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Identification and CRISPR editing of pathogen responsive genes in *Lactuca Sativa*

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Biotechnology, Molecular Biology

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Sammendrag

En av de største utfordringene menneskeheten står ovenfor i dag er å produsere mat til den forventede befolkningen på 10 milliarder innen 2050. Plantepatogener er fremdeles en stor utfordring for landbruket, og en av de mest lovende metodene for en effektiv og bærekraftig forvaltning av plantepatogener er bruken av genredigeringsverktøy for å generere nye og forbedrede kultivarer. Plantepatogenet *Sclerotinia Sclerotiorum* forårsaker råtesopp på en rekke vertsplanter, inkludert *Lactuca Sativa* (salat). Råtesoppen forårsaker betydelige tap for salatavlingene (opptil 30%) og resulterer i landbruks- og økonomiske utfordringer for bøndene. Målet med dette studiet er å gjennomføre en funksjonell analyse av gener som muligens er involvert i *S. sclerotorium* patogenese i salat ved å utvikle CRISPR-Cas9 knockout konstrukter for målrettet mutagenese på kandidatgener. Hensikten er å genere genredigerte *L. sativa*-linjer med forbedret resistens mot plantepatogenet *S. sclerotiorum*. Bioinformatisk analyse, litteraturstudier og RT-qPCR genekspresjonsanalyse av infisert og frisk salat ble brukt til å identifisere målgener. CRISPR-konstrukter med sgRNA, spesifikt designet for tre målgener, ble konstruert for å gjennomføre studiet. Kloningssystemet som ble brukt i denne oppgaven besto av *Escherichia Coli* for transformasjon av CRISPR ekspresjonsvektorer og *Agrobacterium Tumefaciens* binær vektorsystem, som overførte CRISPR-konstruktene til *L*. *Sativa*plantene. CRISPR-konstruktene ble testet med to typer *A. tumefaciens*-mediert plantetransformasjon. Dessverre ble ikke transformasjonseksperimentene fullført på grunn av COVID-19 utbruddet som førte til at den norske regjeringen innførte unntakstilstand som berørte de fleste offentlige institusjoner, inkludert NIBIO.

Abstract

One of the greatest challenges faced by humanity today is to feed the estimated population of 10 billion people by 2050 in the face of climate change. Plant pathogens are still a major challenge for agriculture and one of the most promising ways for efficient and sustainable management of plant pathogens is the use of gene editing tools to generate new improved crop cultivars.

The plant pathogen *Sclerotinia Sclerotiorum* causes a disease commonly known as white mold on a wide range of host plants, including *Lactuca Sativa* (lettuce). This brings significant losses on lettuce yields (reportedly up to 30 %) and results in agricultural and economic challenges for the farmers.

The aim of this study is to conduct functional analysis of genes possibly involved in the *S. sclerotorium* pathogenesis in lettuce, by developing CRISPR-Cas9 knockout-constructs for targeted mutagenesis on candidate genes. Ultimately gene edited *L. sativa* lines with enhanced resistance against the plant pathogen *S. Sclerotiorum* would be generated. Bioinformatical analysis, literature search and RT-qPCR gene expression analysis of infected and uninfected lettuce were used to identify target genes. CRISPR-constructs with sgRNA specifically designed for three target genes were constructed to perform the study. The cloning system used in this thesis consisted of *Escherichia Coli,* for transformation and propagation of the CRISPR expression vectors, and *Agrobacterium Tumefaciens* binary vector system, which delivered the CRISPR-constructs into the *L. Sativa* plants. The CRISPR-constructs were tested in both stable and transient *A. tumefaciens*-mediated plant transformation. Unfortunately, the plant transformation experiments were not completed due to the COVID-19 outbreak which caused the Norwegian government to inflict lock-down on most public institutions, including NIBIO.

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1 Introduction

1.1 Background

To provide food security for a constantly growing population is one of the greatest challenges faced by humanity today. Predictions suggest that by 2050 (1) the world population will reach 10 billion people, and food production needs to increase 60-100% to feed humanity in its entirety. In addition to a growing population farmers and food producers face several other challenges due to climate change, such as extreme weather, reduced agricultural land availability, increasing biotic and abiotic stresses to mention some.

One strategy in facing these challenges is to create new improved crops. However, conventional breeding of such new improved crop varieties is a lengthy, complex, imprecise, unpredictable and expensive process (2). Thus, alternative technologies are required.

A promising alternative to overcome the challenges faced by conventional breeding is the utilization of genome editing tools. Such tools enable the precise and efficient modification of an organism's genome (3). The CRISPR-Cas9 gene editing tool has over the recent years emerged as the overall best technology for genome editing, due to its high precision, affordability and feasibility (4).

Lactuca sativa is a leafy vegetable grown practically all over the world and is one of the largest vegetable crops in terms of economic significance. According to FAOSTAT the estimated gross production value of lettuce in the world was 15692.9 Million Dollars in 2016 (5). In Norway alone, lettuce is traded for approximately 400 million NOK/year (6). The plant pathogen *Sclerotinia Sclerotiorum* is a fungal parasite that causes significant losses (up to 30 % has been reported) on lettuce yields, and thus represent a great agricultural and economic challenge for the farmers. Furthermore, fungal infections in general is a great threat to food security worldwide.

Various approaches have been used to fight such infections in plants, primarily among them are fungicides, that are in general toxic and is possibly harmful to the environment. In Norway alone the estimated use of fungicides in agriculture has been around 100 tons/year since 2001

(7). It is thus preferable to minimize the use of such compounds, and search for alternative approaches to control fungal infections in plants.

One such alternative is development of improved crops with desired characteristics using genetic engineering technologies like traditional GMO (genetically modified organism) and gene editing tools such as CRISPR-Cas9. These technologies can generate crops with desired traits like plants with increased yields and enhanced resistance which in turn may reduce the use of fungicides. Compared to conventional breeding for genetic improvement, the creation of genetically engineered plants is also significantly more efficient in terms of time, labor and general costs (8).

There are several ways to genetically modify plants for increased resistance to fungal infections. The most common approach today is the random insertion of resistance genes (e.g. genes coding for compounds such as enzymes central to defense of infection) into the plant genome. This will increase a plants resistance, as confirmed by several experiments, and is hitherto the most common approach in genetic engineering for increasing resistance in plants (9). However, this approach confers significant limitations (discussed in chapter 1.4), so other strategies are required. Targeted mutagenesis of susceptibility genes (e.g. genes that reduce defense) has emerged as an alternative.

This strategy has already been successfully utilized in an experiment on citrus. Scientists were able to create plants with increased resistance to a disease called citrus-canker using CRISPR-Cas9 targeted mutagenesis on host disease susceptibility genes (9).

As opposed to traditional GMO gene transfer, CRISPR-Cas9 editing can induce targeted mutations and deletions without introduction of foreign DNA within the host genome if the CRISPR-Cas9 editing machinery is eliminated after mutagenesis. Once mutations are obtained, all foreign DNA (e.g. the CRISPR-construct) may be removed through crossbreeding (or other technologies) leaving only the desired mutation(s). Consequently, crops edited in this manner are inseparable form naturally mutated crops. This distinction is imperative for CRISPR edited crops potential to be commercialized, as traditional GMOs are subject to great political opposition (10). The current EU regulation of CRISPR edited plants as GMO are criticized as

CRISPR edited organism do not contain foreign DNA. The current EU laws and regulations causes the commercialization process of CRISPR edited organisms to be long and costly, if approved at all (11).

CRISPR presents significant opportunities for improvements in crop production. The possibilities include reducing biotic/abiotic stresses (e.g. enhancing resistance) and increasing yields to mention some. Furthermore, it may limit use of pesticides/fungicides and save labor, time and money compared to conventional breeding. It is preferable to traditional GMOs as gene editing seems more publicly and politically accepted compared to transgenic modifications in traditional GMO. The greatest challenges of utilizing CRISPRs vast potential in crop improvement includes political opposition and regulations already mentioned and some technical aspects which will be addressed in chapter 1.5.

1.2 Lettuce

Lactuca sativa, commonly known as lettuce, is a domestic annual plant belonging to the Asteraceae family. Lettuce is the common term for all plants of the genus *Lactuca* of the flowering plant family Asteraceae, and especially refers to plants of the commercially important species *L. sativa.* Lettuce is a commercially significant food plant both nationally and globally. The global lettuce production is estimated to be approximately 26. 78 metric tons yearly (12). The term lettuce may also refer to the edible, succulent leaves of *L. sativa,* which commonly are eaten raw in salads. *L. sativa* is the most common salad vegetable, and It is cultivated mainly as a foodplant for its fleshy leaves (13). Four main types are generally recognized, namely asparagus (stem), cos (romaine), leaf, and head lettuce (iceberg lettuce). There are many cultivars within in each type (14). Iceberg lettuce is the most commercially significant one and is thus chosen for this study. The strains Great Lakes, which is the common hobby lettuce, and MATCH, which is the most common commercial lettuce, were selected for this thesis.

Lettuce is a convenient model organism for the purpose of this study, as it is easy to cultivate, has relatively short life spam, completely sequenced genome, requires moderate amount of space and is cheap. Additionally, it is relevant from both agricultural – and economic perspectives.

1.3 *Sclerotinia Sclerotiorum*

S. sclerotium, commonly known as white mold, is a plant fungal necrotrophic pathogen with a broad host range including many important crops, such as lettuce. It can cause a disease called white mold which is easily identified on infected plants due to the characteristic white cottony mycelium of the pathogen formed on the surfaces of infected aerial tissues. This mycelium aggregates itself into sclerotia which are the structures that allow *Sclerotinia* species to survive in soil in the absence of a plant host. Sclerotia have a hard, black exterior rind with a white to light beige interior. They are irregularly shaped, and typically measure 2 to 5 mm in diameter and up to 25 mm in length. These sclerotia may give rise to a fruiting body in the spring that produces spores. Once the hyphae of the fungi forms on infected hosts, it produces various compounds including enzymes and oxalic acid, creating lesions soaked in water, frequently with a distinct margin. However, the infection mechanism in its entirety is not yet clearly understood. Secondary symptoms such as wilting, bleaching, and shredding can be observed on above-ground tissues such as leaves and stems (15).

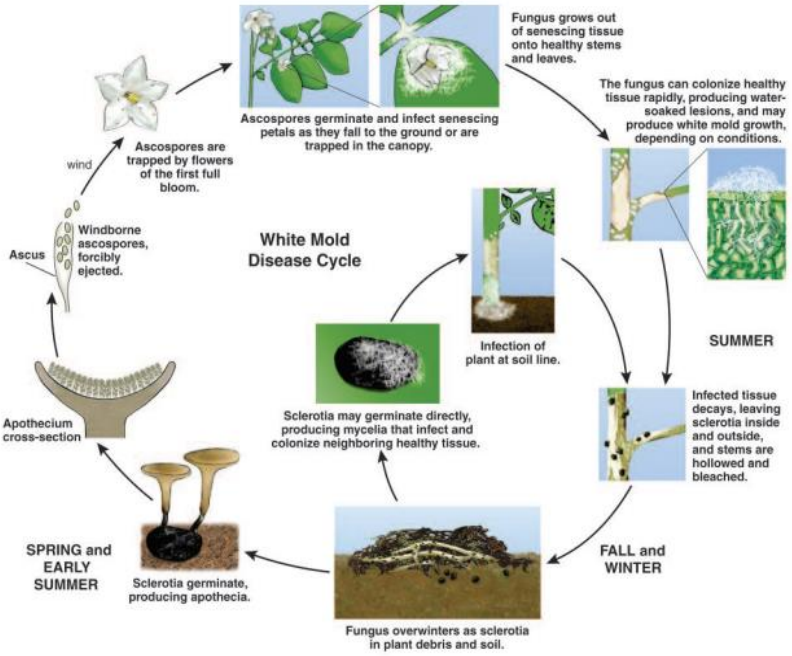


Fig. 1. *Life cycle of S. sclerotium. Illustration from* (16).

In Norwegian commercially grown lettuce, *S. sclerotiorum* is one of the most important pathogens, causing significant yield and economic losses every year. So far, the most utilized method for disease management is chemical fungicides and resistant varieties. The fungicides

available today are, in addition to being generally toxic and possibly harmful to the environment, inconvenient as timing of application is a sophisticated matter and infection sites are often hard to access. Although the total damage caused to the environment by using fungicides is hard to estimate, due to its many ripple effects, it's clearly comprehensive. Fungicides effects a variety of soil organisms and then in turn the crucial functions such organisms conduct, for instance breakdown of organic matter. Hence, any impacts caused by fungicides may have durable impacts on the health of agricultural soils. In a similar fashion, fungicides, which make their way into waters, have the potential to cause adverse effects to the structure and functioning of aquatic ecosystems (17). Thus, it is desired to decrease the use of fungicides due to their toxicity and the risk of pathogens developing resistance to them.

Although the pathogenesis of *S. sclerotium* is not completely understood, research clearly suggest it is associated with production of oxalic acid, which is recognized as a major virulence factor (18). Plants with resistance to oxalic acid are more tolerant to *S. sclerotium* infection. However, the exact role(s) of oxalic acid during infection is not clearly identified, but many of the enzymes secreted by the fungi during infection has low optimal pH. Oxalic acid might increase enzymatic activity by lowering the pH. Possibly oxalic acid chelates calcium, which may in turn compromising the function of calcium-dependent defense pathways and results in weakening of cell walls. Furthermore, oxalate suppresses oxidant biosynthesis which may be disabling the earliest resistance response in plant cells (19).

1.4 Plant defense, resistance and susceptibility

Plants, as opposed to mammals, lack mobile immune cells and an adaptive immune system. Plants respond to pathogen infections using mainly two branches of the innate immune system. One branch uses cell surface pattern-recognition receptors (PRRs) to recognize microbe-associated molecular patterns (MAMPs), common to many classes of microbes, and host-derived damage-associated molecular patterns (DAMPs). The second responds to virulence factors/effector molecules secreted by the pathogen using resistance proteins, coded for by resistance genes (R-genes) in the plant genome (20).

Plant pathogens are a still significant challenge in agriculture. One of the most effective and sustainable ways to manage plant pathogens is the use of gene editing tools (21). Several gene editing technologies exist to enhance resistance to various pathogens in plants, including transfer of R-genes, knock-out of susceptibility-genes and to create RNAi mediated resistance in genetically engineered plants (22). The latter has mainly been utilized for viral resistance but has been effectively used against some pathogenic fungi species as well. However, it is ineffective against many pathogens, and is considered an" additional control strategy" in plant breeding (21). Perhaps the most obvious strategy is to create transgenic plants with enhanced resistance by transferring of R-genes. However, resistance induced in this manner are often not durable (resistance durability increases with stacking e.g. several R-genes inserted simultaneously (23) and many R-genes have a narrow range of resistance, often to only one or a few strains of a single pathogen species (24). Other strategies are hence desired and editing of susceptibility-genes has emerged as a promising alternative.

Disease in plants caused by pathogenic microorganisms involves a compatible interaction between the plant and the pathogen. In this infection process, certain host genes are activated by the pathogen to favor pathogen growth and promote symptom development (25). Such host genes, that facilitates this compatibility and/or are involved in the promotion of the pathogen infection, are considered susceptibility genes (S-genes). Hence, susceptibility genes may be viewed as the opposite of resistance genes. Deploying resistance genes has hitherto been the most obvious and utilized strategy to increase resistance in plants. However, this strategy confers several limitations. First and foremost, resistance is based on the immune system's ability to recognize a single pathogen-derived molecular pattern. Thus, the defense genes involved are highly specific, which in turn means they can easily be bypassed by mutations in the pathogen in question (25).

As pathogen infection disease arise from compatible interaction between the plant and pathogen, altering a susceptibility-gene has the potential to provide a more broad-spectrum and lasting type of resistance. However, as oppose to resistance-genes, susceptibility-genes confer functions outside the realm of pathogen infection. Once a susceptibility gene is mutated to interrupt a pathway in pathogen establishment, all other products from this pathway is also

sacrificed. Thus, the greatest limitation of this strategy is unintended consequences or undesired side effects that must be investigated for ecological and practical application of the genetically modified plant.

1.5 Genome editing and CRISPR-Cas9

Genome editing, also referred to as gene editing or genome engineering, is a group of technologies that enable scientists to precisely and efficiently introduce alterations into an organism's DNA. These technologies can add, remove or alter genetic material at specific sites in the genome. Several such technologies have been developed over recent years and the three most popular and utilized are Zinc finger nucleases (ZFNs), Transcription activator-like effectors (TALEs) and CRISPR-Cas9 (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeat and **C**ascade **9**). All three technologies confer advantages and disadvantages. However, CRISPR-Cas9 has emerged as the most promising one, as it is more efficient, faster, cheaper and more precise compared to the other technologies (26).

The CRISPR-Cas9 system is naturally occurring in the adaptive immune system of some bacteria. Simply put, the CRISPR system chops parts of the invasive organisms' nucleic acids into small pieces and inserts it into the bacteria's own genome, where it serves as a reference/molecular memory to protect against future infections of the same invasive organism. The system creates a protein-RNA complex that recognizes and cuts sequences similar the ones stored in the genome of the bacteria, and thus effectively neutralizes threats posed by the organism that have invaded the bacteria before. The CRISPR-Cas9 system has in recent years been modified in laboratories for genome engineering and is now the most promising gene editing technique. The fact that the system uses a reference sequence that it cuts out in other sequences makes it extremely valuable for targeted genome editing (26).

