Targeting of Genes involved in Egg Cell Development via Artificial microRNAs in *Arabidopsis thaliana*

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Abstract

The formation of gametes is a crucial part of any sexually reproducing organism's life cycle. In plants, the gametes are formed in reproductive organs comprised of reprogrammed somatic cells. The female germ cells are generated in the female gametophyte, also called the embryo sac. Whilst the development of the female gametophyte has been morphologically characterized, the underlying molecular mechanisms are poorly understood. The RK domain (RKD) transcription factors have been associated with female gametophyte development in plants and are involved in egg cell formation, however their precise functions remain unknown. When the AtRKD1 and AtRKD2 genes are ectopically expressed in *Arabidopsis thaliana*, tissue proliferation and the expression of an egg-cell marker are induced. The hope is that understanding how these genes control egg cell development will enable engineered plant propagation through seeds without fertilisation. In loss-of-function experiments of the singular RKD gene in the liverwort *Marchantia polymorpha* phenotypes affecting the egg cell have been identified. In *A. thaliana* however, no such phenotypes could be produced due to a probable functional redundancy amongst its five RKD genes. *A. thaliana* is an important plant model organism for genetics as genes of interest can easily be inserted into its genome via *Agrobacterium tumefaciens*, a species of parasitic bacteria. To better understand the role of the RKD genes in female gametophyte development four different RKD transcription factors were knocked down in *A. thaliana* plants with the help of two genetic constructs. The downregulation of the RKD transcription factors is based on a knockdown strategy using artificial microRNAs, which are driven by a late-stage egg-cell specific promoter. The knockdown transformation lines showed no egg-cell phenotypes. A side-effect phenotype was observed in a single transformation line likely caused by T-DNA induced chromosomal translocation. This data provides novel insight into the regulatory control of female gametophyte development.

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1 List of Abbreviations

The following list provides the meaning of various abbreviations and acronyms used throughout this Matura paper.

Abbreviation	Meaning
amiRNA	artificial microRNA
AS	acetosyringone
CCN	central cell nucleus
cDNA	complementary DNA
Col	Columbia
Col-0	wild-type Columbia
DCL	Dicer-like
ddNTPs	dideoxynucleotide triphosphates
DIC	differential interference contrast
dNTPs	deoxyribonucleotide triphosphates
dsDNA	double-stranded DNA
FG	female gametophyte
FM	functional megaspore
gen	gentamicin
goi	gene of interest
GR	gene ruler
hyg	hygromycin
IAA	indole acetic acid
kan	kanamycin
LB	left border
LB solution	liquid broth solution
Ler	Landsberg *erecta*
MCS	multiple cloning sites
miRNA	microRNA
MM	master mix
MMC	microspore mother cell
MS	Murashige and Skoog
NHEJ	nonhomologous end joining
NIN	nodule inception-like
ori	origin of replication
PCR	polymerase chain reaction
RB	right border
rDNA	recombinant DNA
rif	rifampicin
RISC	RNA-induced silencing complex
RKD	RK domain
RNAi	RNA interference
RT-qPCR	quantitative reveres transcription PCR
ssDNA	single-stranded DNA
T-DNA	transferred DNA
Ti	tumour-inducing
T0	transformant generation 0
T1	transformant generation 1
T4SS	type IV secretion system
VBP	VirD2 binding protein
VIP1	VirE2 interacting protein
vir	virulence
Vir	virulence protein
WT	wild-type

2 Introduction

The ability to alter the code of life has long been a fascination of mine. The breadth of possible applications and uses are seemingly endless, from curing hereditary diseases to accelerating the process of crop breeding to avoid wide-spread food shortages in a rapidly changing climate.

I was first introduced to the various methods that can be utilised to genetically modify an organism during biology class. I was surprised to find that a naturally occurring mechanism consisting of a parasitism between a species of bacteria and plants was being exploited for genetic engineering purposes. To me genetically modifying plants seemed more intriguing but also challenging than transforming single-celled organisms and simultaneously more accessible and legally speaking less troublesome than modifying animals.

The development of new gene manipulation methods over the past few decades has allowed a greater malleability of this code to be achieved and consequently allowed a more thorough understanding of the arguably most important macromolecules for life, nucleic acids. Advances in research technology have drastically decreased the cost of performing deliberate alterations in the genetic code. Genetic engineering has allowed several crops including rice, barley, wheat, and maize to be modified such that their resistance against pests and herbicides is enhanced, traits which are agriculturally desirable.

Genetic engineering has not only pushed advancements in agriculture but also in fundamental research in the realm of molecular biology. *Arabidopsis thaliana* is by far the most broadly studied representative of the plant kingdom. In the year 2000 it became the first plant species to have its entire genome sequenced.[1] Over the past few decades, it has served as a model organism for genetics and molecular biology, particularly for creating transgenic plants.[2] Floral dip has become the standard method for genetic manipulation of *A. thaliana*, which makes use of the pathogenic bacteria *Agrobacterium tumefaciens'* ability to insert foreign genes into its host.-A high number of transgenic lines can be generated in approximately three months' time. This method of transformation is a vital tool for characterizing the function of family of genes or even singular specific genes. Land plants have a complicated life cycle of which not every step is fully understood in detail. One such step is the development of the female gametophyte and is of interest as little is known about the molecular mechanisms of these events. I had the chance to be a part of experiments, wherein the goal was to understand the development of the female gametophyte in *A. thaliana*. In the conducted experiments RKD transcription factors were knocked down in *A. thaliana* plants utilising artificial microRNA (amiRNA) expressing constructs driven by late-stage egg-cell specific promoter, which were constructed by Nadine Eggenberger, who had previously worked on RKD transcription factors during her master thesis.[3]

The RK-domain (RKD) transcription factors, belonging to the RWP-RK domain containing gene family in plants, have been linked to female gametophyte development. The nomenclature refers to a

conserved amino acid motif at the carboxyl terminus, which is shared amongst all members of this class of transcription factors. Previous studies have proposed a few biological functions, e.g., the involvement in egg cell differentiation. The knockdown of the single RKD gene in the egg cells of *Marchantia polymorpha* induces a form of parthenogenesis, where egg cells spontaneously develop into disorganized unviable embryos without fertilisation.[4] Parthenogenesis is a part of apomixis, the process of asexual seed formation bypassing meiosis and fertilisation leading to the generation of maternal clones.[4, 5] The development of technology capable of engineering apomixis in crop plants will revolutionize agricultural food production, enabling efficient and consistent yields of high-quality seeds at lower costs through clonal seed production.[6, 7] Before this can happen however, the mechanisms regulating reproductive development in plants need to be better understood. Investigating what role RKD transcription factors play in *A. thaliana*, an angiosperm, is a crucial step in the development of apomixis technology.

