTCACGATTACTCGCTAGGTAAAAGATAAGATACTAAACAACTACTATAGCCCCTTTAATTCAAGTCCGTTTTTT
TTTGTTCTTCTTCTTTTCAATTATGTGTTAAAGATACAAACTTTTGTCTGATTTGCTTCCACCGGTTTCACCTAA
GATACTCAATTTTCTTACTTTTTGTGTGTTTTGTAATTCTAATTCTTTTATAGCTTCAATTTTTAGATTCATTGA
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TAAGACAAGAACAAGGAGGAGGAGTACTGCTGGTCCTTTGCAGGTATCTACCTTTCTGGTTTTTAACTTGGAGTT
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AACTGCAAAGTACCTGGCTGATGCAGGCCACAAACCTCTGTTGCTTGAAGCAAGAGATGTTCTTGGTGGAAAGGT
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TACCATGCTGTCTTGTGGGTTTAACAATTCATCTGGTATCCTGCTTATTAATATGTTGTTTATGTTTTTCTTGTA
GTCGATATCCTGAAGCTCCTTTTACCAGATCCCTGGAAAGAAATACCGTACTTCAAGAAATTGGATAAATTAGTT
GGAGTACCAGTTATTAATGTTCATATATGGTTAGTGAAACCGTTATCTCCCTTGTTTTCTTTTGGCCATTATGTG
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TCAATTTTATAATTTTTAAAAAGTAATTACTGTCGACCTTTTGTAATCATAGTGATTTATCATTATGTCTCTCTT
TTTAAAACCTTTTCTGGTACAAATTATAAAA

**The nucleotide sequence of pUC57-sgRNA expression vector:** The sequence was retrieved from addgene. BsaI restriction sites are highlighted in pink and DraI restriction sites are highlighted in yellow. The revers complement sequence of M13 primer are highlighted in blue. T7 promotor sequence is highlighted in green.

>pUC57-sgRNA expression vector

tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaag
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