The CRISPR-Cas9 system in bacteria naturally consists of three components: small CRISPR RNAs (crRNAs), auxiliary trans-activating crRNAs (tracrRNAs) and Cas9. Cas9 is an enzyme (site specific endonuclease) that can cut double stranded DNA (dsDNA) at specific locations in the genome of the invasive organism (Cas in the natural context) guided by the crRNA/tracrRNA complex.

Engineered CRISPR systems contain two components: a synthetic fusion of crRNA/ tracrRNA, called single guide RNA (sgRNA) or gRNA and a CRISPR-associated endonuclease (Cas protein). Among the many Cas proteins, SpCas9 (from *Streptococcus Pyogenes*) is the most common and will be used in this thesis. The Protospacer Adjacent Motif (PAM) sequence (in the target genome) serves as a binding signal for Cas. The sequence varies in length and composition depending on the corresponding Cas protein used, but for SpCas9 the PAM sequence from *S. pyogenes* is 5'-NGG-3' where "N" is any of the 4 nucleotide bases.

sgRNA contains a small pre-designed RNA sequence (20 bp) called a spacer, attached to a longer RNA scaffold in the 3’ end. Once the Cas9 is expressed, the Cas9 protein and the sgRNA form a ribonucleoprotein complex through interactions between the sgRNA scaffold and the surface of Cas9. This causes Cas9 to undergo a conformational change that shifts the protein from an inactive, non-DNA binding conformation to its active DNA-binding conformation, while the spacer region of the sgRNA remains free to interact with the target DNA (27). The pre-designed sequence guides Cas9 to a genomic target site, while the scaffold is necessary for Cas9 to bind to DNA. The spacer defines the genomic target, and one can thus change the genomic target by changing the spacer sequence. It is important that the spacer sequence is unique to prevent off-target effects (the Cas9 protein cutting sequences outside of the intended locus). As mentioned, another target component is required for Cas9 to bind to both strands of DNA namely the PAM sequence situated 3-5 bp downstream of the target site. In the bacterial immune system the PAM sequence is used to distinguish the bacterial “reference” DNA from the actual invasive viral sequence or plasmid sequence, to ensure that only foreign genetic material containing the PAM sequence, and not the CRISPR locus host DNA, is destroyed. The PAM dependency is limiting factor of the CRISPR system, as the PAM sequence is required for the system to function, but not all target sites are adjacent to a PAM sequence.

Once the sgRNA-Cas9 complex is formed and Cas9 has undergone its conformational shift to its active form, the sgRNA of the complex guides the complex to the target site. The scaffold proteins are necessary for binding of the complex to the target DNA. Once bound, the Cas9, which is an endonuclease, will enzymatically break the double stranded DNA at a specific location (defined by sgRNA), causing the cell to activate one of its two main double strand break(DBS) repair systems to repair the DBS-break: NHEJ (non-homologous end joining) and HDR (homologous directed repair). The core difference between these repair pathways is that NHEJ-break ends may be ligated without template DNA, whereas HDR requires template DNA (the template cause it to be the most precise of the two, as bases are not randomly added but added according to the template). Preferentially, cells use the NHEJ pathway to repair DBS-breaks, which frequently causes insertions or deletions (random bases added as there is no template DNA available), which in turn leads to frameshift mutations creating loss of function alleles (referred to as knock-out). Fig. 2. illustrates a simplified overview of the mechanisms of the CRISPR/ Cas9 system, which may be used to insert, delete or edit DNA at specific genomic sites.

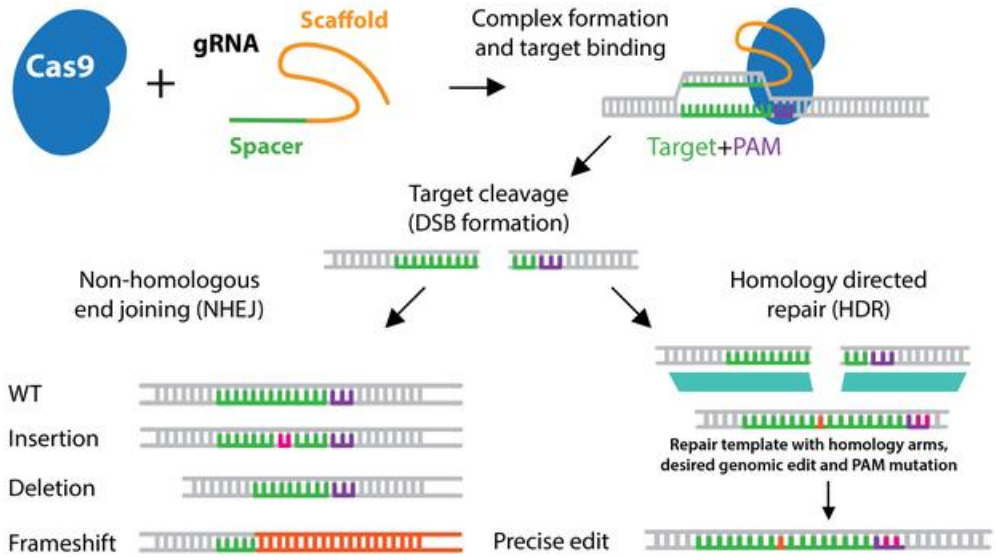


Fig. 2. *Illustration of the CRISPR-Cas9 system. Firstly, Cas9 and sgRNA form a complex, which bind to the target site in the genome. Cas9 induces a DBS-break, leading the cell to repair the DBS-break using either NHEJ or HDR. NHEJ leads to frameshift mutations caused by insertions or deletions, whereas HDR with template DNA may be used for specific genomic changes (Precise edit). Figure from* https://www.addgene.org/crispr/cut/.

There are several ways to modify genes with CRISPR-Cas gene editing systems including knock-out, knock-in, knock-down (in promoter, e.g. lowering expression level) and small edit of genes. Moreover, there are many Cas proteins, and thus many CRISPR-Cas systems, such as CRISPR/ Cas 12a (also known as Cpf1). Cas12a is slightly different from Cas9 in that it requires only one RNA, cuts target DNA further away from the PAM sequence (protospacer adjacent motif), it is a smaller and simpler endonuclease, in addition to a few other distinctions, which may favor it over Cas9 in some applications (28). However, hitherto CRISPR-Cas9 is the best studied and commonly used of the CRISPR-Cas systems. A knockout is achieved by deactivating the gene and this may in turn give rise to new loss of function phenotypes enabling functional analysis of the gene. As illustrated in in previous fig. 2. this can be done using CRISPR-Cas9, as it creates double strand breaks that are mostly repaired through NHEJ. The DBS induced by the CRISPR-Cas9 in combination with the error prone NHEJ repair system in general results in a loss of function through either significant loss of codons or frameshift. Knockouts can be done both conditionally (requires different CRISPR system including development specific promoter), which means knocking out genes in certain tissues later in development, and constitutively by knocking out genes in the early stages before the differentiation of tissues has begun. CRISPR-Cas9 induced knock-out is the genetic modification strategy in this study, hence the other CRISPR-Cas9 applications will only be described briefly.

Knockdowns are alterations in DNA that reduce the expression of one or more genes. It resembles knockout in the way that it seeks to impede already existing functions, but contrary to knockout it cannot destroy the gene, since doing so would silence the gene's function entirely. Merely reducing the expression requires modification of the translational activity. This is most often done by introducing a catalytically inactive Cas9 protein. This protein still contains a sgRNA that finds precise locations in the genome, but lacks the activity used to create dsDNA breaks. Hence, the protein will only bind to the target site, and not initiate a break. This kind of Cas9 complex works in reference to knock-down as a blockade for translational factors. This has been very effective in *Escherichia coli*, efficiently repressing the target gene expression without any off-target effects (29). However, several other factors are involved in knockdowns and it is beyond the scope of this study to go into more detail.

Knock-in is the process of placing a coding sequence precisely into the genome at a specific location, often with a gain of function as desired result. Previously this has been done with homologous recombination and non-homologous end joining (NHEJ) by using zinc finger nucleases (ZFN) or Transcription Activator-like Effector Nucleases (TALENs) with good results (30). However, recently scientist have developed improved protocols for efficient CRISPR-Cas9-mediated gene knock in and gene repair using improved protocols of CRISPR technology (31)Adding a template sequence flanked by regions homologous to the target region, enables the plant cells to use HDR precisely.

In order to use CRISPR-Cas9 for gene editing, CRISPR-constructs (expression system) must be made specifically for the model organism. Due to time limitations a specific lettuce promotor was not designed, and the standard Arabidopsis Atu6-26 was used instead (Discussion). The essential components of the CRISPR expression system (CRISPR-construct) is depicted and explained in fig. 3. A complete plasmid (CRISPR-construct) map, containing all components is provided in results.

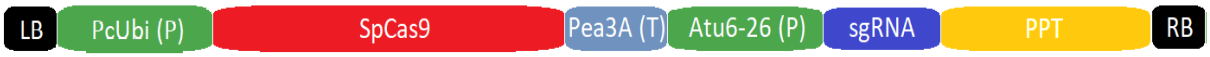


Fig. 3. *Schematic illustration of the engineered CRISPR-Cas9 system. Cas9 is expressed under the parsley ubiquitin promotor (PcUbi) and transcription is terminated by the Pea3 terminator. SgRNA is expressed under AtU6- 26 promotor (Arabidopsis promotor) and forms a sgRNA-Cas9-complex. The sgRNA guides the complex to target site, where Cas9 induces a DBS-break on the target DNA 3-5 bp upstream for the PAM sequence which (together with sgRNA) ensures the correct genomic site. The PPT cassette is for plant selection (BASTA). LB and RB denotes left border and right border, respectively.*

The most important technical challenges with CRISPR-Cas9 is off-target effects, PAM sequence dependency, transformation efficiency and regeneration of transgenic plants. Off-target effects are a limiting factor in CRISPR technology as the sgRNAs, which guides the CRISPR complex to the target site, is only 20 bp long, and only requires 15 bp match near 3’ on target site, several

sites that match the sgRNA may exist in a single genome, and if so, off-target effects may occur (32). This may be soplved by designed other sgRNAs. In a broader sense there are of course other challenges worth mentioning like political/legal, ethical and the current incomplete understanding of genetics. However, these will not be discussed as these aspects are beyond the scope of this thesis.

1.6 Plant transformation

CRISPR-Cas9 gene editing is based on altering an organism's genome using engineered CRISPR expression system described previously. This implies the introduction of the CRISPR-constructs into the host plant cells, referred to as plant transformation. Plant transformation may be divided into two major groups (33) 1) **indirect gene transfer**, which transfers DNA to plant cells with biological vectors and 2) **direct gene transfer**, which transfer genes by physical or chemical means like a gene gun. There are numerous methodologies within both groups, but indirect gene transfer using *Agrobacterium tumefaciens* mediated transformation to introduce CRISPR-constructs to plant cells is the most common method and will be used in this thesis. *Agrobacterium tumefaciens*-mediated may further be divided into stable and transient transformation where both techniques utilize the plant pathogen *A. tumefaciens* to transfer DNA to plant cells (34).

A. tumefaciens-mediated CRISPR-Cas9 stable plant transformation is a four-step process: T-DNA transfer and integration into the plant's genome, callus induction, regeneration of shoots and rooting. A suspension of *A. tumefaciens* transformed with CRISPR-constructs, is used to infiltrate plant cells that subsequently are induced for callus development (see methods for detailed explanation). In the stable transformation of plants, the germline transgene transmission is inheritable, hence providing a basis for the development of fully transgenic plants, where every cell contains a T-DNA (transferred DNA) copy integrated into its genome (germline mutations) ((35).

In the transient transformation the same transformant *Agrobacterium* suspensions is injected with a syringe (without needle) into the leaves of plants. Transient transformation is mostly

used to confirm expression of the CRISPR-constructs and eventually the subsequently induced mutation(s). Transient expression is predominantly derived from the transcription and translation of non-integrated T-DNA (35).

As already mentioned, several methodologies within each transformation techniques exist. Both other cultivars of *Agrobacterium tumefaciens* and other bacterial species may be used for delivering the CRISPR-constructs to plant cells (36). It appears that different methods yield different transformation efficiencies depending on several variables such as *Agrobacterium* species, and the model plant 's genotype. Furthermore, plant transformation efficiency and regeneration of transgenic plants are the main technical bottlenecks in plant genome editing for crop improvement hitherto.

1.7 Objective of the experiment

The primary objectives of this study are to discover, functionally analyze and edit genes involved in *L. sativas* resistance against *S. sclerotiorum* to ultimately generate lettuce lines with enhanced resistance using bioinformatics and literature studies.

Secondary objectives to accomplish for this thesis:

- Identify candidate genes and ideally susceptibility-genes (S-genes) that contributes to reduced defense from RNA-sequencing data from lettuce infected with *S. sclerotiorum*.
- Determine changes in gene expression of candidate genes that potentially contributes to reduced defense, and thus possibly identify S-genes in lettuce, using RT-qPCR.
- Functional analysis of highly upregulated genes by developing CRISPR-Cas9 knock-out constructs and gene edited lettuce lines.
- Study phenotypic effects on resistance in edited plants in green-house experiments, where transgenic plants will be infected by *S. sclerotiorum.*

2 Materials

An overview of all equipment, kits and chemicals used in this is experiment are listed in Table 2.1 to 2.11.

Table 2.1: Equipment.

Equipment	*Model*	*Supplier*
Centrifuge	5810 R	Eppendorf, Hamburg, Germany
Centrifuge	Heraeus Fresco 21	Thermo Fisher Scientific, Waltham, MA, USA
Gel electrophorese visualizer	Gel Doc™ EQ	Bio-Rad, Hercules, CA, USA
Heatblock	Thermo-Shaker PSC24	Thermo Fisher Scientific, Waltham, MA, USA
PCR machine	T100™ Thermal Cycler	Bio-Rad, Hercules, CA, USA
RT-qPCR machine	CFX96™ Real-Time System	Bio-Rad, Hercules, CA, USA
Spectrophotometer	NanoDrop™ 2000	Thermo Fisher Scientific, Waltham, MA, USA
Water bath	Isotemp® GPD 05	Thermo Fisher Scientific, Waltham, MA, USA

Table 2.2: Software**.**

Software	*Manufacturer*
Benchling	Benchling Inc., San Fransisco, CA, USA
Bio-Rad CFX manager	Bio-Rad, Hercules, CA, USA
Excel	Microsoft, Redmond, WA, USA
gRNA design tool	https://crispr.cos.uni-heidelberg.de (37).
Primer design tool	http://www.primer3.ut.ee/

Table 2.3: Size marker ladders for gel electrophoresis.

Ladder	*Supplier*
100 bp	New England BioLabs, Ipswich, MS, USA
1 kb	New England BioLabs, Ipswich, MS, USA

Table 2.4: Competent cells and their suppliers.

Competent cells	*Supplier*
NEB® 5-alfa Competent *E. coli*	New England BioLabs, Ipswich, MS, USA
One Shot™ Top 10 Chemically Component *E. coli*	Thermo Fisher Scientific, Waltham, MA, USA
***Agrobacterium tumefaciens* LBA4404**	Provided by Magne Skårn, NIBIO.

Table 2.5: Primers.

Oligo Name	*Oligo sequence (5' – 3')*
LS_PPO_1F	CATGCGAATATCGACAGAATG
LS_PPO_1R	TCTCATCGTAGAACAAGAACGAAG
LS_PPO_2F	TTGTGAACGATGAAGATGACG
LS_PPO_2R	TCTTCACATTCTTCCCATGC
LS_C2calcium_1F	TTGCATTAGTGCCTTTACGTG
LS_C2calcium_1R	AACAAACCCAGATGGAGAATG
LS_C2calcium_2F	CATCGGATCTTCACCATTTG
LS_C2calcium_2R	CCATGATCTCGTTTTCGTTC
LS_beta-caryo_1F	TATTGCTGGCCACAAGAAAG
LS_beta-caryo_1R	TTCCATGCATCTTCGATTTG
LS_beta-caryo_2F	AACTTTCCTCCTGCCATTTG
LS_beta-caryo_2R	AATCTTTCCGCACTTGTTCC
LS_Tubulin_1F	TAGTTCCATATCCGAGGATTCAC
LS_Tubulin_1R	TGTTGGTTATCTCAGCAACTGAC
TPC F	TCTTGAATTGGTTTGTTTCTTCAC
TPC R	GAGCACGACACGCTTGTCTA
Atu6 F	TCTTCAAAAGTCCCACATCG
SS43	GCATATAAGAAACCCTTAGTCG
SS61	GAGCTCCAGGCCTCCCAGCTTTCG
pChimera F	GCCACGTGTCTTGTCCAGAG
pChimera R	CTGTTTCCTTGCGTATTGGG

Table 2.6: sgRNAs.