The goal of this paper is on one hand to demonstrate all the steps required to obtain transgenic *A. thaliana* plants via the floral dip method and on the other hand to exemplify the utility of transgenic plants for gene function characterization based on the downregulation of RKD transcription factors in mature female gametophytes. All the experiments were conducted in the laboratory of the Department of Plant and Microbial Biology of the University of Zurich under the supervision of Dr Hannes Vogler, PhD student Nicholas Desnoyer and PhD student Alex Plüss. Besides the experiments performed on *A. thaliana*, various experiments were also conducted with *Nicotiana benthamiana* and *M. polymorpha*, other plant model organisms, which play an important role in plant molecular biology. To keep the scope of this paper in check however, these experiments are not described in this paper.

3 Theoretical Framework

3.1 *Arabidopsis thaliana*

3.1.1 Classification and Ecology

Arabidopsis thaliana, also known as the thale cress, mouse-ear cress or just simply *A. thaliana*, is a small flowering plant, which falls under the Brassicaceae family. Other members of this family include horseradish, cabbage, rapeseed, and broccoli.[3] *A. thaliana* was first described by Johannes Thal in the 16th century, from whom the name is derived. The plant can be found throughout Europe, Asia, and North America. *A. thaliana* often grows in rocky, sandy often calcareous, nutrient-poor soil.[a] Many different ecotypes have been collected from natural populations and are available for experimental analysis. Ecotypes are genetically distinct varieties, populations, or races within a species, which are genotypically adapted to specific environments.[b] Nowadays, the Columbia (Col) and Landsberg *erecta* (Ler) ecotypes form the broadly accepted standard for both genetic and molecular studies (see figure 1). However, it is worth mentioning that due to the wide use of *Agrobacterium*-induced transformation methods, many mutant lines have been generated and characterized, which are also at *Arabidopsis* researchers' disposal. Mutants in this case refer to lines, where the function of certain genes was engineered to be turned off or the insertion of transgenes took place.[3, 8, c]

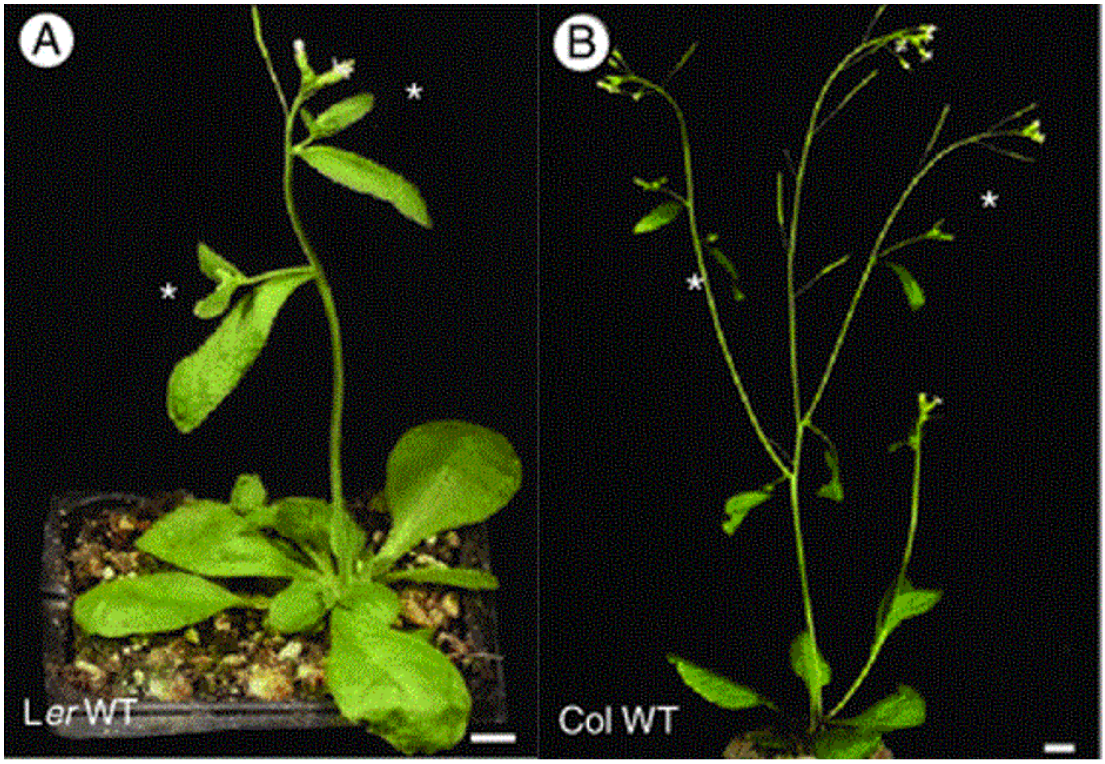

Figure 1 (A) A Landsberg *erecta* (Ler) wild-type (WT) plant with a primary inflorescence and two secondary inflorescences (asterisks). (B) A Columbia (Col) WT plant with a primary inflorescence and two secondary inflorescences (asterisks). Scale bars: 5 mm.[99]

3.1.2 Life Cycle of *Arabidopsis thaliana*

The life cycle of land plants is known as sporic or dibiontic, which exhibits an alternation between two heteromorphic generations, namely between a diploid sporophytic (spore-producing plant) generation and a haploid gametophytic (gamete-producing) generation (see figure 2). Spores are cells, which can grow into a new organism without combining with another cell, whilst gametes are the haploid egg and sperm cells, which fuse during fertilisation.[3, 9, 10, d]