Oligo name	*Oligo sequence (5' – 3')*
Ls_ppo_grna1f	attgTCCGGCTGACTTACCCCAAGGGG
Ls_ppo_grna1r	AAACCCCCTTGGGGTAAGTCAGCCGGA
LS_ppo_grna2F	attgTACGGCGCCGCCGCCACTTTTGG
LS_ppo_grna2R	AAACCCAAAAGTGGCGGCGGCGCCGTA
LS_C2calciumgRNA1F	attgTGAGTTGACTCGGTTGATTCCGG
LS_c2calcium_gRNA1R	aaacCCGGAATCAACCGAGTCAACTCA
LS_c2calcium_2F	aatgACAATTTCAAGGTTTTCTTGTGG
LS_c2calcium_2R	aaacCCACAAGAAAACCTTGAAATTGT
LS_beta-caryo_grna1F	attgAACTTTCCTCCTGCCATTTGGGG
LS_beta-caryo_grna1R	aaacCCCCAAATGGCAGGAGGAAAGTT
LS_beta-caryo_grna2F	attgCACAGCATACAAACTTGTTGAGG
LS_beta-caryo_grna2R	aaacCCTCAACAAGTTTGTATGCTGTG

Table 2.7: Kits.

Kits	*Supplier*
DNase I Amplification Grade Kit	Sigma-Aldrich, St. Louis, MO, USA
DNeasy® Plant Mini Kit	Qiagen, Hilden, Germany
iScript™ Advanced cDNA Synthesis Kit	Bio-Rad, Hercules, CA, USA
Qiagen® Plasmid Midi Kit	Qiagen, Hilden, Germany
QIAprep® Spin Miniprep Kit	Qiagen, Hilden, Germany
Spectrum™ Plant Total RNA Kit	Sigma-Aldrich, St. Louis, MO, USA
Wizard® Gel and PCR Clean-Up System	Promega, Madison, WI, USA
QIAprep® Spin Maxiprep Kit	Qiagen, Hilden, Germany

Table 2.8: Chemicals, hormones and antibiotics.

Chemical	*Supplier*
Agarose	Sigma-Aldrich, St. Louis, MO, USA
Boric acid	Sigma-Aldrich, St. Louis, MO, USA
Chlorine	Orkla, Norway
Chloroform:Isloamylalcohol (24:1)	Thermo Fisher Scientific, Waltham, MA, USA
dNTP nucleotides	Thermo Fisher Scientific, Waltham, MA, USA
Ethanol 96%	VWR Chemicals, Radnor, PA, USA
Ethidium bromide (EtBr)	Merck KGaA, Danmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Merck KGaA, Danmstadt, Germany
Nitrogen (liquid)	AGA, Norway

SOC Outgrowth Media	New England BioLabs, Ipswich, MS, USA
Sodium chloride (NaCl)	Merck KGaA, Danmstadt, Germany
Tris-base	Sigma-Aldrich, St. Louis, MO, USA
Trizma® hydrochloride (Tris-HCl)	Sigma-Aldrich, St. Louis, MO, USA
Tween® 20	Sigma-Aldrich, St. Louis, MO, USA
β-aminobutyric acid (BABA)	Sigma-Aldrich, St. Louis, MO, USA
β-mercaptoethanol (ME)	Sigma-Aldrich, St. Louis, MO, USA
BAP (6-Benzyl Amino Purine)	Sigma-Aldrich, St. Louis, MO, USA
NAA (1-Naphthaleneacetic acid)	Sigma-Aldrich, St. Louis, MO, USA
BASTA	Sigma-Aldrich, St. Louis, MO, USA
Cefotaxime	Sigma-Aldrich, St. Louis, MO, USA
Spectinomycin	Sigma-Aldrich, St. Louis, MO, USA

Table 2.9: Solutions.

Solution	***Reagent***	***Volume***
1 X TBE buffer	Tris-base	108 g
	Boric acid	55 g
	EDTA (0.5 M)	40 ml
	Distilled H_2O	Up to 1L
Luria- Bertani (LB) Broth	Tryptone	10 g
	NaCl	10 g
	Yeast extract	5 g
	Distilled H_2O	Up to 1L
Luria- Bertani (LB) Agar	Tryptone	10 g
	NaCl	10 g
	Yeast extract	5 g
	Agar	15.0 g
	Distilled H_2O	Up to 1L
1 X SOC Outgrowth Media	Vegetable Peptone	2 %
	Yeast Extract	0.5 %
	NaCl	10 mM
	KCl	2.5 mM
	MgCl2	10 mM
	MgSO4	10 mM
	Glucose	20 mM

Table 2.10: Enzymes.

Enzymes	*Supplier*
FastAP Thermosensitive Alkaline Phosphatase	Thermo Fisher Scientific, Waltham, MA, USA
AmpliTaq DNA Polymerase	Applied Biosystems, Foster, CA, USA
iScript Advanced Reverse Transcriptase	Bio-Rad, Hercules, CA, USA
AvrII (XmaJI)	Thermo Fisher Scientific, Waltham, MA, USA
SsoAdvanced™ Universal SYBR® Green	Bio-Rad, Hercules, CA, USA
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, MA, USA
BpsI	Thermo Fisher Scientific, Waltham, MA, USA

Table 2.11. List of target-and -reference genes. (Corresponding RNA sequencing data with upregulation etc. is provided in appendix).

Gene	*GenBank accession number*	*Putative function*
PPO polyphenol oxidase	XM_023907626.1	Acting on diphenols and related substances as donors
C2 calcium-dependent membrane targeting	XM_023904808.1	Membrane targeting
F. L Fungal lipase-like domain containing protein	XM_023892636.1	lipid metabolic process; C:membrane; F:hydrolase activity
B.C beta-caryophyllene synthase-like	XM_023895118.1	F:magnesium ion binding; F:terpene synthase activity; P:terpenoid biosynthetic process
Tubulin1 alpha tubulin 1	XM_023900018.1	Reference. Nucleoside-triphosphate phosphatase

For convenience, target-genes will be referred to as *PPO*, *C2*, *F.L* and *B.C*.

3 Methods

Experiments

The entire study was conducted at NIBIO (Norwegian Institute of Bioeconomic Research) in Ås, Akershus, Norway, where all equipment, materials, protocols and laboratory/ green-house facilities were provided/ located.

3.1 Selection of candidate genes

The strategy used in this study for finding candidate genes and ideally S-genes was based on data from analysis of RNA sequencing data (provided to the start of this project) in combination with existing literature. RNA from lettuce was extracted and sequenced at three stages: uninfected, 24- and 48hours after infection with *S. sclerotiorum*. Genes were then listed and organized according to their upregulation during the infection process. Highly upregulated genes after 24hours and 48hours of infection were then screened and categorized for potential susceptibility-genes based on GO-terms and information on functionality (of homologous genes) from and compared to existing literature to identify potential candidate genes.

Once all highly upregulated genes were identified and organized chronologically (in terms of upregulation) in a table (appendix), the most upregulated candidates were blasted in the NCBI Blast web tool to obtain functional information on the genes. Once main characteristics for the genes were obtained, genes not matching the following criteria were weeded out; genes with known essential functions in the organism, genes displaying high activity in the uninfected lettuce plants and genes with several known functions.

The remaining genes were then compared to existing literature and the three considered most promising in terms of contributing to reduced defense were chosen for the actual experiment. Initially eight candidate genes were selected (Appendix). This list was narrowed down to three candidate genes (Materials) to limit the extent of the experiment.

3.2 RNA extraction and gene expression analysis

The RNA sequencing and extraction were done prior to the start of this thesis. RNA sequencing was done on uninfected and *S. sclerotiorum* infected lettuce to identify candidate genes, as described in the previous paragraph.

The RNA sequencing data was provided by Torgeir Tengs (NIBIO). RNA sequencing data for target and- reference genes are listed in appendix. Both the RNA extraction and RNA sequencing were done prior to this study.

Reverse transcription qPCR (RT-qPCR) was used to confirm the quantitative gene expression data from the RNA sequencing, described in following paragraphs.

3.3 DNA and RNA isolation

DNA from both *Agro*-transient transformed and stably transformed Lettuce plants were isolated to examine expression of the CRISPR- constructs. However, due to COVID-19 outbreak it was not sequenced. 100 mg of leaves or leaf discs (cut after transient transformation) were homogenized and grounded to fine powder in liquid nitrogen using mortar and pistil. From this powder DNA was isolated using DNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany), following the manufacturer's protocol. Isolated DNA was stored in -20°C.

RNA from the same material were isolated, cDNA was synthesized and used for RT- qPCR (and sequenced) to investigate the gRNA-scaffold expression. The isolation was done using SpectrumTM Plant Total RNA Kit (Sigma-Aldrich®, St. Louis, MS, USA), following the manufacturer's protocol.

3.4 cDNA synthesis and RT- qPCR

PCR (polymerase chain reaction) is a common method used in molecular biology to copy and amplify (generate many copies) a particular DNA segment of a larger DNA-molecule (e.g. a chromosome). It amplifies DNA using temperature stable DNA polymerase, primers, buffer and

nucleotides (dNTP). The purpose is to synthesize enough of the DNA segment in question, in this case the target genes, to do further analysis, cloning etc.

To confirm that the expression levels of the selected target genes correspond to RNA sequencing data RT-qPCR was done, using primers for target- and -reference genes. cDNA from the RNA samples (cDNA synthesis etc. will be explained in the next paragraphs) is used as template, with primers for the genes in question, including a reference gene which is used for normalization. These expression level data were then compared to RNA expression data to verify the original RNA sequencing.

RT-qPCR is the most common method for quantitative gene expression analysis. For normalization of the expression, typically good "housekeeping" genes are selected as reference. Such genes expression is assumed to remain unchanged over a wide range of conditions, thus serving well as reference for expression. In this thesis, *TUB1* (Tubulin1) was used as reference gene.

The C_q value (cycle quantification value) is the PCR cycle number at which a sample's reaction curve intersects the threshold line. This value tells how many cycles it took to detect a signal from a sample. In other words, the C_q value is inversely proportional to the expression level; the higher the C_q value, the lower the expression. The C_q values, are the basis for the $2-\Delta\Delta Ct$ method, used to present RT-qPCR data in a fold change diagram.

To run RT-qPCR, cDNA (complementary DNA) had to be synthesized.

cDNA is synthesized from isolated RNA through the reverse transcription reaction. Reverse transcriptases (RTs) are enzymes using short primers and RNA template to synthesize cDNA which in turn may be used as template for the RT-qPCR.

The cDNA was synthesized using the iScript TM Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A). The reactions were done according to the manufacturer reaction setup and protocol. To limit background the optional step of DNase treatment was done.

The samples were combined in PCR plates. 1 µg RNA/µl was added to each reaction. Two samples were made without reverse transcriptase enzyme, serving as negative controls.

Solution:

4 µl 5x iScript Advanced Reaction Mix

1 µl iScript Advanced Reverse Transcriptase

1 µl RNA template

14 µl Nuclease-free h_2o

20 µl Total volume

The samples were placed in a S100™ Thermal Cycler PCR machine (Bio-Rad, Hercules,CA, USA) and was run by the following program:

Reverse transcription 20 min at 46°C followed by inactivation of the enzyme 1 min at 95°C.

Once synthesized, cDNA was diluted 10 folded and used as template for RT-qPCR.

Gene expression analysis was performed in duplicates in two 96-well reaction plates using SsoAdvanced™ Universal SYBR® Green dye system (BIO-RAD, Hercules, CA, USA) in a CFX96TM Real-Time System (BIO-RAD, Hercules, CA, USA). Reactions were carried out according to manufacturer's protocol. The reaction solution is listed below (see appendix for the sheet for the reaction setup).

Solution:

10 µl SsoAdvanced™ Universal SYBR® Green

1 µl Primer forward + reverse (2 µl total)

2 µl cDNA template

6 µl Nuclease free h_2o

20 µl Total volume

qPCR was run using the following program:

Initial denaturation at 95°C for 3minutes, followed by 40 cycles of amplification/denaturation at 95°C for 10seconds, and primer annealing at 60°C for 30 seconds.

The data from the expression analysis was used to make a graphic representation (see results). For all duplicates, an average was made before making the graphic representation.

3.5 Primer design for target genes

Primers for candidate genes were made using the Primer3 web tool.

mRNA sequences for the target genes from the RNA sequencing were confirmed by the NCBI mRNA search tool and pasted into the primer3 tool with the following parameters for optimizing the primers:

1. 20- 30 bp long
2. Amplicon from 100- 200 bp
3. G/C content 40- 45%
4. TM 58- 60^{o} C
5. G/C clamp (e. g sequence ends with G or C the enhance ligation)

From here, the two best primer sets (forward/ reverse) per gene were selected**.** Only one primer set (e.g. f1/ r1 for each gene) per gene were used, whereas the second served as backup. All primers with sequences are listed in materials.

3.6 Designing sgRNA

The design of sgRNA was done using the CCTop –CRISPR-Cas9 target online predictor, sited in materials. In short, this program suggests possible sgRNA for a selected host genome, and scores candidates according to off-target sites. Off-target site scores indicate the likelihood of a stable sgRNA/DNA heteroduplex (“Based on experimental evidence this likelihood decreases the closer the mismatch is to the PAM sequence”) (37).

Parameters used:

Reference genome: Lactuca Sativa

Custom: attg, aaac overhang.

Max 500 bp, find gRNA in the first half of the gene and Score <0.74.

sgRNA sequences are listed in Materials.

3.7 Design and cloning procedure of CRISPR-constructs (plant expression vectors)

The cloning system used in this study consisted of *Escherichia coli* for transformation and propagation of the vectors and *Agrobacterium tumefaciens* binary vector system for plant transformation, which deliver the CRISPR-constructs into the *L. Sativa* plants. The system is called binary as two vectors are used together where one vector contains the CRISPR- construct and the other is a "helper plasmid" which contains *vir* genes derived from the Ti plasmid of *Agrobacterium*. A detailed explanation of the different *vir* genes and their many functions is beyond the scope of this study. However, it's worth mentioning that the *vir* genes essentially code for enzymes/ compounds that helps cut T-DNA from the CRISPR-construct at left and right borders, before facilitating the transduction of T-DNA from the CRISPR-construct into the host genome (38). The T-DNA integrates at a random site on of the host plant chromosome. Once integrated, the CRISPR components are (hopefully) expressed, and the sgRNA-Cas9 complex is formed. The sgRNA guides the complex to the correct genomic site, binds the complex (together with the PAM sequence). This binding cause Cas9 to undergo a conformation change to its active form, and the DBS-break may occur.

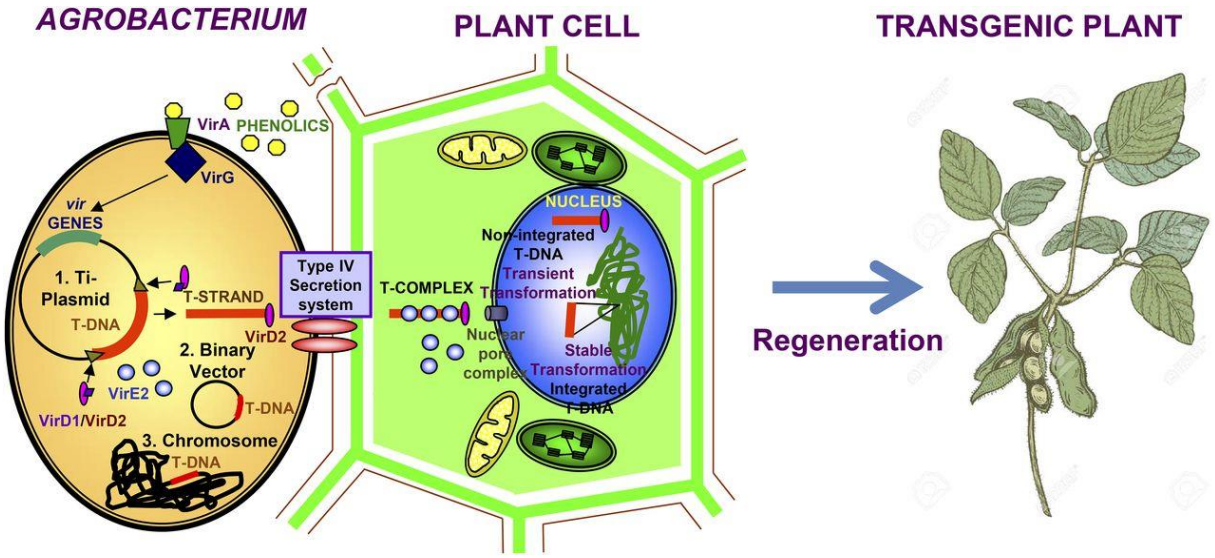


Fig. 4. *simplistic illustration of Agrobacterium mediated plant transformation. Picture from www.plantcell.org.*

The following paragraphs describes the entire cloning procedure in detail.

3.8 Cloning of sgRNA into pChimera

First *E. coli* JM109 (Promega,) was transformed with the pChimera plasmids to produce enough pChimera plasmid to continue the cloning procedure and cultivated on selective LB-medium containing ampicillin (pChimera confers resistance to ampicillin). Plasmid isolation was done with Qiagen® Plasmid Midi Kit (Qiagen®, Hilden, Germany) according to manufacturer's protocol.

Two sets of sgRNAs from each of the three genes were annealed to oligonucleotides and cloned into empty pChimera plasmids according to the protocol outlined below.

The designed sgRNAs were confirmed by sequence alignment to not contain any restriction sites for neither *Bpi*I (*Bbs*I) or *Xma*JI (*Avr*II).

Annealing of oligos

2 µl of fwd oligo 1 (100 µM)

2 µl of rev oligo 2 (100 µM)

16 µl nuclease free h_2o

20 µl total volume

Annealed in a thermocycler using the following parameters:

95^o C for 5 minutes (no cooling at the end).

Cooling at room temperature for 20 minutes.