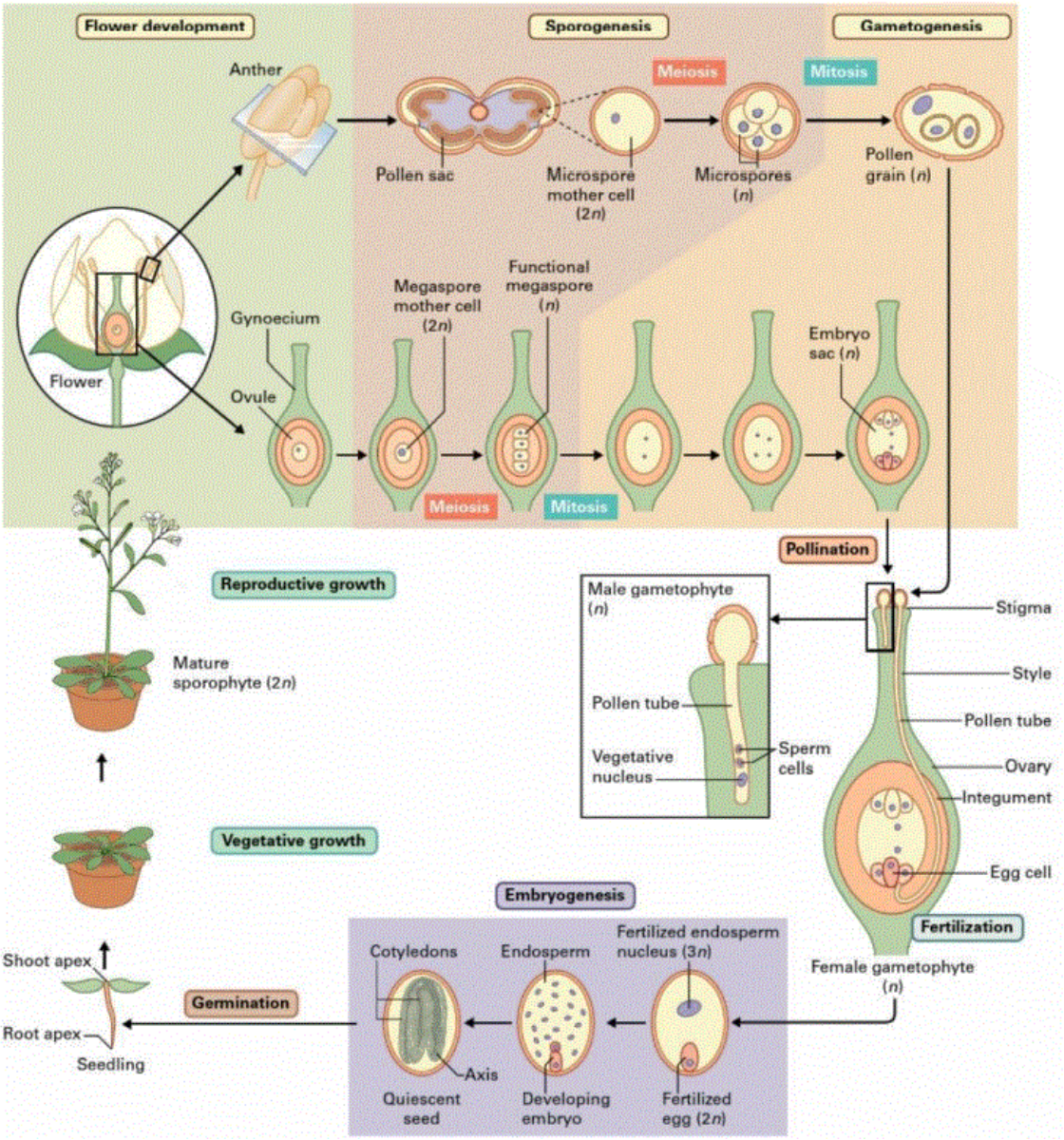

Figure 2: Life cycle of *A. thaliana*.[3]

In flowering plants or angiosperms, such as *A. thaliana* the sporophytic generation is the dominant phase in the life cycle, whereas only a few cells make up the gametophyte, which is reliant on the sporophyte.[10]

In the first stage of the life cycle, the sporophyte or seed goes through germination, which is succeeded by a vegetative period during which it produces several leaves. The transition between

vegetative and reproductive growth is marked by the process of flowering, which is dependent on various factors such as water and nutrient availability, along with photoperiod and environmental temperature. The shoot apical meristem, undifferentiated tissue, which previously was responsible for vegetative growth is reprogrammed to an inflorescence meristem, which produces multiple floral meristems.[9] In the developing flower the transition between the sporophytic phase to the gametophytic phase takes place. The process of sporogenesis consists of the formation of the megaspore mother cell in the ovules and the microspore mother cell in the anthers, which are both diploid.[3]

Through meiosis of the mother cells the haploid microspores and the haploid functional megaspore are formed respectively in the anthers and the ovule. Next, during a phase termed gametogenesis the gametophytes consisting of the pollen grain and embryo sac are formed through mitosis (see figure 2).[9] *A. thaliana* is usually self-pollinating. meaning the pollen fertilises the egg cell of its own flower. The tip of the carpel, known as the stigma, receives the pollen during pollination. The carpel is the female reproductive organ enclosing the ovules. Then the pollen germinates on the stigma surface and forms a pollen tube, which grows towards the ovules. Each pollen tube contains two sperm cells and one vegetative cell. Once the pollen tube bursts in the ovule, the egg cell and the central cell are fertilised by the two sperm cells, a process termed as double fertilisation (see figure 2). The final stage of the life cycle is termed embryogenesis, and it leads to the formation of a seed, ultimately leading to another plant upon germination, thus closing the life cycle.[3, 9, 10, d]

3.1.3 Female Gametophyte Development in *Arabidopsis thaliana*

To understand subsequent chapters, it is vital that the development of the female gametophyte in *A. thaliana* is taken a closer look at. The development of pollen is briefly mentioned in chapter 3.1.2; however, it is not as relevant for the purposes of this paper and will not be described in detail.

As mentioned in the previous chapter (see 3.1.2) the female reproductive development of *A. thaliana* can be distinguished in two phases, megasporogenesis and megagametogenesis. More than 70% of all angiosperms undergo what is called monosporic or Polygonum-type development.[11] This pattern is exhibited by all members of Brassicaceae including *A. thaliana*.[3, 12, 13, 14]

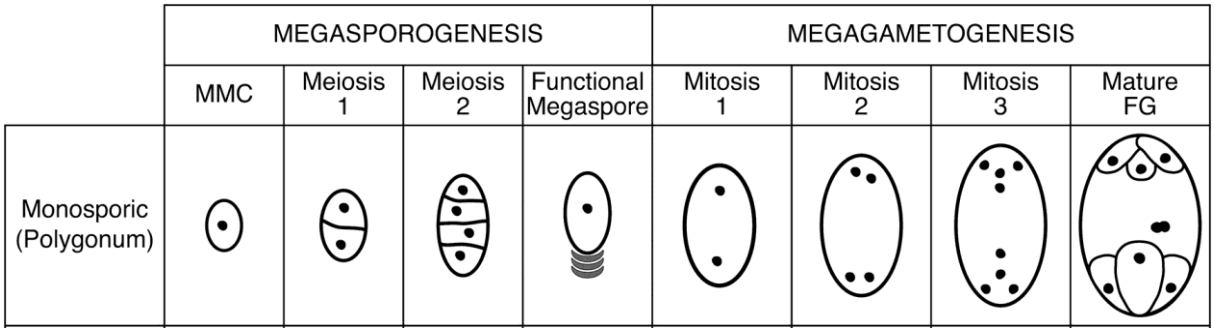

Figure 3 Graphical representation of the stages of monosporic female gametophyte development. Each dot represents a nucleus. MMC; microspore mother cell, FG; female gametophyte.[11]

Megasporogenesis can be divided into three stages: The formation of the megaspore mother cell, followed by the formation of the haploid megaspores through two meiotic divisions and the selection of the functional megaspore (FM), which will lead to the entire female gametophyte.[15] In the case of the monosporic pattern one of the four megaspores is selected, whilst the other three undergo cell death (see figure 3).