*Digestion of pChimera with Bpi*I *(Bbs*I*)*

2 µl 10x Buffer G

15µl nuclease free h_2o

2 µl of pChimera (1 µg in total)

1 µl of *Bpi*I (*Bbs*I) (10 units/µl)

20 µl total volume

Digested at 37^o C for 2 hours in a thermocycler.

Ligation of pChimera with annealed oligos

Ligation components was added directly to digest.

2.5 µl 10x T4 DNA ligation buffer (use aliquoted buffer)

1 µl of annealed oligos

1.5 µl T4 DNA ligase (1 unit/µl)

20 µl Nuclease free h_2o

25 µl total volume

Ligated at 37^o C for 1.5 hours in a thermocycler and put on ice.

Transformation of cells

Used 2 µl from the above reaction to transform *E. Coli* JM109 cells.

PCR-screening of colonies

Primers used: sgRNA1(for each gene) and pChimera r.

Annealed at 56^o C, 30 sec elongation and 30-40 cycles.

Expected band size approximately 370.

3.9 Transformation of bacterial cells with pChimera-sgRNA and pCas9-TPC

After insertion of sgRNA fragments, positive transformants of pChimera plasmids confirmed by sequencing were transformed into NEB® 5-alfa competent *E. coli*.

Plasmids from positive transformants (confirmed by sequencing, results) were isolated with the "QI Aprep spin minprep kit" according to manufacturer's protocol.

pCas9-TPC plasmids was transformed into One Shot™ Top 10 Chemically Component *E. coli* (Thermo Fisher Scientific, Waltham, MA, USA), cultivated on selective LB- medium containing spectinomycin (pCas9- TPC confers resistance to spectinomycin). Successful transformants were confirmed by PCR and gel electrophorese and plasmids were isolated by QIAprep® Spin Maxiprep Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol.

Yields of DNA after isolation

PChimera: 475, 9 ng/µl

PCas9-TPC: 745.5 ng/µl

Cloning products were confirmed by sequencing (Results).

3.10 Digestion

After plasmid isolation, both plasmids were cut with restriction enzymes, to linearize the circular structure of the plasmids. This open structure is necessary for the following steps. The reactions are described below:

Reaction setup for pChimera (one for each clone/ target gene)

10 µl pChimera (*PPO*, *C2* and *B.C*)

3 µl *Avr*II

5 µl 10x Buffer Tango

32 µl Nuclease free h_2o

50 µl in total

Reaction setup for pCas9-TPC

20 µl (10ng) pCas9-TPC

3 µl AvrII

5 µl 10x Buffer Tango

22 µl Nuclease free h_2o

50 µl in total

The digestion reactions were done in a S100™ Thermal Cycler PCR machine (BIORAD, Hercules, CA, USA) at 37°C for 10 hours.

3.11 Gel purification

Gel purification is a technique to isolate and purify desired DNA fragments, based on size. The procedure starts with standard agarose gel electrophoresis, which separates DNA fragments according to their size (in base pairs). The volt difference across an agarose gel matrix causes the negatively charged DNA to migrate through the gel towards the positive pole in a buffer solution. Large fragments of DNA will migrate slower than smaller fragments due to the pores in the gel. It is thus possible to visualize and identify individual fragments of DNA by comparing it to known bp ladders visualized through UV light and Ethidium-Bromide (EtBr) staining (39). Following electrophoresis DNA bands were cut out of the agarose gel and purified using Wizard® Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

Gel preparation for standard 1% Agarose Gel

1.1 g of agarose was added to 100 mL X TBE buffer solution in a glass bottle.

2. Microwaved for 1- 5 minutes until the agarose is completely dissolved

3. The solution was cooled down to approximately 50°C and one drop (0, 05 ml) of EtBr (0.5 µg/ml) was added per 50 ml solution.

4. The solution (only 70ml used) was carefully mixed and poured into a gel tray.

Running of the agarose gel

pChimera with sgRNA: 48µl of DNA from each sample was combined with 10 µl of loading buffer to a total volume of 58 µl. The 70 ml 1% agarose gel were run at 70V for 45min and visualized under UV light. The three (one for each gene) gRNA cassettes (which includes promoter, 20 bp sgRNA, scaffold and terminator) from the cloned pChimera vectors were

clearly separated and were found at the expected size of approximately 700 bp. The fragments were then cut out from the gel using a sterilized scalpel. The gel slice was weighted and put in 1.5 ml tubes. After gel slicing, DNA was cleaned with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

For pCas9-TPC the same procedure was done. However, the gel was run for 80 min before the fragments arrived at the expected size of 14 kb.

The 1% agarose gel is standard. To better separate small fragments one can increase the concentration of agarose in the gel.

3.12 Dephosphorylation

pCas9- TPC was dephosphorylated by the phosphatase enzyme FastAP(Thermo Fisher Scientific, Waltham, MA, USA) in order to prevent the vector from re- ligation during the ligation reaction. The phosphatase enzymes remove the 5' phosphate ends of the vectors DNA, thus preventing it from "melting" back together. Instead, the phosphate ion (PO_4)$^{3-}$ and -OH from the sgRNA fragment is used to restore the DNA backbone through formation of covalent phosphodiester linkages and thus ensuring the sgRNA cassette is placed at the desired site in the vector.

For pChimera, the dephosphorylation is not necessary as the restriction sites are not complementary. The enzyme (AvrII) cuts 4 bp upstream of the two restriction sites. Hence, the probability that the sticky ends of each restriction site are complementary are close to zero. pCas9-TPC has only one restriction site for AvrII and hence had to be dephosphorylized.

Dephosphorylation of pCas9-TPC

37 µl (4µg) pCas9-TPC

5 µl buffer (10x reaction buffer for FastAP)

2.5 µl FastAP

5.5 µl Nuclease free h_2o

50 µl in total

The dephosphorylation reaction was done in a S100™ Thermal Cycler PCR machine (BIORAD, Hercules, CA, USA) at 37°C for 10 min followed by 5 min at 75°C for inactivation of the enzyme.

3.13 Ligation

The final step in the construction of the CRISPR-constructs was to connect the insert DNA (sgRNAs fragments with promoter, scaffold and terminator, Results) with the backbone of the dephosphorylated pCas9-TPC vector. This was accomplished by ligation. The ligation reaction is facilitated by the enzyme T4 DNA ligase, which catalyzes the formation of covalent phosphodiester linkages between the nucleotides, permanently joining them together. Once ligation is completed, the insert DNA is physically attached to the vector, resulting in a complete recombinant plasmid (39).

The ligation reaction was done in a PCR machine at 16°C for 16hours.

Ligation reaction (six reactions, one for sgRNA1 and 2 for all three genes)

1 µl T4 DNA ligase

1 µl Ligase buffer

1 µl pCas9-TPC dephosphorylated (100 ng)

6.5 µl sgRNA cassette from pChimera (100 ng)

0.5 µl nuclease free h_2o

10 µl in total

Negative control solution

1 µl T4 DNA ligase

1 µl Ligase buffer

1 µl pCas9-TPC

7 µl nuclease free h_2o

10 µl in total

The negative control was ligation of the recipient plasmid DNA (pCas9-TPC) without any insert to map level of background of uncut or self-ligating recipient plasmid backbone.

The ligation reactions were confirmed by sequencing.

3.14 Transformation of bacterial cells with CRISPR-constructs

The following protocol was used to transform the ligation mix (CRISPR-constructs) into NEB® 5-alfa competent *E. coli* cells

Transformation:

1. 1 µl of ligase mix (solution from ligation previous paragraph) was added to 20 µl NEB® 5-alfa competent *E. coli* cells (in total six, two for each target gene) and kept on ice for 30 min followed by a heat shock at 42°C for 45 seconds in a water bath.

2. After heat shock, tubes were immediately put on ice for 2 min, and 600 µl of SOC Outgrowth Medium (room temperature) (New England BioLabs, Ipswich, MS, USA) was added to each tube.

3. Tubes were then incubated at 37°C in a heat chamber with shaking at 225 rpm for 1 hour.

4. After incubation, 20 µl of the culture was spread out on Petri dishes containing LB agar with spectinomycin (50 µg/ml) (pCas9-TPC confers resistance).

5. The remaining culture was centrifuged for 15 seconds at 10.000 rpm and resuspended in approximately 100 µl of the liquid. 100 µl of the resuspended culture was spread out on the petri dish. All petri dishes were incubated over night at 37°C for bacterial colony growth

3.15 PCR and gel electrophoresis

Bacterial colonies from the transformation were PCR-screened and ran on agarose gel electrophoresis for visualization of the CRISPR-constructs with the inserted sgRNA cassette. Firstly, PCR was done to amplify the inserted DNA, using specific primers for the insert cassette, listed below. The PCR solutions were then run on gel to confirm presence of the vectors.

PCR-screening for colonies

7 µl of nuclease free h_2o was added to the wells of a PCR plate. 18 randomly selected colonies, six for each CRISPR-construct, were picked carefully from its petri dish, using a pipette tip. The pipette tip was dipped in the well and carefully removed, and then spread out on marked sites on a selection media, e.g. a petri dish with LB containing spectinomycin (50 µg/ml). This procedure yields fresh cultures of single colonies and makes it easy to track successful transformants (bacteria transformed with CRISPR-constructs) after gel electrophoresis. 1 µl of positive control, a pCas9-TPC plasmid without gDNA cassette, was added to one well in addition to a negative control, consisting of nuclease free h_2o only.

The PCR solution

11 µl of nuclease free h_2o

2,5 µl PCR buffer

2 µl dNTP

1 µl of each primer (sgRNA1 f and pCas9-TPC r)

0, 2 µl AmpliTaq DNA Polymerase

16, 2 µl in total

The PCR solution was added to all wells, before it was run on the following PCR program:

94°C 5 min, (94°C 30 sec, 56°C 30 sec, 72°C 45 sec) x 40, 72°C 7 min and 4°C ∞

S100™ Thermal Cycler PCR machine (BIORAD, Hercules, CA, USA).

The PCR products were run on gel (prepared like described previously) to identify successful transformants.

3.16 Preparation of bacterial cultures

Once confirmed by gel electrophoresis, bacterial colonies of verified transformants for each CRISPR-construct were used to make fresh bacterial cultures.

2 cultures were prepared for each target gene CRISPR-construct (PPO sgRNA1 and sgRNA2, C2 sgRNA1 – and sgRNA2 and *B.C* sgRNA1 and sgRNA2). Each culture was prepared by adding five ml LB with spectinomycin (50 µg/ml) to 50 ml falcon tubes. Bacterial cultures were added to the tubes, using a sterile pipette tip, and incubated at 37°C overnight at 160 rpm. 2 ml of the overnight cultures were transferred to 2 ml tubes and centrifuged at 10000 rpm for 3 minutes to obtain a pellet and used for mini preps.

The plasmid DNA isolation was performed using the QIAprep® Spin Miniprep 250 Kit (Quiagen, Hilden, Germany) according to manufacturer's protocol. Once isolated, DNA concentrations were measured by NanoDrop™2000, and set to be in the 30-100 ng/µl range, for all six samples. 20 µl of the DNA solution was prepared and sent for sequencing to Eurofins Genomics (Germany) accompanied with primers (sgRNA1f and pCas9-TPC r).

Once confirmed by sequencing, the transformation of the CRISPR-constructs into *A. tumefaciens* began.

3.17 Agro transformation

Sequencing confirmed the six CRISPR-constructs to be correct, but to limit the extent of the experiment only the construct with the highest ranking sgRNA for each target-gene was selected. For all three genes sgRNA 1 was selected. The CRISPR-constructs (plasmid vector pCas9-TPC with sgRNA1 for *PPO*, *C2* and B. C) were transformed into a strain of *A. tumefaciens* named LBA4404 (originally from Invitrogen, provided by Magne Skårn at NIBIO) in the following manner:

Freeze-thaw transformation of *A. tumefaciens* competent cells:

1. Tubes of approximately 50 µl frozen competent cells were thawed on ice for 30-50 min. 250 ng of plasmid DNA was added to the tubes, and tubes were incubated on ice for 5 minutes for the plasmid DNA to stick to the outer side of the cells.
2. Tubes were frozen in liquid nitrogen for 5 min, and immediately transferred to a heat shock in 37°C water bath for 5 min, then returned to ice for 5 min. This temperature treatments makes the cells take up the plasmid DNA.
3. 1 ml of room tempered LB broth was added to each tube and incubated at 28°C with shaking at 200 rpm for 3-4 hours.
4. After incubation, 50 µl and 200 µl of each culture was plated out on LB agar plates containing rifampicin (50 µg/ml) selection agent for *A. tumefaciens* combined with spectinomycin (50 µg/ml) for pCas9-TPC for culturing.

Plates were incubated for colony growth at 28°C for 3 days.

Transformation was confirmed by PCR and gel electrophorese.

3.18 Cultivation of lettuce

Great Lakes seeds were planted on petri dishes containing germination media for 4-7 days before transformation. All medias used at all stages of this experiment are listed below. The coated MATCH seeds were cultivated on petri dishes containing only distilled h_2o for 14 days. For the transient transformation, Great Lake seeds were cultivated by Vinh Hong Le in soil for 30 days before transformation.

Lettuce Medias

1. **CCM media** –liquid for co cultivation of plant and Agro

1 x MS (Murashige and Skoog)

3 % sucrose, pH 5.8

Added 0.5M acetosyringone freshly in media. Acetosyringone induces the *vir* gene in the Ti plasmid of Agrobacterium and enhances infection (40).

2. **Seed germination media**

1 x MS (Murashige and Skoog) (See next table)

0.8% agar pH 5.8

3. **Lettuce callusing medium** – solid.

	STOCK	**1000 ml**	
1 x MS Macro	10x	100 ml	= MS powder
1 x MS Micro	100x	10 ml	
1 x Vitamins	500 x	2 ml	
1 x Iron	200x	5 ml	
3 % Sucrose		30 g	
0.8 % TC agar		8 g	
0.2 mg/l BAP	2.0 mg/ml	100 µl	
0.05 mg/l NAA	1.0 mg/ml	50 µl	

pH was adjusted to 5.5-5.8 with (KOH 1M and 0.1M) before agar was added.

Antibiotics and hormones: solutions were prepared in sterile distilled water, filter sterilized and stored in -20C. Antibiotics and hormones were added to the autoclaved media after cooled down to approximately 50° C, just before pouring.

Antibiotics:

BASTA - 4µg/mL or 4mg/ L

Cefotaxime – always 300mg/ L

4. **Lettuce rooting medium** – solid.

   1 x MS (Murashige and Skoog)

   0.8% agar pH 5.8

   Antibiotics:

   BASTA - 4µg/mL or 4mg/ L

All medias were made according to respective protocols mentioned above. All were made with distilled h_2o. The pH was measured and adjusted to 5.5- 5.8 before agar was added. Once the mixture was ready, approximately 30 ml was poured into petri dishes and kept in room temperature until solid (except CCM liquid media) and then stored in fridge at 4° C.

The hormone BAP (6-Benzyl Amino Purine) is a synthetic cytokinin which together with auxins elicits plant growth and development responses (41). NAA (1-Naphthaleneacetic acid) is one of the synthetic auxins, used in plant propagation It can induce the formation of lateral and adventitious root (42).

The pCas9-TPC vector confers resistance to BASTA so this antibiotic was used as a plant selection agent in the medias, whereas the antibiotic Cefotaxime was used to kill remaining *Agrobacterium* after transformation.

3.19 Plant transformation

The plant transformation method used in this thesis was provided by NIBIO. In total 6 separate transformations were performed during this study. Four on Great Lakes callus' (using CRISPR-construct sgRNA1 for all three genes), one on MATCH callus' using only CRISPR-construct *PPO1*. The MATCH transformation was conducted to identify possible differences on transformation efficiency among different lettuce strains. The sixth and last transformation was transient transformation on Great Lakes lettuce leaves using CRISPR-constructs for all three target-genes.

For the four transformations of *L. sativa* seeds (non-coated, Great Lakes) were rinsed and sterilized as described in the following procedure. For the first of these transformations, a CRISPR-construct for the gene Nced4 provided by Artie Rai (NIBIO) was used to serve as control.

Washing steps

1. First lettuce seeds were sterilized with 70% EtOH for 1 min.

2. Lettuce seeds were then sterilized with 20.01% tween20 and 2.5% NaOCl for 7 minutes.

3. Seeds were rinsed with distilled water three to four times and plated on MS media plates.

4. The seeds were then germinated on Petri dishes containing MS media with 0.8% agar for 4-7 days. After Germination, cotyledons were used for transformation

The washing and sterilization of the MATCH seeds (coated) was done using the following procedure:

Coated seeds germination and sterilization

Seeds were germinated on soaked filter paper in Petri dishes for 14 days.

After 14 days of germination, germinated cotyledons were washed with 70% EtOH for 30 seconds.

Then washed with 20.01% tween20 and 2.5% NaOCl for 7 minutes, before rinsed with distilled water three to four times and proceeded on to *Agro*- transformation.

For the first three transformations of the Great Lakes genotype, seeds grew for 4 days. The last transformation was done on seeds that grew for 7 days, to investigate if grow time had any effect on transformation frequency.

After germination the transformations were conducted according to the protocol listed below.

Agrobacterium culture preparation and transformation

Prepared overnight culture of Agrobacterium (broth, with Rifampicin and spectinomycin 28 degrees shaking at 200 rpm). OD 600 nm of bacterial suspension was set between 0.5-1.0 in MS media containing 0.5 M acetosyringone (freshly added) and 3% glucose.

Cotyledons were cut out from seedling dipped in (moving) *Agro*- suspension media for 15 min.

The explants were dried on autoclaved filter paper and placed, using a sterilized tweezer, on Petri dishes with MS medium containing 3% sucrose (NAA 0.05mg/L and BAP 0.2 mg/L) for 48 hours in dark.

Then explants were washed in sterilized water and 250 mg/L cefotaxime for 1 hour to remove *Agrobacteria*. Plant pieces were again washed with sterilized water 2-3 times to remove remaining *Agrobacteria*.

Then explants were dried on filter paper and placed on Petri dishes with MS media containing 3% sucrose (NAA 0.05mg/L and BAP 0.2 mg/L) + 250mg/L cefotaxime + 4 mg/L Basta.

Proliferating calli arising from the explants subcultured into the same fresh medium every alternate week. Emerging shoots were individualized and cultured regularly on MS media containing 3% sucrose (NAA 0.05mg/L and BAP 0.2 mg/L) + 250mg/L cefotaxime + 4 mg/L Basta.

The following overview shows the number of transformed cotyledons (two leaf stage) per gene for each transformation (#1-4, and Match). In total 473 cotyledons (946 leaves) were transformed.

	#1	**#2**	**#3**	**#4**	**MATCH**
PPO	25	20	21	75	35
C2	26	25	19	73	
B.C	28	25	22	58	
Nced4	21				
Total	100	70	62	206	35

3.20 Transient transformation

In addition to *Agrobacterium*-mediated stable plant transformation, transient transformation was performed. The transient expression of the CRISPR-constructs in lettuce leaves were done according to the following protocol. Transformation was done on six leaves, two leaves for each CRSPR- construct (*PPO*, *C2* and *B.C*).

1. *Agrobacterium* was inoculated overnight in the same manner as for the plant transformation.
2. The bacteria were precipitated by centrifuge at 4000 rpm, 20 °C for 7 min. Then the pellets were resuspended in 5 ml *Agro*-infiltration buffer containing; 10mM MgCl2, 10mM MES- KOH at pH 5.7 and 200 mM Acetosyringone and dH2O.
3. OD 600 nm was set to 0.5 by dilution with additional buffer, and left on bench for 3 hrs.
4. The infiltration was done on 30 days old lettuce (Great Lakes), using a 2ml syringe. The syringe was pressed to the underside of the leaves, and counter- pressure was exerted by pressing fingers on the upside of the leaves.
5. After 4 days the leaves were harvested, and DNA was isolated using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol, and PCR screened. RNA was also isolated using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol.

3.21 Greenhouse conditions

All seeds and explants were grown indoor at NIBIOs plant growth room. Growth conditions are listed below.

Light: Lights on from 08:00 to 00:00.

Temperature: 22°C.

Humidity: 60%

4 Results

4.1 Selection of candidate genes

Candidate genes, and ideally S-genes, possibly involved the in the pathogenesis of *S. sclerotiorum* were selected from the provided RNA sequence data, based on elevated expression upon infection, known functions and existing literature(Methods). All three candidate genes expression were zero or close to zero (*C2* had a few reads, *B.C* and *PPO* had 0, see 4.2) in uninfected lettuce and was highly upregulated upon infection which indicates involvement in the pathogenesis. Furthermore, it was assumed that knock-out of genes that are not expressed in despite of infection would be preferential considering unintended consequences.

Initially a list of 8 candidate- and reference genes (listed with RNA seq data, Appendix) were selected. In order to limit the extent of this thesis, this list was narrowed down to the three candidate genes (*PPO, C2* and *B.C*) for the actual plant transformation (list in materials). *B.C* replaced the gene *F.L* as it was recognized as a better candidate after the initial list was made.

Studies on *S. sclerotiorum* (cited previously), concludes that oxalic acid secretion and chelation of calcium are important factors in the pathogenesis. In the same study it was postulated that *S. sclerotiorum* may secrete oxalic acid to precipitate Ca^{2+} ions released by the plants cell wall as it degrades during infection. Due to this study and the high upregulation of expression upon infection, the gene encoding *C2* calcium-dependent membrane targeting protein (*C2*), was selected. Moreover, similar genes have been shown to elevate expression upon pathogen infection (43). Based on this knowledge it was hypothesized to might be involved in the pathogenesis. However, the exact role(s) is currently not identified, and its specific function in the pathogenesis can potentially be as an innate immune response gene, it may be used by the pathogen in the calcium chelation process, which protects *S. sclerotium* from the toxic levels of Ca^{2+} ions in plant cell walls during the infection or it may simply be activated merely as a consequence of the increased Ca^{2+} ions during infection, without further involvement. *C2* is known to interact with Ca^{2+} ions and membranes (like cell walls) so a functional study by a knockout would provide a better understanding of its function in the pathogenesis. However,

C2 may of course have other important roles not identified and a knock-out may cause undesired effects, phenotypic or otherwise.

Polyphenol oxidase (PPO) was also selected due to its elevated expression upon infection and because other studies have shown it is exhibiting a central role in the rotting process (e.g. browning (44)) of several crops including potatoes, tomatoes and apples. Both suggests its involvement in the pathogenesis. Although it is not considered a susceptibility gene per se, it was selected as a candidate gene primarily to yield easily detectible phenotypic effects. It would hence serve as a form of positive control in evaluating the effect of the CRISPR-construct in the phenotypic study transformed plants. Knockout of *PPO* has been done in several crops yielding phenotypes displaying no signs of browning, like the famous example of Artic apples (45).

One study showed that oranges with highly downregulated *B.C (Beta-caryophyllene synthase)* was displaying resistance against the fungus *P. citricarpa* (46). Furthermore, its expression was highly upregulated upon infection. Based on this knowledge, it was selected as a candidate gene and potentially a s-gene. Moreover, *B.C* belongs to the terpene synthase gene family. Overexpression of the protein d -limonene synthase (which is encoded for by a gene belonging to the same gene family) encodes seems to stimulate growth of some pathogenic fungus, including *C. graminicola* (44), which further supports this hypothesis. However, the same study concluded it is also involved in the biosynthesis of terpenes which may have an opposite function:" some terpenes may have a defensive role in green fruits, thus protecting the immature seeds...".

4.2 Validation of gene expression levels of RNA sequencing data using RT-qPCR for relative gene expression.

To verify candidate genes expression levels from the RNA sequence data RT-qPCR on cDNA synthesized from RNA isolated from lettuce at all three stages of *S. sclerotiorum* infection (K/ uninfected, 24hours- and 48hours upon infection) was conducted. Gene specific primers

(materials) for the candidate- and reference genes were designed and used for the RT-qPCR. Fig. 5. shows relative gene expression of target genes.

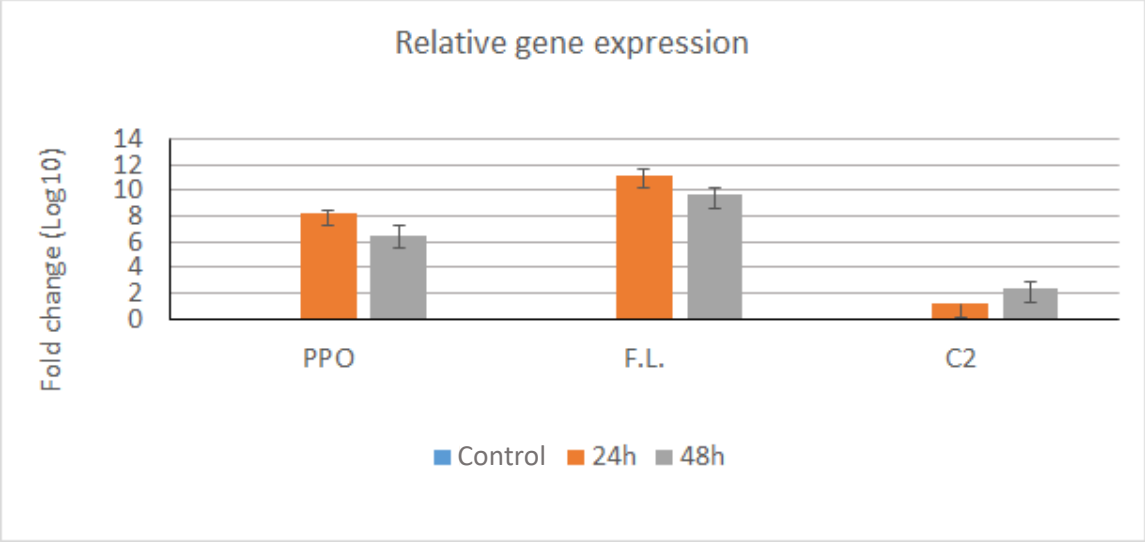


Fig. 5. *Relative gene expression of target-genes using RT-qPCR, based on the 2−ΔΔCt method (fold changes of expression log10, error bar: 95% confidence int).*
TUB(Tubulin1) was the reference gene used for calculating fold change. RT-qPCR was run in technical duplicates and biological triplicates (K1-3, 24H1-3 and 48h1-3) for each target-gene, and the average of the technical and biological replicates was calculated. The foldchange diagram illustrates increased expression of target genes during infection time. The x-axis shows gene and infection time with the corresponding fold change values (Log10 scaled) at the y-axis.

For *PPO* and *F.L* there was no expression in K samples, indicating that these two genes expression level were too low to be detected in the uninfected samples which was in coherence with the RNA sequencing data (*PPO* had 0 expression, while *C2* and *F.L* had close to 0 expression, cf. RNA sequencing data). Hence, this verification is not 100% accurate, but close enough to confirm the elevated expression tendency of target genes upon infection. The low but positive *C.2* expression in the control sample caused its fold change to be significantly lower than the other target genes. *F.L* denotes the gene *Fungal lipase* (from the initial list of candidate genes) which was replaced with *B.C* a while after this expression analysis was conducted.

Hence, *B.C* was not included in the analysis. However, the expression level analysis confirms the elevated expression levels upon infection of the other two target genes (*PPO, C2*) from the original RNA sequencing data (Appendix) to be correct. RT-qPCR was run on all the initial target- and –reference genes (Appendix). However, for convenience only the final target genes were included here.

4.3 Construction of CRISPR-constructs

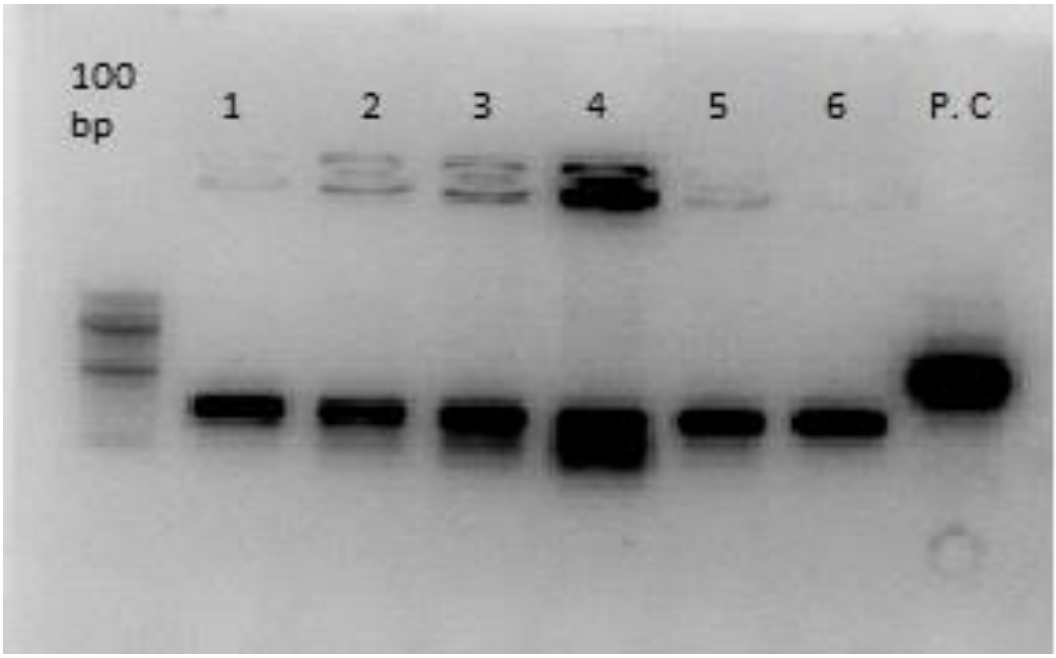
CRISPR-constructs for each target gene were designed to knock-out the three candidate genes confirmed by RT-qPCR to be induced by *S. sclerotiorum* infection. The following overview will clarify terminology and summarize the entire cloning procedure. Firstly, sgRNAS were annealed into oligonucleotides and cloned into a sgRNA-CRISPR expression vector (pChimera). Then the sgRNA cassette from pChimera (chimeric sgRNA (sgRNA and scaffold) and AtU6- 26 promotor) were subcloned into a plant expression vector (pCas9-TPC) containing Cas9 for plant transformation. The final plant expression vectors, referred to as CRISPR-constructs, was then used to transform *A. tumefaciens,* which in turn was used for the actual plant transformation. Finally, one CRISPR-construct for each target gene containing sgRNA1 was used for the actual plant transformation.

The components of the cloning procedure (vectors) and the final CRISPR-constructs are explained and illustrated in the following chapters.

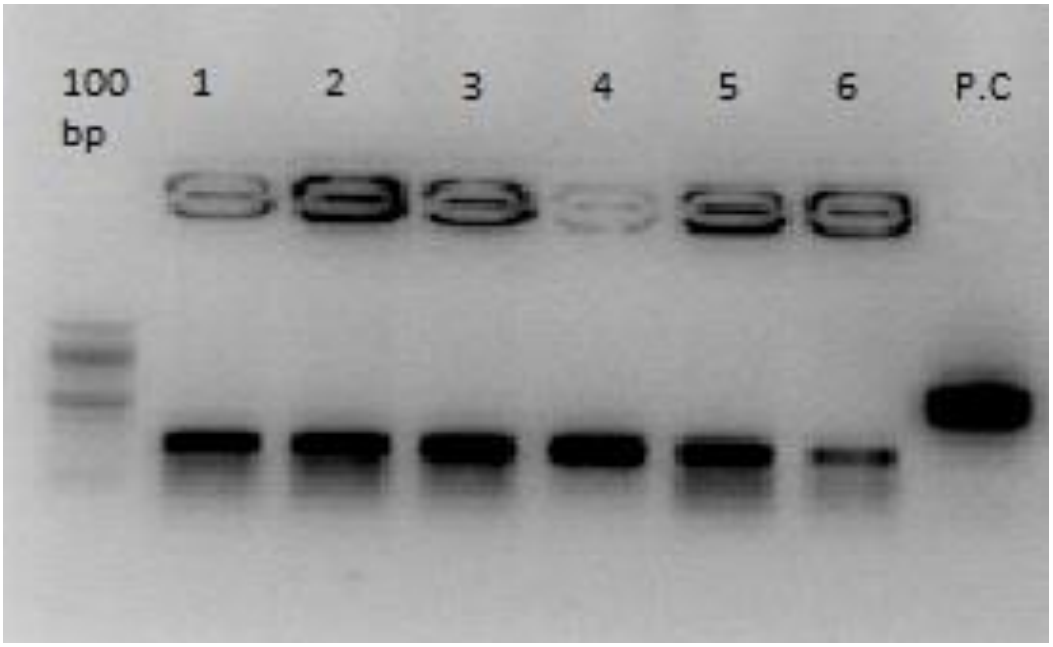
4.4 Verification of sgRNAs cloned into pChimera

The first step in the construction of the CRISPR-constructs was to design gene specific sgRNAs for each target gene (Methods). The two complementary sgRNAs for each gene were then annealed to form oligonucleotides and subsequently cloned into the linearized pChimera vector at the BpsI restriction site (fig. 7). After the cloning products were confirmed by gel documentation of colony PCR products (*E. coli t*ransformed with sgRNA-pChimera vectors, Methods) (fig. 6) and sequencing (fig. 8), the sgRNA cassette from pChimera was digested with the pCas9-TPC vector (which contains the Cas9 expression cassette (47)using AvrII (chapter 4.5). As illustrated in fig. 6. the cloning of the oligonucleotides formed by sgRNA1 (for each target gene) into pChimera was verified by gel documentation of PCR products.