Once the FM has been selected, it continues to the phase of megagametogenesis, which can also be divided into three stages. Firstly, the FM undergoes 3 mitotic divisions without cellularization, bringing forth the formation of a vacuole and 8 nuclei in the embryo sac. Secondly, cellularization occurs, where two nuclei from opposite poles (polar nuclei) fuse to give rise to the homodiploid central cell whilst the other six nuclei are packed into individual cells. Finally, the cells differentiate to form the mature, seven-celled embryo sac, which is comprised of the central cell, the egg cell, 2 synergid cells and 3 antipodal cells (see figure 4).[3, 16, 17]

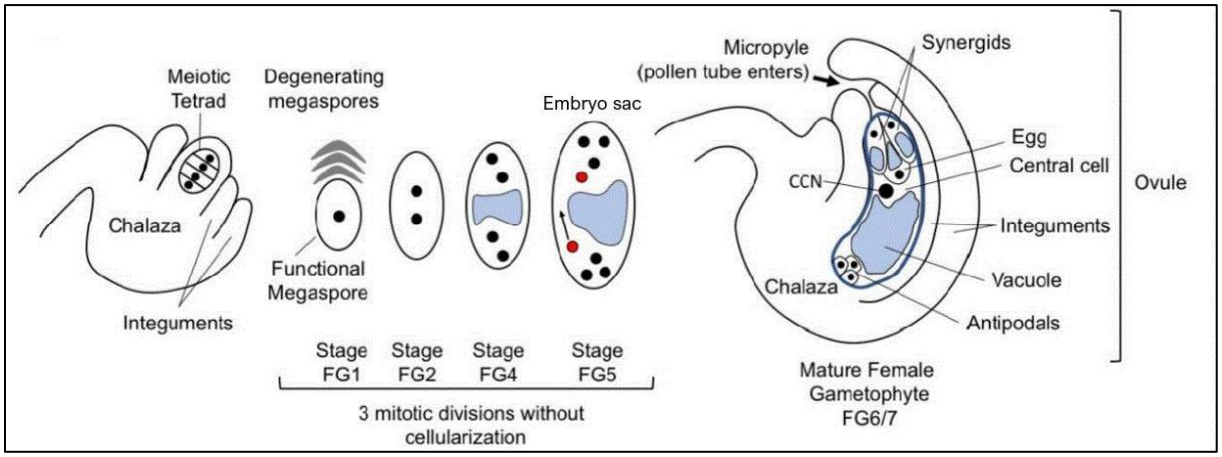

Figure 4: A schematic representation of the seven stages of female gametophyte (FG) development shown from left to right. After meiosis, one of the four haploid megaspores enlarges forming the functional megaspore (FM), whilst the other three megaspores degenerate. The first five stages consist of the three mitotic divisions and the formation of a vacuole (blue), which separates the nuclei. Contemporaneously to the third mitotic division (FG5 stage), cellularization begins. The two polar nuclei, which are marked in red migrate to the centre of the megaspore and fuse to form the homodiploid central cell. In the FG6/7 stages the remaining cells differentiate into the three antipodal cells, the egg cell, and the two synergid cells forming the mature, seven-celled embryo sac alongside the central cell with large diploid nucleus (central cell nucleus, or CCN). The embryo sac is encompassed by two epidermal integuments that serve as a protective layer. They almost completely envelop the embryo sac apart from a small opening called the micropyle through which the pollen tube may enter.[3, 16, 17]

3.1.4 Model Organism

A. thaliana has been established as the model organism of choice for research in plant biology and is by far the most thoroughly studied plant species.-There are numerous reasons as to why *A. thaliana* is an efficacious model organism:

(i) It has a relatively short life cycle for an angiosperm. The entire life cycle, which includes the germination of seeds, rosette plant formation, bolting of the main stem, flowering, and maturation of the first seeds is completed in 6 weeks.[8]

(ii) It is small and easy to cultivate, even in restricted space. Its flowers are self-pollinating but can also be crossed by applying pollen to the stigma surface. Furthermore, the plants can be grown under various conditions, such as in petri dishes or in pots with soil and either in a greenhouse or under fluorescent lights in the laboratory, making *A. thaliana* a rather versatile plant.[8]

(iii) Mature plants produce a large number of offspring; as each plant usually produces several hundred siliques, which aggregately contain more than 5000 seeds. After drying the seeds, either further experimenting and studying can be conducted on them or they can be simply stored at room temperature for later use. Seeds remain viable for several years, which minimizes efforts to maintain lines over long periods of time, as genetic stocks can be saved.[8]

(iv) The genome of *A. thaliana* is small and has been sequenced and mapped. The genome contains roughly 27'600 protein-encoding genes and roughly 5200 non-encoding genes.[e] Even though its genome is small, its molecular complexity remains high enough such that conclusions drawn from studying *A. thaliana* can be applied to other larger plants.[3, 18]

(v) Arguably the most important characteristic of *A. thaliana* as a model is its simple and efficient transformation system utilising *A. tumefaciens*. *Agrobacterium*-mediated transformation is used for many different plant species. What makes the transformation of *A. thaliana* so simple is the floral dip method, floral tissue is dipped into a dipping solution containing sucrose and *A. tumefaciens* cells, holding the respective construct which the plant is to be transformed with. The *A. tumefaciens* cells transform the seeds, and the transformed seeds can be selected by plating on herbicide media. For other plant species only the tissue culture regeneration method works, which is much more laborious and time-consuming (see chapter 3.5 for advantages of the floral dip method).

When considering these attributes of *A. thaliana*, it does not come as surprise that it was the first plant species to have its entire genome sequenced in the year 2000.[1] The genome consists of roughly 125 megabase pairs, which are organized into five chromosomes. This pioneered the subsequent sequencing of the genomes of over a hundred other plant species.[19]

3.2 *Agrobacterium tumefaciens*

3.2.1 Classification and Ecology

Agrobacterium tumefaciens is a soilborne gram-negative bacterium belonging to the family of Rhizobiaceae. Gram-negative bacteria unlike gram-positive bacteria do not retain the crystal violet stain, which is used in the Gram staining method of bacteria classification.[f] The primary difference between these two types of bacteria is the makeup of their cell walls. Other members of Rhizobiaceae include nitrogen-fixing legume symbionts. *A. tumefaciens* however, does not form a symbiosis with plants, instead it is best known for its capabilities as a parasitical pathogen, which causes crown gall disease. It is a serious pathogen for grape vines, stone fruits, nut trees, horse radish, sugar beets and rhubarb.[20, f]

Figure 5 (Left) Healthy blackberry plant. (Right) Neoplastic growth at a branch of a blackberry plant, a characteristic symptom of crown gall disease caused by *A. tumefaciens*

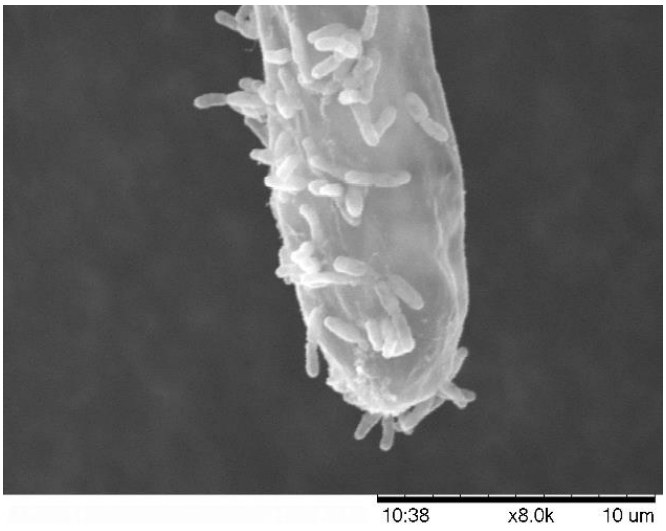

Figure 6: Scanning electron microscope image of *A. tumefaciens* attached to a root of a Col-0 *A. thaliana* plant. The 0 denotes that it is a wild-type plant. The scale bar is indicated at the bottom right.[100]

The disease is characterized by neoplastic growth at the site of infection, which is a result from oncogenic transformation of the host's cells.[21] The transfer of genetic material between *A. tumefaciens* and related species (*A. rhizogenes, A. rubi, A. vitis*) to their host plants is the first known example of active horizontal gene transfer from prokaryotes to eukaryotes, commonly referred to as interkingdom DNA transfer.[20, 22] The tools that provide *A. tumefaciens* with this ability reside on the large tumour-inducing (Ti-) plasmid, which contains a region that holds the *vir* (virulence) genes, which encode the majority of proteins, which are required to mediate the transfer of the essential T-DNA (transferred DNA).[20]

The ability of interkingdom DNA transfer for both transient gene expression and stable genetic transformation has established *A. tumefaciens* as an invaluable tool for both genetic research and technology (see figure 7). For example, several crops including rice, barley, wheat, and maize have been modified to enhance their resistance against pests and herbicides, traits which are agriculturally desirable. Transformation methods have been developed, which capitalise on the T-DNA's transformative properties whilst disabling the oncogenes, which cause crown gall disease (see figure 5). It is worth mentioning that the host range of *Agrobacterium* species has been artificially expanded in the pursuit of innovation in the realm of biotechnology. Several non-plant organisms such as yeast, filamentous fungi, and human cells were successfully transformed with genetically altered strains of *Agrobacteria*.[23, 24, 25] Although the general mechanism of T-DNA integration has been widely studied such as in plants like *A. thaliana*, the fine intricacies of the process such as the molecules, which are involved in the transfer pathway and integration are still not completely understood.[20]

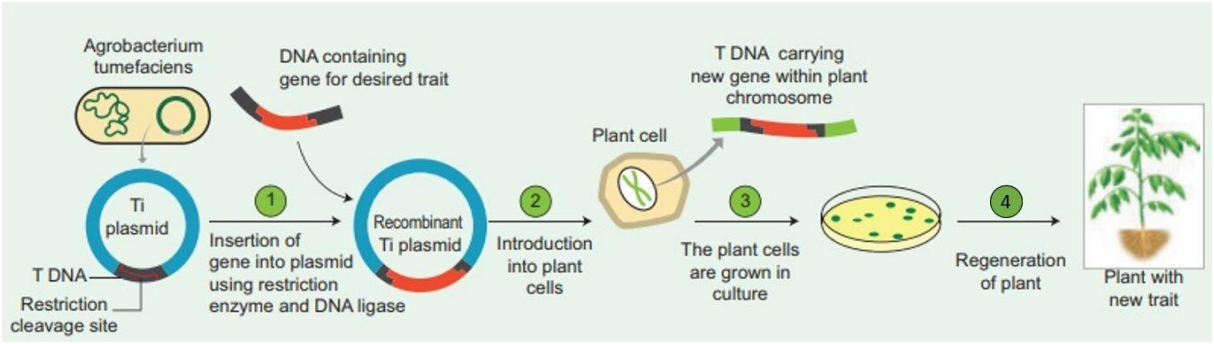

Figure 7: Graphical representation of *Agrobacterium*-mediated transformation of plants. 1) The Ti-plasmid is isolated from *A. tumefaciens* using restriction enzymes and with DNA ligase a gene of interest is inserted into the plasmid (recombinant plasmid). Often the plasmid is then cloned using *E. coli* (not depicted). 2) Next, the plasmid is inserted back into the *A. tumefaciens* cells, which mediate the transfer into the genome of plant cells (T-DNA holding the gene of interest is inserted into a plant chromosome). 3) The plant cells, in the case of the floral dip method the seeds, are grown on selection media, containing an herbicide or antibiotic to select for transformants. 4) The selected transformant cells or seeds are then grown, which lead to plants with new traits.

3.2.2 The Ti-Plasmid

As stated before (see chapter 3.2.1), Tumour-inducing (Ti-) plasmids can be found in the parasitical bacteria species *A. tumefaciens* and are big circular genetic structures, roughly 200 kb in size, which facilitate the means for interkingdom DNA transfer. In nature, this leads to the formation of neoplastic, undifferentiated tissue at the site of infection, after the oncogenic transformation of the host. There are two main regions to be distinguished, namely the T-DNA and *vir* region (see figure 8).[26, 27]