A) *PPO*



B) *C2*



C) *B.C*

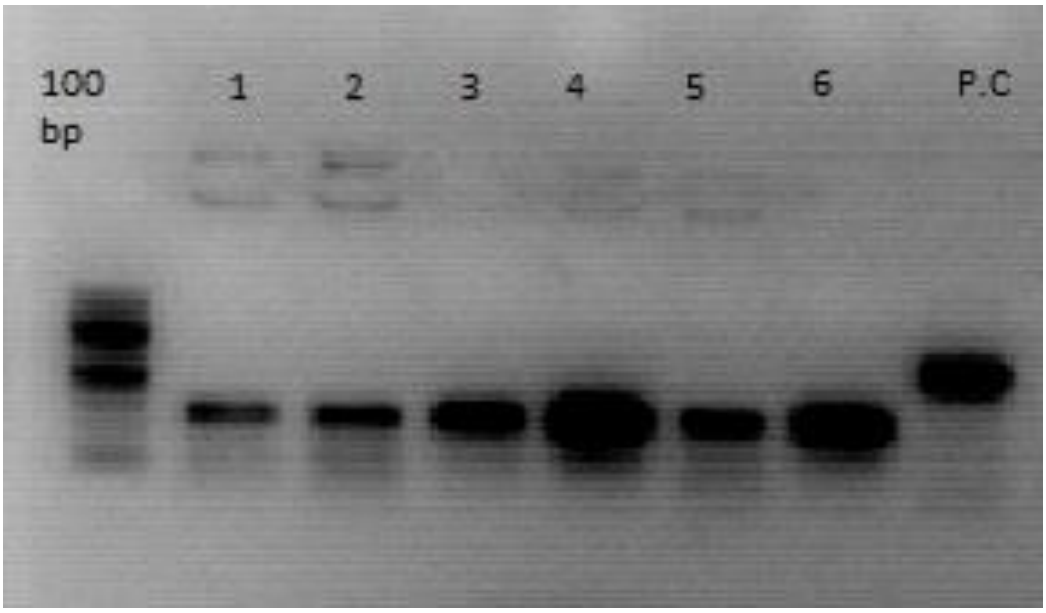


Fig. 6. *Gel documentation of colony PCR products of E. coli transformed with sgRNA1-pChimera plasmids (one for each gene). 100 bp ladder was used for fragment size determination. Well 1- 6 represents six different colonies using sgRNA1f and pChimera r as primers with expected fragment size of approximately 265 bp. In the P.C (positive control) pChimera f and r primers with empty pChimera plasmid was used, with expected fragment size of 650 bp.* ***A, B, C*** *represents PPO, C2 and B.C respectively.*

Fig. 7. shows the plasmid representation of the pChimera vector after cloning with sgRNAs.

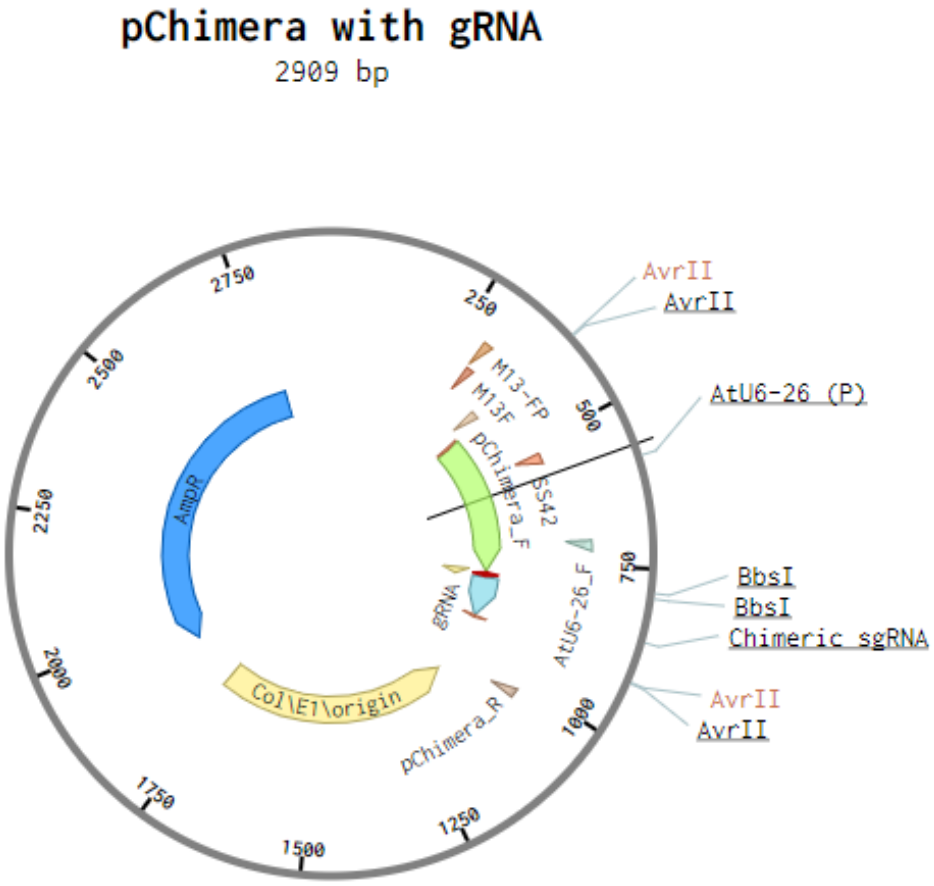


Fig. 7. *Illustration of the pChimera plasmid with cloned sgRNA, Atu6-26 promotor, relevant primers, ampicillin resistance genes for bacterial selection and restriction sites. The sgRNA cassette from pChimera used to clone into pCas9-TPC is the segment confined between the two AvrII restriction sites.*

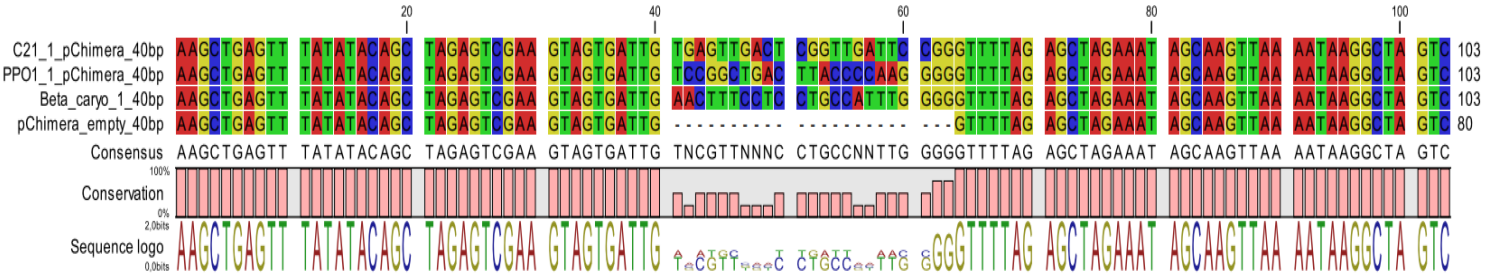


Fig. 8. *Schematic representation of the sequencing data confirming successful cloning of sgRNAs into pChimera for all target-genes and aligned with an empty pChimera plasmid.*

4.5 Verification of the final CRISPR-constructs

The final CRISPR-constructs were the result of cloning the pChimera sgRNA cassettes into a prepared (phosphorylated etc. Methods) pCas9-TPC vector. The sgRNA cassettes were cloned into the prepared pCas9-TPC vector at the AvrII restriction site. All constructs were confirmed correct by sequencing. However, due to the size of these sequence files, they were not included, but may be provided on request. Fig. 9. illustrates the final constructs and fig. 10. shows the orientation of sgRNA cassettes in the final constructs.

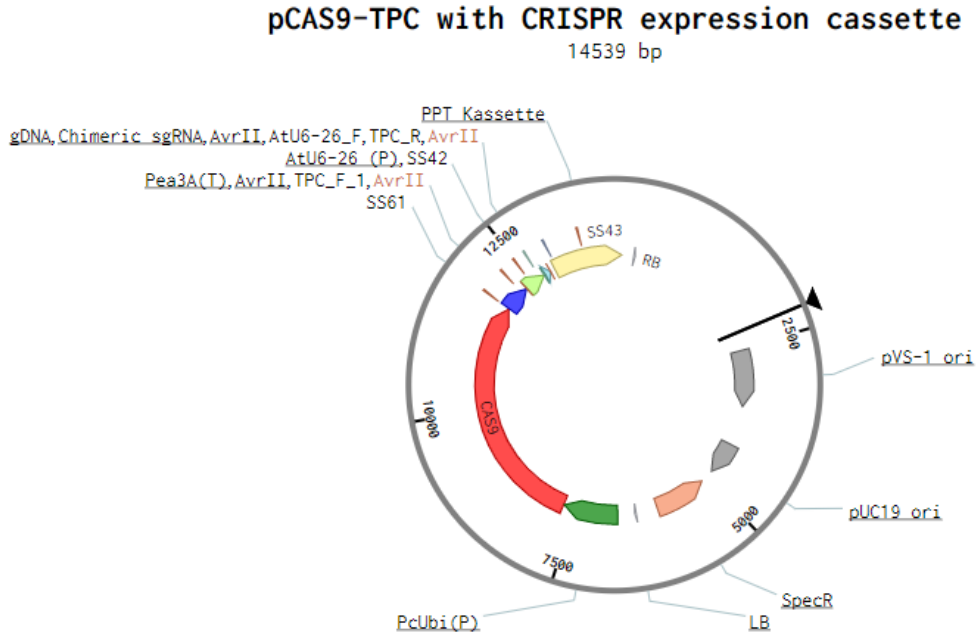
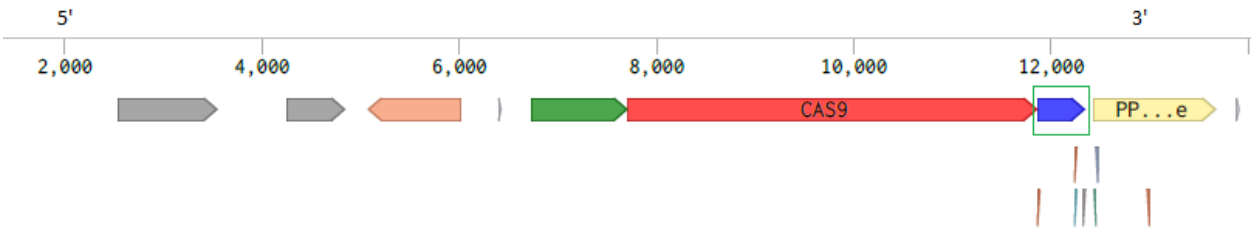


Fig. 9. *The final CRISPR-constructs consist of pCas9-TPC vector including CRISPR-Cas9 expression components, primers (Materials), spectinomycin resistance gene for bacterial selection, PPT*

cassette for plant selection (BASTA) and the sgRNA cassette from pChimera. The sgRNA cassette of the *CRISPR-construct consists of AtU6- 26 (Arabidopsis promotor), sgRNA, scaffold, parsley ubiquitin promotor (PcUbi), Cas9 (endonuclease) and the Pea3 terminator* (48).

Schematic view of sgRNA cassettes orientation in the final CRISPR-construct after cloning

A) *PPO*



B) *C2* and *B.C*

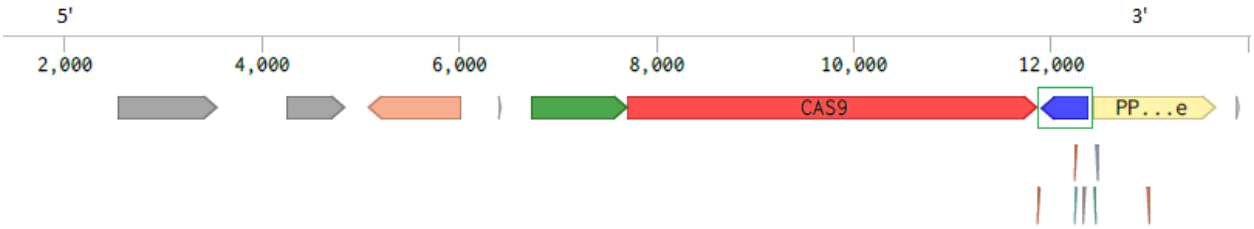


Fig. 10. *Schematic illustration of the orientation of the pChimera cassette cloned into the final CRISPR-constructs. Orientation is highlighted in the green box.* ***A*** *is for PPO and is in the 5'- 3' direction.* ***B*** *is for C2 and B.C and is 3'-5' direction.*

4.6 PCR-screening of colonies

Once the sgRNA cassettes were cloned into pCas9-TPC to form the final CRISPR-constructs, these constructs were used to transform *One Shot™ Top 10 Chemically Component E. coli* cells which in turn were grown on selection media (Methods). Putative positive colonies were PCR screened with primers SS43/ 61. Fig. 1. shows gel documentation of PCR products from *E. coli* colonies transformed with CRISPR-constructs (pCas9-TPC- sgRNA1 for all three target-genes).

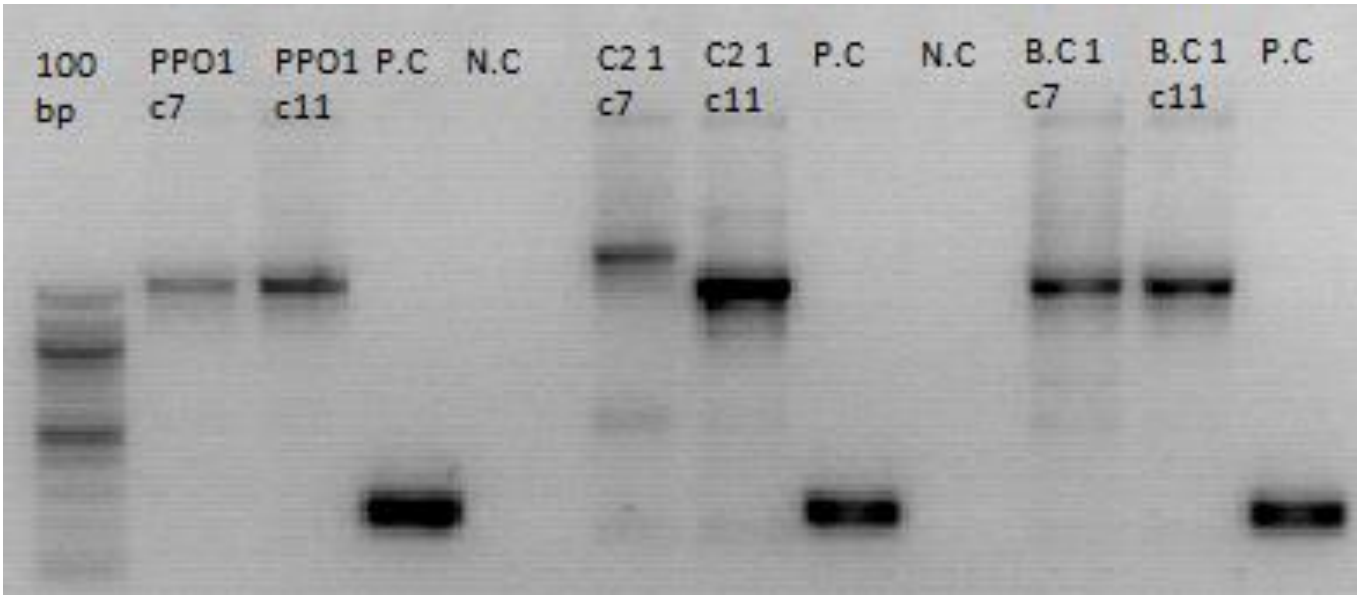


Fig.11. *Gel documentation of PCR products following transformation of One Shot™ Top 10 Chemically Component E. coli with CRISPR-constructs. Positive control (P.C) was empty pCas9-TPC using primers TPC f /r with expected bond size of 200 bp. Negative control (N.C) was dH2O. For all construct's primers SS43/ 61 were used with expected bond size of approximately 1600 bp. c7 and c11 denotes colony 7 and colony 11 respectively.*

After gel documentation the samples were sent for sequencing to confirm the final CRISPR-constructs (and thus the prior ligation and digestion steps) and the orientation of the sgRNA cassettes in the final constructs (fig. 10.).

PPO1 c7, *C21* c11 and *B.C1* 11 was chosen for continuation. C21 c7 was not correct and naturally excluded.

4.7 Verification of transformation of *A. tumefaciens* with the CRISPR-constructs

Once confirmed by sequencing, CRISPR-constructs for the three target genes were used to transform *A. tumefaciens.* Transformed *A. tumefaciens* were grown on selection media (Methods), and putative positive colonies were PCR screened with primers from the CRISPR-constructs (FvU6 f and TPC r) to verify the transformants. Fig. 12. shows gel documentation of successful transformants.

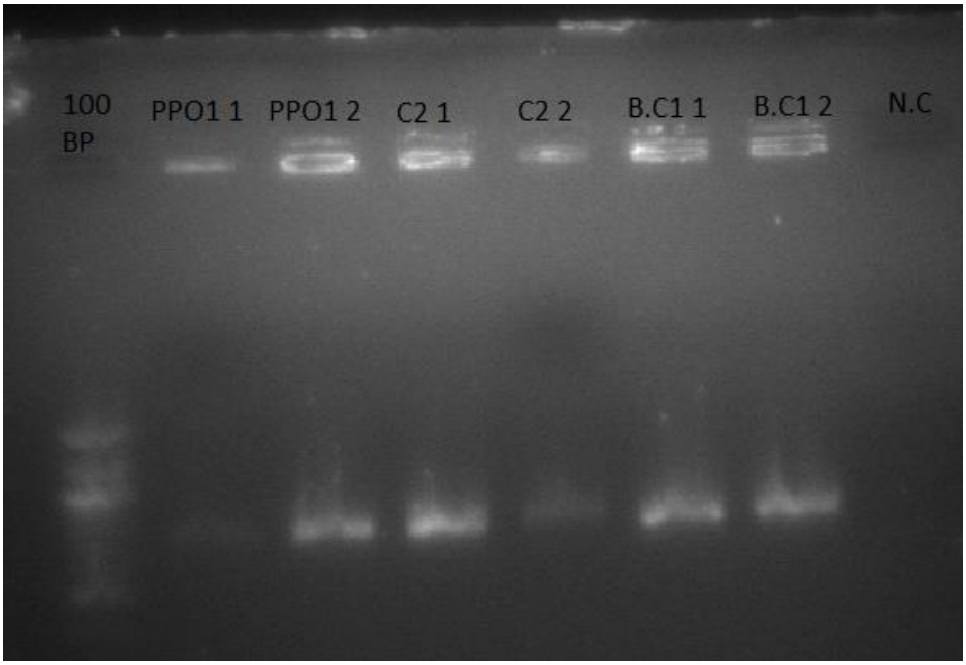


Fig. 12. *Gel documentation of colony PCR of A. tumefaciens transformed with CRISPR-constructs. Two samples of each target-gene. FvU6 f and TPC r primers were used with expected bond size 300 bp. N.C (negative control) was nuclease free h_2o. 100 bp ladder was used to determine fragment size.*

4.8 *Agro*-mediated stable transformation of *L. sativa* "Great Lakes"

Agro-mediated stable transformation was done with CRISPR-constructs for all three target genes on a total 876 Great Lakes leaves, over the course of four separate transformations (Methods for details). Unfortunately, all transformants from the first three transformations died, and by the time of the submission of this thesis merely (approximately) 20 leaves from the fourth transformation with CRISPR-construct for PPO are still alive. Fig. 13. shows the development of Great Lakes leaves from germination to after *Agro*-mediated transformation. Only pictures of leaves transformed with CRISPR-construct for target gene PPO is included.

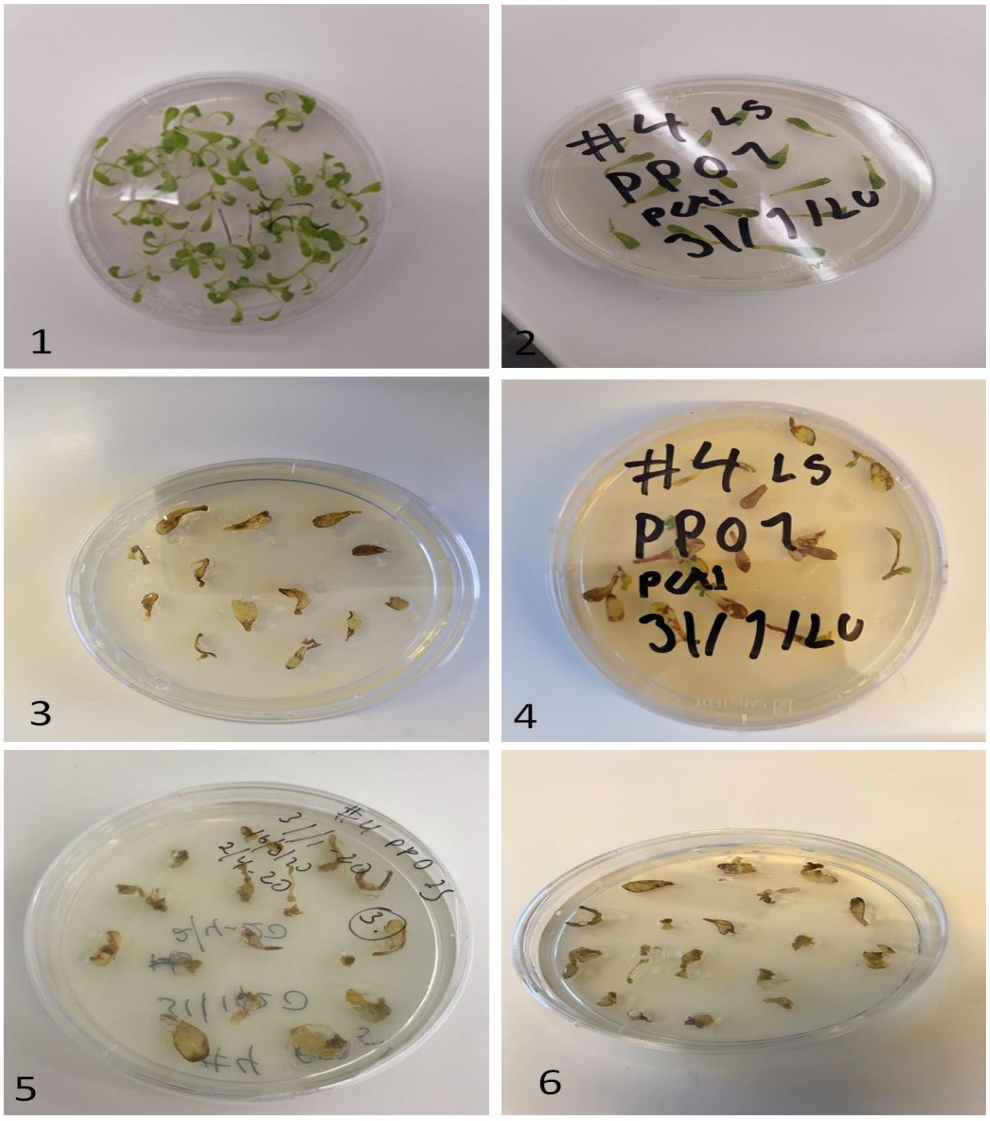


Fig. 13. *Pictures 1-6 shows development of leaves from germination (1), and 2-6 shows development of leaves after Agro-mediated transformation with 2 weeks intervals. All pictures are from the transformation using CRISPR-construct for target gene PPO on Great Lakes leaves.*

4.9 *Agro*-mediated stable transformation of *L. sativa* "MATCH"

Agro-mediated stable transformation was done with CRISPR-construct for target gene PPO on 70 MATCH leaves, to investigate possible genotype effect on transformation frequency. Unfortunately, all transformed leaves died within two months after transformation. Fig. 14. shows the development of leaves from germination to after *Agro*-mediated transformation of MATCH lettuce leaves.

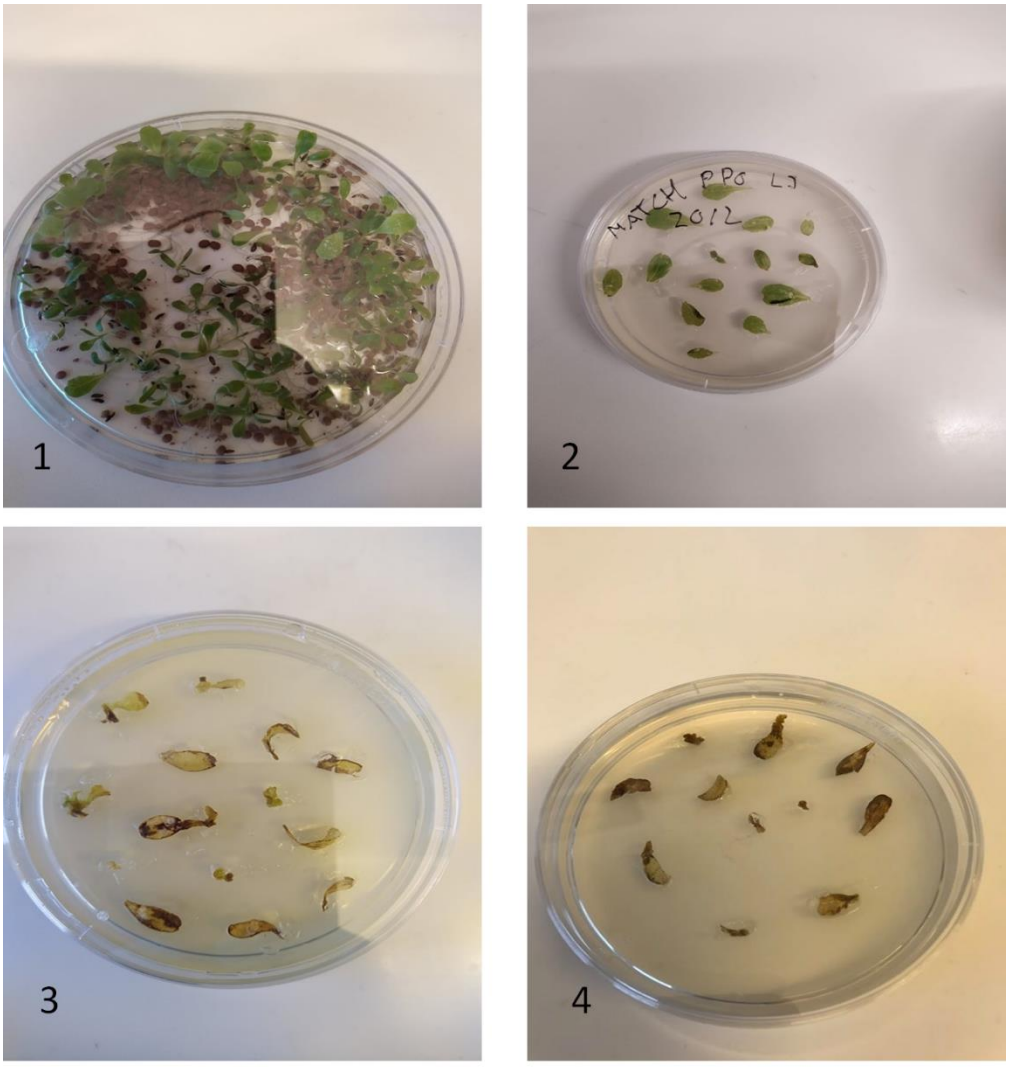


Fig. 14. *shows development of leaves from the germination stage (1) and 2-4 shows development with 14 days intervals after transformation with CRISPR-construct for target-gene PPO.*

4.10 Transient transformation

In addition to *Agrobacterium*-mediated stable plant transformation, transient transformation was performed to investigate expression and performance of the CRISPR-constructs. Leaves of 30 days old lettuce were infiltrated with *Agro*-suspension using a syringe without needle (Methods). Fig. 15. verifies presence of the CRISPR-constructs in the DNA isolated from lettuce leaf material four days after transient transformation through gel documentation of PCR products. However, this was merely the first step in this investigation, which unfortunately were ended before completion, due to the COVID-19 outbreak. The remaining steps are addressed in the discussion.

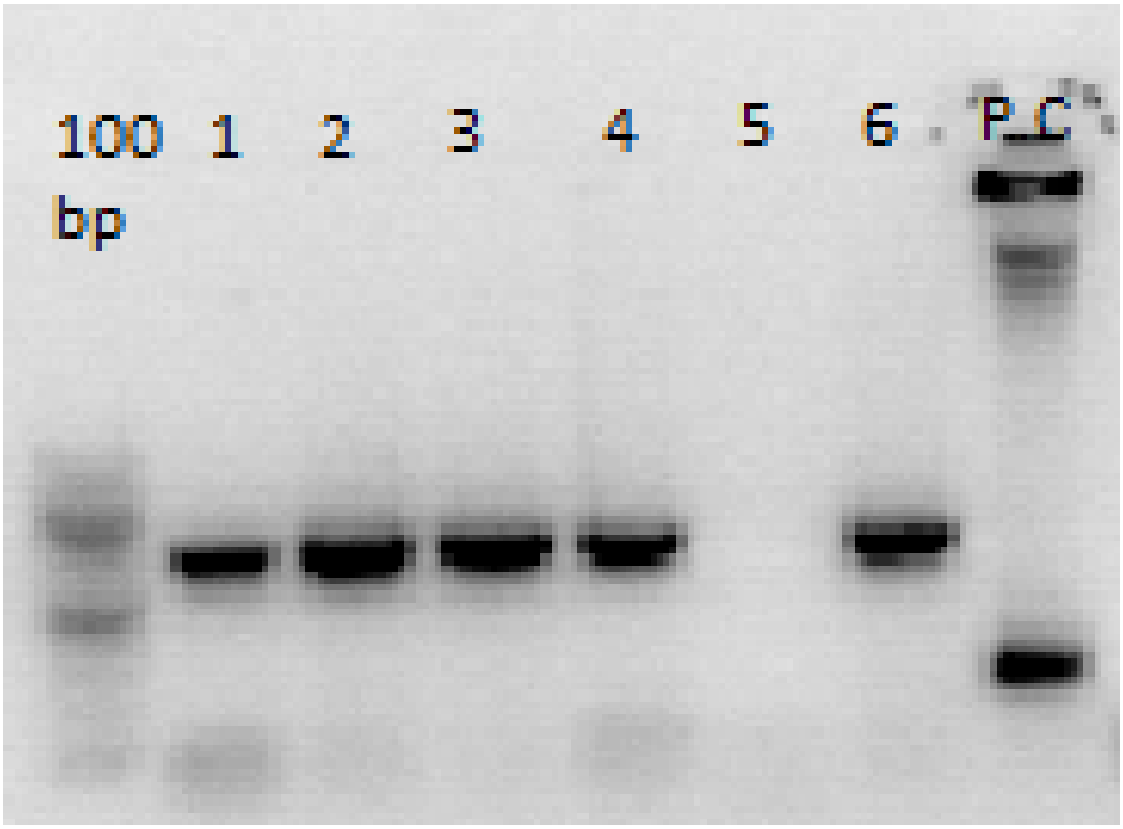


Fig. 15. *Gel documentation of PCR products from transient transformation. pCas9-TPC empty plasmid using primers TPC f/ r with expected bond size of 200 bp was used as positive control (P.C). For all sample's primers TPC f / r was used with expected bond size of 700 bp. Well 1 and 2 was for PPO, well 3 and 4 for C2 and well 5 and 6 for B. C. Sample 1 (the first of the two for each gene) was diluted 1:10 with nuclease free h_2o, and sample 2 was undiluted (there was no band for the diluted B.C sample, probably due to inaccurate pipetting).*

5 Discussion and future perspectives

The COVID-19 outbreak caused the Norwegian government to induce lockdown on most public facilities, including NMBU and NIBIO. This occurred in the middle of the plant transformation process which inhibited the completion of transient transformation analysis. Additionally, due to the comprehensiveness of this study, the initial objective regarding the phenotypic study of the stable transformants would not have been completed independent of the COVID-19 outbreak as the transformed cotyledons did not reach the rooting stage by the time of the submission of this thesis. Hence, it is impossible to draw any solid conclusions. However, several topics and challenges considering CRISPR-Cas9 and plant transformation are worth discussing and will be addressed in the next chapters.

5.1 RNA sequencing, expression levels and candidate genes

The primary objective of this experiment was to enhance *L. sativas* resistance against *S. sclerotiorum*, infection using CRISPR-Cas9 to knock-out genes that possibly are involved in the development and growth of the pathogenic organism. This strategy has successfully been used in several studies among different plant species including tomato, maize, and potato (32). The function of many genes is still putative "the overwhelming majority of genomes are annotated in an automated, transitive fashion based solely on sequence similarity or motif or domain presence, resulting in coarse, inaccurate estimations of gene function. Few, if any, genes are characterized for molecular, biochemical, or biological function. When coupled with gene duplication and neo- or sub functionalization, transitive annotations can be even more erroneous." (49). Hence, functional analysis of candidate genes will be obtained by CRISPR-Cas9 knock-out, based on putative function.

Of the 7000 genes in the RNA sequence data (Unpublished, Thorstensen, T.) approximately 1500 were upregulated upon infection. Highly upregulated genes were then screened for functions. Among them, eight genes were selected as candidate genes based on elevated expression levels upon infection, function and literature search. This list was further narrowed down to three candidate-genes in order to limit the extent of the experiment. Gene expression

analysis is a powerful tool for identifying gene function and has been used in other studies to identify susceptibility-genes (50). As expected, and shown in results (4.2), the quantitative gene expression analysis (RT-qPCR) confirmed the expression levels of the target genes from the RNA sequencing data. This verifies the target genes elevated expression upon infection and thus supports the initial hypothesis that these target genes may be involved in reducing *L. sativas* resistance upon *S. Sclerotiorum* infection, and thus possibly be S-genes. However, this was merely the first step in the process of investigating the target-genes role in the *S. sclerotiorum* infection. Elevated gene expression may of course also be innate plant immune responses genes or a consequence of secondary effects, and is not evidence of genes' possible role in reducing defense per se.

The next step in this assessment was not achieved as transformants did reach required size to either be sequenced to screen for mutations (transgenic plants) or subsequently be phenotypically studied. If plants were to be confirmed transgenic (e.g. desired mutations caused by the *Agro*-mediated transformation confirmed by sequencing) the next step would be a phenotypic study of the transgenic plants. Transgenic plants would be inoculated with *S. sclerotiorum* once suitable size was reached (after rooting with a few fully expanded leaves). Leaves of edited plants would been infected with *S. sclerotiorum* using detached leaves assay and phenotypes scored based on diameter of necrosis at the infection site, plant growth etc. If the transgenic plants displayed increased resistance (or immunity) compared to wild type lettuce inoculated with *S. sclerotiorum*, it would constitute a strong support for the hypothesis that the target-genes were S-genes. Unfortunately, the transformed explants did not reach suitable size for conducting a phenotypic study, which were a premise for functional analysis of target genes.

5.2 Cloning and CRISPR-constructs

In the beginning of this study, it was planned to design a promotor for the CRISPR-constructs specific for *L. Sativa.* However, as several challenges were faced during the study and due to the magnitude of the experiment, this turned out to be too optimistic in terms of time. Consequently, the standard Arabidopsis promotor (Atu6-26) was used. It would of course be

preferable to use a lettuce specific promotor. However, other researchers (51) have used the Atu6-26 promotor in lettuce (and many other species) successfully. The promoter may influence expression of sgRNA, which in turn may influence the mutation frequency, so a specific promoter could have elevated the mutation frequency to some extent. However, the promotor does not influence the transformation efficiency. Another technical aspect worth mentioning is the direction of the sgRNA cassettes from pChimera cloned into the final CRISPR-constructs. Only the *PPO* construct had 5'-3' orientation, although this should not be of any significance as the components of the cassettes internal order was correct.