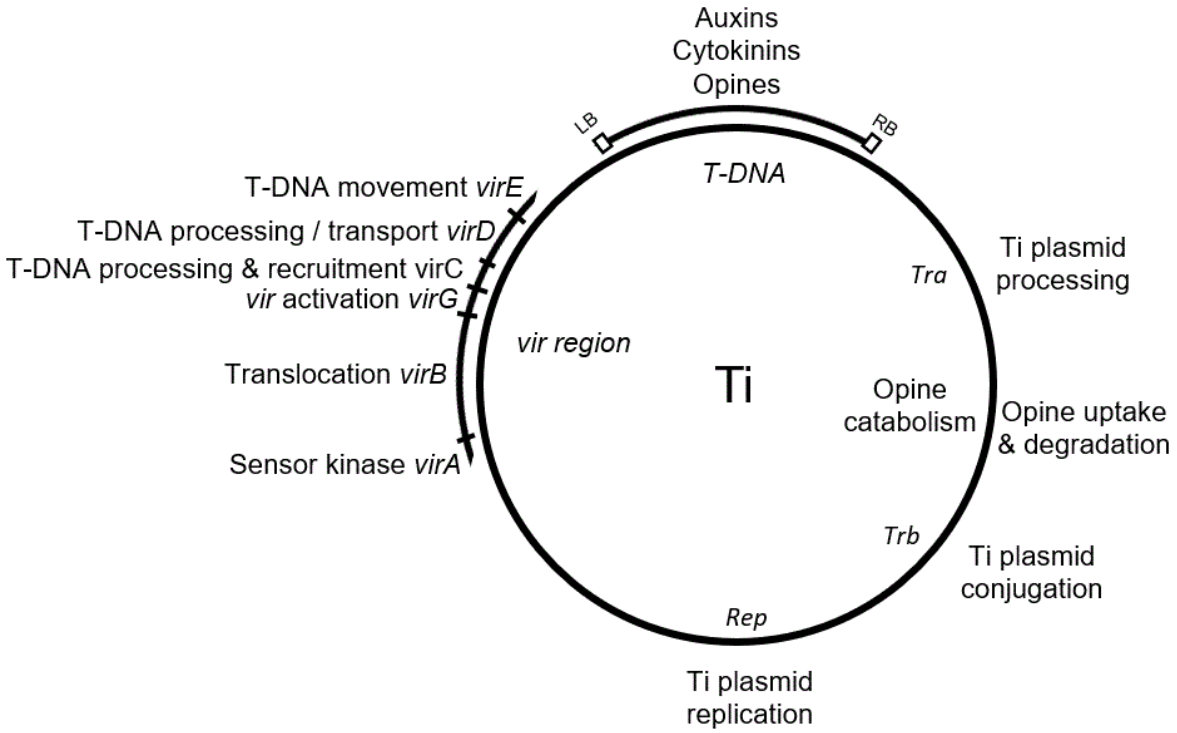

Figure 8: A schematic representation of a Ti-plasmid. The locations of the genes encoding for plasmid maintenance (*rep*), infection of plant cell (*vir* region, T-DNA), cell survival once entered in the host (opine catabolism) and conjugative transfer of the Ti-plasmid to recipient *A. tumefaciens* cells (*tra* and *trb*) are shown. The functionalities of the various *vir* gene products are listed. The T-DNA is delimited by the right border (RB) and left border (LB) marked as white boxes.[26]

The T-DNA is a small region on the Ti-plasmid, making up roughly 5-10%, which will eventually be integrated into the host's genome. It is here where the oncogenes reside, but also genes, which regulate the plants resources for the synthesis of opines. The T-DNA is delimited by two short 24 to 25 base pairs (bp) direct repeat sequences, dubbed the left border (LB) and right border (RB). The oncogenes encode for plant hormonal growth regulators such as auxins and cytokinins, which promote uncontrolled cell division, ergo these genes are responsible for the cancer like callus growth. The remaining genes encode for enzymes that produce opines, a small group of molecules often consisting of an amino acid and an organic acid or a carbohydrate.[28, 29] These molecules serve as a source of carbon, energy and in some cases as nitrogen and phosphorous sources for *A. tumefaciens* cells, which are essential during colonization inside the plant.[21, 30] One of the central adaptations of *A. tumefaciens* as a plant pathogen is this T-DNA-directed biosynthesis of a chemically diverse group of metabolites,

which occur rarely in nature and establish a suitable environment for bacterial proliferation. It points to a co-dependant evolution between plants and *A. tumefaciens*.[26, 27, 31]

The *vir* region consists of approximately 10 operons, termed *Agrobacterium vir* genes. Under normal conditions the expression of the *vir* region is repressed and is only activated when plant-derived signals from wound sites are sensed by the bacteria. The virulence activation leads to the production of the *vir* proteins, which are responsible for four major functions (see chapter 3.2.3 for detailed description of mechanisms).

(i) The VirA/VirG two-component system is responsible for the virulence activation and is able to sense phenolic compounds, secreted by wounded plants. The wounded areas serve as excellent sites for bacterial entry. Some of the *vir* genes are also responsible for chemotaxis towards regions, where such phenolic compounds are most highly concentrated.

(ii) The processing of the Ti-plasmid, which leads to the export of the single-stranded T-DNA and effector proteins via the formation of the T-complex.

(iii) Many of the *vir* genes are involved in the type IV secretion system, a protein complex for translocating macromolecules out of the bacterial cell and involved in T-DNA transfer (see step 2, chapter 3.2.3)

(iv) Many *vir* genes contribute to processes such as cytoplasmic trafficking, nuclear targeting, and integration into the host genome once the T-complex has entered the host.

Genetically, these genes all play an important role in the virulence of *A. tumefaciens*. Additional to the T-DNA and *vir* regions, there are 4 other regions, which play important roles for the maintenance of the Ti-plasmid. A region is responsible for opine uptake and degradation, termed opine catabolism. The *tra* and *trb* regions are required for conjugative plasmid transfer, a process, where mobile genetic elements are transferred between bacteria (see figure 9).[32] The *rep* region supports autonomous replication of the plasmid (see figure 8 for location of the various regions on the Ti-plasmid).[26, 27]

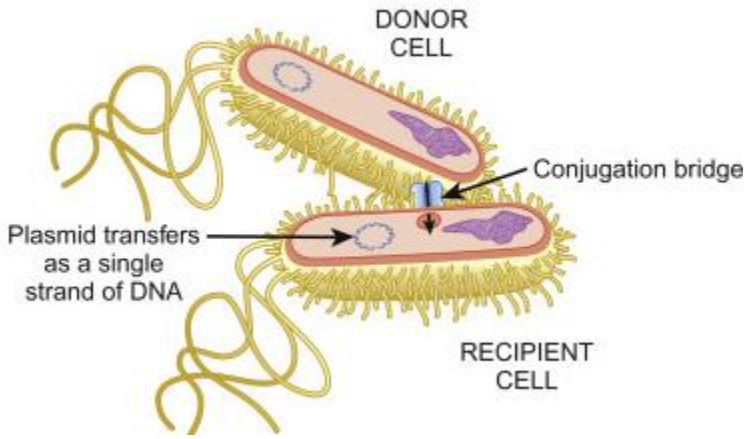

Figure 9: Schematic representation of bacterial conjugation. The rod-like extension labelled as conjugation bridge (blue) connects the cytoplasm of the two bacterial cells, such that the plasmid from the donor cell can transfer a copy of itself to the recipient cell.

3.2.3 The Mechanism of *Agrobacterium*-mediated DNA Transfer and Integration

To understand how *Agrobacterium*-mediated transformation methods work, which allow the insertion of specific genes into plants avoiding the side-effect of callus growth by using binary transformation systems and biotechnologically engineered plasmids, it is necessary to understand how the wild-type Ti-plasmid functions (see chapter 3.2.2). For the purposes of this paper, the mechanism of T-DNA transfer and integration into the host cell's genome was simplified into four steps, shortly summarized here, and described in further detail below. The activation of the *vir* genes occurs via a low pH and acetosyringone. It is the initialising process for the T-DNA transfer. The secondary plant substance acetosyringone is excreted by wounded root cells, which attracts mycorrhiza, symbiotic fungi, which help protect the root cells. *A. tumefaciens* capitalises on this secondary metabolism pathway.[20, 33]

The *vir* genes encode for proteins important for the generation of the single-stranded T-DNA (see figure 10.1), export out of the cell (see figure 10.2), the transport and nuclear targeting in the host cell (see figure 10.3) and the integration of the T-DNA into the plant's genome (see figure 10.4). After the T-DNA has been processed, it is referred to as the immature T-complex. The T-complex is exported out of the parasite and into the host, after *A. tumefaciens* has attached to its host by embedding itself in the biofilm of the plant. Additionally, effector proteins are exported, which help with the entry into the host cell and targeting the nucleus. Once the immature T-complex has interacted with the effector proteins it is referred to as the mature T-complex. After arriving at the host's nucleus, the T-complex is integrated preferentially into damaged areas of the genome, utilising the host's repair systems for integration.[20]

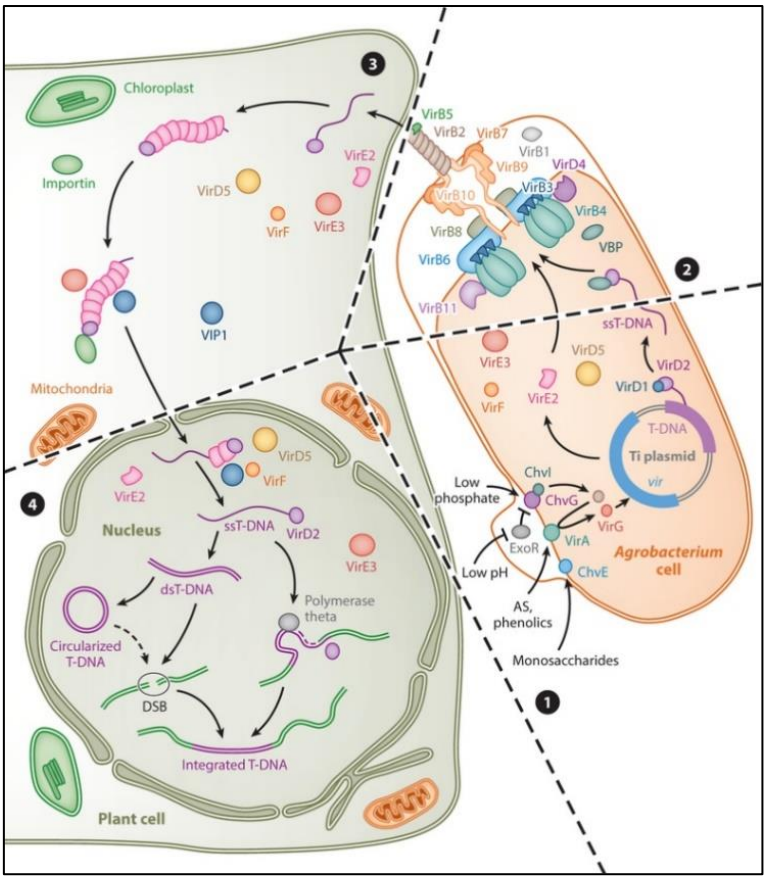

Figure 10 Schematic representation of the four main steps of T-DNA transfer from and *A. tumefaciens* cell into the genome of a plant cell. 1) Signals derived both from the plant and the environment activate the bacterial virulence system, which leads to virulence induction through *vir* gene expression, which is followed by the generation of the ssT-DNA. 2) The immature T-complex (VirD2 covalently bonded to the ssT-DNA) alongside several effector proteins (VirD5, VirE2, VirE3 and VirF) are exported out of the bacterial cell via the VirB/VirD4 T4SS. 3) Once the T-complex and the effector proteins are in the plant cell, they are targeted towards the nucleus. 4). Upon entry in the nucleus the T-DNA is processed and integration into the plant cell chromosomal DNA occurs. AS; acetosyringone, Chv; chromosomal virulence protein, DSB; double-stranded break, dsT-DNA; double-stranded transferred DNA, Exo; exocellular, ssT-DNA; single-stranded transferred DNA, T-DNA: transferred DNA, T4SS; type IV secretion system, Ti-plasmid; tumour-inducing plasmid, *vir*; virulence gene region, Vir; virulence protein. VBP; VirD2 binding protein, VIP1; VirE2 interacting protein 1.[20]

Step 1: Induction of Virulence and Generation of Single-Stranded T-DNA

A. tumefaciens cells can sense phenolic compounds such as acetosyringone, which originate from wounded plant cells, e.g., in the roots. These compounds activate the expression of *Agrobacterium vir* genes, which results in the generation of the single-stranded T-DNA and the synthesis of proteins required for DNA transfer.[20]

Furthermore, a low pH is required for virulence induction. Given these two conditions, two specific *vir* genes *vir*A and *vir*G genes are activated, which form the VirA/VirG two-component system, which results in a strong induction of all the other *vir* genes and probably also the chemotaxis of the bacteria towards plant-derived signals.[34, 35, 36] The expression of *vir* promoters is first observed a few hours after the virulence induction via acetosyringone and usually reaches a plateau after 12 to 24 hours.[20, 37]

The T-DNA is transferred from the Ti-plasmid into the host cell as a single-stranded DNA (ssDNA) intermediate, commonly referred to as the T-Strand.[38] There are two essential proteins for the processing of T-DNA, namely VirD1, a DNA topoisomerase, which unwinds the double-stranded DNA, and VirD2, an endonuclease, which cuts out the T-DNA.[39, 40, 41, 42] The collaboration of both proteins regulates the mobilisation of the T-DNA from the Ti-plasmid via a mechanism, which regulates complementary strand replacement of the T-DNA on the plasmid, whilst it is being cut. Importantly, VirD2/VirD1 only needs to recognise the LB and RB borders of the T-DNA for the system to function, thus the sequence between the borders can to a certain extent be altered arbitrarily (see chapter 3.4 on how binary transformation systems function as a vital tool for genetic engineering). This is why it is a particularly powerful tool to introduce specific transgenes into plants for biotechnological reasons. After T-DNA processing, the VirD2 endonuclease remains covalently bonded to the 5' end of the T-strand, forming what is called an immature T-complex, also referred to as the single-stranded transferred DNA (see Figure 10.1).[20, 43]

After 12 to 24 hours a successful infection of the host plant by the *A. tumefaciens* cells should have taken place, after which the virulence system is shut off. It has been observed that during the activation of the virulence system the bacteria population growth decreases as a result of the high energy cost.[44] Indole acetic acid (IAA) is suspected to function as a competitive inhibitor to acetosyringone (AS) for binding to VirA.[45] Not only does it inhibit another further infection by the initial strain, but it also prevents any additional transformation from competing bacterial strains.[20]

Step 2: Export of the T-DNA and Effector Proteins and Host-to-Pathogen Interactions
For the transfer of T-DNA and effector proteins, i.e., for the activation of virulence via the *vir* genes to occur it is thought that a close-range interaction and attachment has to take place between the bacterial and host cells. Under laboratory conditions bacterial virulence can be induced without such an interaction by adding acetosyringone to the bacteria colony.[46] In nature, these two processes are connected with each other. Furthermore, the virulence induction leads to a change in the bacterial lifestyle. The once free bacteria in the rhizosphere attach to the surface of the host cell and are embedded in its biofilm, adhering to the surface of the host cell.[20]