Initially two CRISPR-constructs with two different sgRNAs for each of the three target genes were made. The second served as a backup in case it for some reason would not perform desirably with the highest ranking sgRNA (sgRNA for all genes). In retrospect, it was probably too optimistic to do transformations with three different constructs. It turned out many transformations were required to generate transgenic plants (5.3). This is confirmed by many studies, including (52) which states "However, in a highly optimized condition the transformation efficiency through organogenesis vary between 4 to 20 % and often false positives, putative transformants and chimeras are encountered". Hence, it would been wiser to make only one or two construct(s) and devoting more time to transformations. A considerable amount of time was spent on making constructs, which was part of why the study unfortunately was not completed.

The CRISPR-constructs were successful, confirmed both by sequencing and gel documentation of PCR products. Transient transformations were conducted to evaluate the expression of the CRISPR-constructs. However, the COVID-19 outbreak inhibited completion of this investigation and expression of the CRISPR-constructs was not verified (5.4).

5.3 Plant transformation

Although CRISPR-Cas9 is widely accepted as the most promising of the gene editing tools there are still several challenges to overcome in order to utilize its full potential. As mentioned in the introduction off-target effects, plant transformation efficiency and regeneration of transgenic

plants constitutes the main technical bottlenecks in plant genome editing for crop improvement (53). This was also the case in this thesis as all transformants from the first three transformations and the MATCH transformation died, and only approximately 10% of the last transformants were still alive by the time of the submission of this thesis. In total 473 cotyledons (946 leaves) were transformed over the five stable transformations, and only approximately 20 leaves are still alive, and may not be transgenic or even survive. Thus, the major challenge in this thesis was plant transformation efficiency.

The two most common plant transformation methods are *Agrobacterium*-mediated transformation, which was the method used in this thesis, and direct gene transfer (for example through electroporation/gene gun). The *Agrobacterium*-mediated transformation is a process subject to a wide range of variables/factors that all may influence the transformation efficiency to different extends. To the very least these include laboratory skills/experience of the researcher and the optimization of protocol for the model organism. The latter includes several sub parameters such as choice of bacterial strain and its affinity to the crop genotype, selection regime (concentrations of chemicals/antibiotics), to control the overgrowth of *Agrobacterium,* (54), and several explant factors like germination time. As the timespan of this thesis did not allow for a complete troubleshoot on all these parameters, it is impossible to conclude the individual factors contribution to the disappointingly low transformation frequency. Two of these parameters were experimented with, genotype and germination time, however not to the extent necessary to draw any conclusions, and the following discussion is thus merely speculations.

Hitherto, *Agrobacterium*-mediated transformation is the preferred tool for stable plant transformation. One study concluded, (54), that *Agrobacterium* mediated transformation is the most efficient method for gene targeting. However, in the same study, it was also shown to be dependent on the choice of *Agrobacterium* strain. In other words, the choice of strain and its affinity to the crops genotype is of great significance to the transformation efficiency. Thus, one can speculate that the choice of Agrobacterium strain in this thesis may be one of the factors

that may not have been optimal for the genotypes of Lettuce used in this thesis, and hence may be a limiting factor of the plant transformation.

As transformation efficiency varies great among different genotypes (54), one transformation (only with CRISPR-construct for *PPO*) on Match lettuce was conducted in order to investigate the possible impact genotype difference had on transformation efficiency. However, it's hard to conclude as all the of the Match transformants also died few weeks after the transformation. Furthermore, due to the time confinement of the experiment, only 35 Match cotyledons (70 leaves) were transformed. To make a statistical legitimate comparison of the two cultivars regarding transformation efficiency an equal amount of transformants from both genotypes would be required. This was done to see if it had a significant effect e.g. if success were achieved in only transformation of Match it would be legitimate to assume this method suited Match better than the Great Lakes genotype.

The transformation protocol requires 4-7 days germination time. For the first three transformations of the Great Lakes genotype, seeds grew for 4 days. The last transformation was done on seeds that grew for 7 days, to investigate if grow-time influenced the transformation frequency. When explants are transformed after only days of growing, explants are very small and may be too fragile for the harsh treatment it suffers during transformation. The optimal germination time within 4-7 days interval of the protocol probably varies with lettuce genotype. As only leaves from the last transformation are still alive by the time of the submission of this thesis, one can speculate that 7 days of germination time would be optimal for Great Lakes. However, there are only approximately 20 leaves from the fourth transformation with the CRISPR-construct for *PPO* that is still alive, and most are turning yellow or brown, which is not a healthy sign. The stable transformation is a lengthy process, and months are required to generate a transgenic line (55). During the transformation, explants are exposed to harsh mechanical stress, and may spend a considerable amount of time to recover. Hence, the surviving explants from this experiment may recover and turn out to be transgenic, but this thesis ended before they eventually reached the rooting stage, which is a premise for

further investigation, so no conclusion may be drawn. Theoretically, if the rooting stage were achieved, the next step would be to transfer the transformed plants to rooting medium containing MS basal medium (selection media, Methods) until rooting was completed with a few fully expanded leaves. Then DNA would be isolated and used for qPCR and sequenced to screen for mutations in transgenic plants.

In addition to experiment with different *Agrobacterium* strains, germination time/plant size and lettuce genotypes, it is possible that experimenting with different concentrations of hormones and antibiotics could enhance the transformation frequency. Different genotypes may perhaps require different amounts of hormones and antibiotics would influence transformation efficiency. However, these are merely speculations and not based on other studies.

A last factor to consider is the technical performance of the protocol itself. This is a vastly delicate matter and is thus a challenge for an inexperienced scientist. Subtle human mistakes, such as handling the leaves a bit too harsh may be fatal. This is not verifiable, and it is also possible that the execution of the protocol was impeccable.

To summarize, it is impossible to conclude which of, and eventually how much, each of the previously discussed factors contributed to the low transformation frequency. As transformation frequency in general is low, and vary considerably with both plant species and genotype, it can't even be concluded that transformation frequency in this study in fact was low. Even under optimal conditions the transformation technique used here may demand more transformations than conducted in this study, as editing frequency of *A. tumefaciens*-mediated transformation is still low in many plant species. However, confirmed by other studies as well, plant transformation is the greatest bottleneck and many transformations are necessary to successfully generate transgenic plants. Moreover, even more transformations are probably needed to get edited plants, as mutation frequency within the transgenic plants vary to a

significant degree. For example, one study reported editing efficiency to vary from 14-100% in CRISPR-edited tomatoes (56). The logical conclusion is hence that plant transformation was the major limiting factor in this experiment. This bottleneck is already recognized by the scientific community, and great efforts are already in place in the field of biotechnology to improve the utilization of CRISPR-Cas9 in plant genome editing for crop improvement. In the future researches may optimize all the parameters discussed here: optimal germination time for different species, *Agrobacterium* strain and affinity to genotype, amount of chemicals added in growth media, introduction of new chemicals with transformation inducing properties and explants pre-treatment/conditions. Furthermore, developing of new and genetically improved *Agrobacterium* strains is already being done. One example is Super-*Agrobacterium* ver. 4, (57). "We succeeded in producing an *A. tumefaciens* strain with improved potential for transformation by imbuing it with the ability to remove ethylene and GABA, which are negative factors in the *Agrobacterium*-plant interactions. *A. tumefaciens* with AcdS and GabT increased the T-DNA transfer and stable transformation frequency. Especially in tomato, this newly bred bacterium (Super-*Agrobacterium* ver. 4) enables us to decrease the number of cotyledons used for transformation and allows us to reduce 72% of the time and labor required for transformation".

5.4 Transient transformation

The transient transformation was done to evaluate of the efficiency of the CRISPR-constructs. Transient transformation is a simple technique to show if CRISPR-constructs were expressed, and if so, if they caused the desired mutations. Unfortunately, due to the COVID-19 outbreak, this analysis was not completed. Only the first step, namely verifying the presence of the CRISP-constructs in the DNA isolated from transient transformed lettuce leaves were conducted. Only the presence of the CRISPR-constructs was confirmed in the DNA isolated from the infiltrated leaf material and documented by gel electrophorese (see, results). This was expected as leaves were infiltrated with the CRISPR-construct through *Agrobacterium* suspension. Thus, it is

impossible to conclude whether the CRISPR-constructs were expressed, and in turn causing the desired mutations as the analysis was not completed.

The next step was to make cDNA from isolated RNA and run RT-qPCR to analyze expression of the CRISPR-constructs using sgRNA f primer and scaffold reverse. If the components of the CRISPR-constructs were expressed, the isolated DNA would be sent for sequencing to screen for the desired mutations, and if mutated, gene edited plants would be confirmed.

Gene editing vs traditional GMO

If this experiment ultimate objective of generating gene edited *L. sativa* lines with enhanced resistance to *S. scleretorium* was accomplished, the commercialization process would be long and expensive, if approved at all, due to the EUs strict GMO laws.

The ruling delivered by the Court of Justice of the European Union in 2018 stated "Organisms obtained by mutagenesis are GMOs and are, in principle, subject to the obligations laid down by the GMO Directive. However, organisms obtained by mutagenesis techniques which have conventionally been used in a number of applications and have a long safety record are exempt from those obligations, on the understanding that the Member States are free to subject them, in compliance with EU law, to the obligations laid down by the directive or to other obligations" (58)In other words, organisms obtained by new techniques of directed mutagenesis, such as CRISPR, is classified as GMO and hence subject to the strict laws of the GMO directive. This judgement is controversial on many levels due to its many important implications and consequences, and a complete discussion could constitute an entire thesis on its own. Hence, only scientific aspects in the context of this study will be discussed, while political and economic consequences will be addressed briefly.

From a scientific perspective this judgement is ambiguous in several regards. First and foremost, EUs definition of GMO "an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or

natural recombination" is vague. To include transgene free CRISPR edited organisms in this definition is contradictive as such organisms are inseparable from naturally mutated crops (1.1). The method of which targeted mutagenesis is achieved may of course be considered unnatural, if one defines human efforts as unnatural. Mutations are as natural as nature herself and occur in plants independent of human intervention. In fact, mutations are (at least from the current scientific understanding) the underlying mechanism of evolution. Mutagenesis, which simply means to generate genetic mutations, encompasses both targeted- and random mutagenesis. Targeted mutagenesis, which includes gene editing tools like CRISPR, can precisely alter DNA of an organism at specific genomic sites. Random mutagenesis on the other hand, refers to the alteration of an organism's DNA a multiple genomic site in a non-targeted way by physical or chemical treatments. The latter is exempt from EUs GMO obligations based on its long and safe record of use in agriculture. This decision has been criticized by the scientific community for not reflecting current knowledge and scientific evidence (59). With regards to food safety, it seems clear that precise targeted mutagenesis would cause less unintended consequences/off-target effects compared to random mutagenesis, and hence be safer in the context of food safety.

That is not to argue that regulations of CRISPR edited organisms are not appropriate. The motivation behind these regulations was (hopefully) to benefit EU citizens, ensure product safety and protect the environment. However, the current laws appear ignorant from a scientific perspective and doesn't respect its initial objective in several concerns. Obviously, GE is not risk free, but legislations should be evaluated scientifically and in the context of hazard in comparison to existing and realistic alternatives like the use of herbicides and random mutagenesis in conventional breeding. EUs current ignorance of scientific arguments has been paralleled to the starving in Ukraine caused by Stalin on ideological advice from his agronomist Lysenko (59).

Other aspects should be considered as well. The current regulations will likely retard the development of these technologies because of diminished investment incentives due to the unfavourable commercialization process imposed by the GMO directive. Furthermore, the GMO

obligations will impact international trade and the regulations are almost impossible to implement and control as GEs may be inseparable from natural mutated crops.

From a humanitarian perspective, one can argue that not utilizing whatever technologies available, and especially GE tools, to feed humanity in the predicted food crisis is unethical. This perspective is also shared by the Danish Council of Ethics which recommends a re-evaluation of Europe's anti-GMO stance. Furthermore, the current regulations appear ignorant in terms of respecting the very laws' initial objective which should be debated and re-regulated according to scientific knowledge. However, the food and population crisis are mainly consequences of the mismanagement of the planet and are hence political/economical in nature. Ruling the world by economy without regards to ecology causes indescribable harm to humanity (and nature), evident by the current world situation. The fact that some countries struggle with obesity while populations in other countries are starving, clearly illustrates the political nature of the food crisis. Hence, I conclude that CRISPR should be utilized to secure the worlds food supply in the predicted food crisis as it is both safer and more efficient than the other options currently available. However, although GE is a powerful tool for improving crops, it will not solve the worlds coming (and existing) food crisis alone and many other measures are necessary as well.

Appendix I

Sheet for reaction setup of RT-qPCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	K1	K1	K2	K2	K3	K3	24T-1	24T-2	24T-2	24T-3	24T-3	Tubulin1
B	48T-1	48T-1	48T-2	48T-2	48T-3	48T-3				NEGATIVE	NEGATIVE	Tubulin1
C	K1	K1	K2	K2	K3	K3	24T-1	24T-2	24T-2	24T-3	24T-3	PPO
D	48T-1	48T-1	48T-2	48T-2	48T-3	48T-3				NEGATIVE	NEGATIVE	PPO
E	K1	K1	K2	K2	K3	K3	24T-1	24T-2	24T-2	24T-3	24T-3	C2
F	48T-1	48T-1	48T-2	48T-2	48T-3	48T-3				NEGATIVE	NEGATIVE	C2
G	K1	K1	K2	K2	K3	K3	24T-1	24T-2	24T-2	24T-3	24T-3	B. C
H	48T-1	48T-1	48T-2	48T-2	48T-3	48T-3				NEGATIVE	NEGATIVE	B.C

Appendix II

RNA sequencing data

Nr	4-24T1_S4	5-24T2_S5	6-24T3_S6	7-48T1_S7	8-48T2_S8	9-48T3_S9	1-K1_S1	2-K2_S2	3-K3_S3	Sum reads
1	1648	5178	450	18076	19398	15693	0	0	0	60443
2	85	157	62	167	158	212	2	1	2	846
3	75	90	36	310	213	287	1	2	9	1023
4	600	2338	311	3584	10020	9565	18	6	17	26459
5	701	1763	354	8568	4550	5979	0	0	0	21915
6	1930	6641	1213	3626	7165	8533	0	0	0	29108
	Reference genes:									
I	7952	8219	3730	928	2999	1675	6416	8504	7157	47580
II	3551	5009	2578	146624	100970	214647	3079	553	3006	480017

Nr	GenBan_acc	C_vs_24H	C_vs_48H	24H_vs_48H	Length	GenBank_annotation	Blast2GO_annotation
1	XM_023907626.1	14,14	17,529	3,59	1954	polyphenol oxidase, chloroplastic-like (LOC111911881), mRNA	polyphenol oxidase, chloroplastic-like
2	XM_023892636.1	5,943	7,332	1,391	1415	uncharacterized LOC111896668 (LOC111896668), mRNA	Fungal lipase-like domain containing protein
3	XM_023904808.1	4,152		2,625	1596	uncharacterized LOC111909011 (LOC111909011), mRNA	C2 calcium-dependent membrane targeting
4	XM_023875671.1	6,302	9,738	3,422	1884	germacrene A synthase short form-like (LOC111879199), mRNA	germacrene A synthase
5	XM_023872974.1	12,842	16,128	3,488	2091	alpha-dioxygenase 1-like (LOC111876447), mRNA	alpha-dioxygenase 1-like
6	XM_023895118.1	14,628	15,997	1,572	1939	beta-caryophyllene synthase-like (LOC111899253), transcript variant X1, mRNA	beta-caryophyllene synthase-like
I	XM_023900018.1		-1,4	-1,378	1667	tubulin alpha-2 chain (LOC111904238), mRNA	alpha tubulin 1
II	XM_023897768.1		6,873	5,941	1421	extensin-1 (LOC111901914), mRNA	putative extensin domain-containing protein

Nr	GO_Names	Enzyme_Codes	Enzyme_Names
1	F:catechol oxidase activity; C:chloroplast; P:pigment biosynthetic process; F:metal ion binding; F:hydroquinone:oxygen oxidoreductase activity; P:oxidation-reduction process	EC:1.10.3; EC:1.10.3.2; EC:1.10.3.1	Acting on diphenols and related substances as donors; Laccase; Catechol oxidase
2	lipid metabolic process; C:membrane; F:hydrolase activity		
3			
4	F:magnesium ion binding; F:germacrene-A synthase activity	EC:4.2.3.23	Germacrene-A synthase
5	F:peroxidase activity; P:response to oxidative stress; F:heme binding; P:oxidation-reduction process; P:cellular oxidant detoxification	EC:1.11.1.7	Peroxidase
6	F:magnesium ion binding; F:terpene synthase activity; P:terpenoid biosynthetic process		
I	F:GTPase activity; F:structural constituent of cytoskeleton; F:GTP binding; C:cytoplasm; C:microtubule; P:cytoskeleton organization; P:microtubule-based process	EC:3.6.1.15	Nucleoside-triphosphate phosphatase
II	F:structural constituent of cell wall; P:plant-type cell wall organization		

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