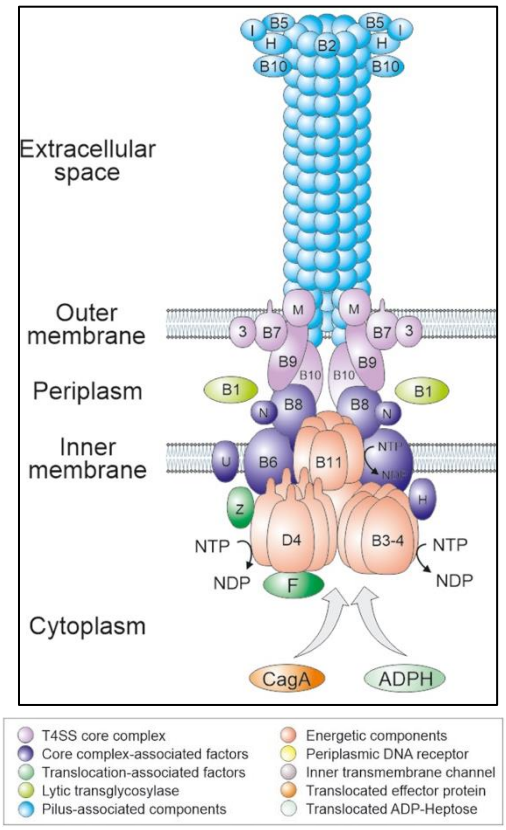

Figure 11: Schematic representation of a type IV secretion system (T4SS).[101]

The cellular interaction between the parasite and host is thought of to happen in two stages. The initial contact usually relies on certain specific host cell-surface receptors. This process can be reversed, as the bacteria have not made themselves indefinitely sessile. In a second stage the bacteria stabilise their attachment to the host cell surface via the synthesis of cellulose fibrils with which it anchors itself in the plant's biofilm.[20]

In *A. tumefaciens* cells there is a very specific secretion system called the T4SS (type IV secretion system), which is a large molecular complex composed of various proteins encoded by the *vir*B operon and *vir*D4. Operons are a cluster of genes, all under the regulation of a single promoter. T4SSs contain a channel through which proteins or protein–DNA complexes, such as T-complexes can be translocated out of the cell membrane and cell wall. [20, 98]

T4SS is thought to be assembled at the cell poles and it has been suggested that VirC1, VirC2 and other VBPs (VirD2 binding proteins) assist in the targeting of the VirD2-T-Strand-complex to the T-DNA export machinery.[47] Independently of the T-DNA export, the T4SS system also exports effector proteins encoded by various *vir* genes, which are important for processes, which occur in the host plant cell. For the purposes of this paper further details of T4SSs are omitted but for illustration purposes the structure of the protein complex is shown (see figure 11).[20]

Step 3: Entry and Guiding of T-DNA to the Nucleus of the Host Cell

The exact mechanism of how the immature T-complex (see step 1) and effector proteins pass through the host cell wall and plasma membrane remain unknown. Several different mechanisms have been proposed, of which three are presented below.[20]

(i) The mechanism of T-DNA entry in the host cell, might be similar to that proposed for bacterial conjugation (see figure 9, chapter 3.2.2) where an exchange of genetic material through a bridge-like connection occurs. Membrane fusion between the parasite and the host might allow the transfer of cargo.[48] It is speculated that VirE2 might act as a protein channel, which allows DNA to pass through the plant's cytoplasmic membrane.[49] On the other hand, VirE1 may mediate the transfer of the VirE2 protein into the host plant cell.[20, 50]

(ii) Pili are hair-like structures found on the outer surface of numerous bacteria and archaea.[g] *A. tumefaciens* has such appendages. A pilus might act as a needle, where macromolecules pass through.

When the pilus interacts with the host membrane, or its proteins cargo is able to pass through. There is however no data to support such an interaction between the host membrane proteins and bacterial factors for the formation of a pore.[20]

(iii) Macromolecules might first be exported into intercellular space, where they remain before being incorporated into the host cell by a mechanism similar to endocytosis, which would then likely require the host to recognize the exported macromolecules with a specific receptor.[20]

Before the integration can occur, both the T-DNA and the effector proteins, vital for targeting the T-DNA to the nucleus of the plant and certain nuclear functions, must be imported into the cell and further into the nucleus. For example, the effector protein VirE2 is thought to interact with the immature T-complex and protects the T-strand from degradation after entry into the host's cytoplasm. Together they form the mature T-complex. It is likely that both VirE2 and VirD2 participate in T-DNA nuclear import, where VirD2 targets the T-strand to the nuclear pores, whereas VirE2 is responsible for packaging the T-Strand and its movement through host cell cytoplasm and through the nuclear pores.[20, 51]

Step 4: Integration of T-DNA in the Host Chromosomal DNA

Similar to the entry process of the T-DNA and the effector proteins, the process of T-DNA integration into the chromosomal DNA of the host in its entirety is unknown. Certain aspects however remain less obscure. It has been shown that the T-DNA integrates randomly into the chromatin, although there might be a tendency toward certain epigenetic markers.[20, 52, 53]

Once targeting has occurred, there are other host factors, which interact with VirD2 and other translocated effector proteins by *A. tumefaciens*, which directly mediate integration.[54] Unlike retroviruses, *A tumefaciens* does not encode an own integrase, a protein which would catalyse the integration of its own DNA into the host's genome. Instead, the T-DNA integration relies mostly on the host cell pathways, specifically the host cell DNA repair pathways for double strand breaks (DSBs) were suggested to be preferred sites of T-DNA integration.[20, 55, 56, 57]

The T-DNA is brought into the nucleus as ssDNA and there are two possibilities as to what occurs next. (i) The T-DNA is converted to a double-stranded DNA (dsDNA) before integration or (ii) the ssDNA partially anneals to the hosts DNA via microhomologies, after which the second strain is synthesized, and ligation occurs. For the latter suggestion, nonhomologous end-joining (NHEJ) is believed to be the main pathway for integration of foreign DNA in plants, as the alternative form of integration, homologous end-joining, occurs at extremely low rates.[58, 59] The host's NHEJ machinery might be manipulated by the *Agrobacterium* effector proteins.[20]

3.3 *Escherichia coli*

3.3.1 Classification and Ecology

Escherichia coli is a gram-negative, facultative anaerobic bacterium and a member of the Enterobacteriaceae family, which includes several harmless symbionts but also various pathogens like subspecies of *E. coli* or *Salmonella*.[h] *E. coli* is found in the microflora of humans and other mammals, especially in the gut. Typically, the gastrointestinal tract of human infants is colonized by *E. coli* within a handful of hours after birth. There it coexists in a mutually beneficial symbiosis between itself and its human host and is seldom the cause of disease. Nevertheless, there are several strains of *E. coli*, which are known to cause three distinct illnesses after infection. Namely, enteric diseases, urinary tract infections and sepsis/meningitis.[60]

In the realm of genetic engineering technologies, *E. coli* has been broadly utilised as a cloning host to produce recombinant proteins. There are several reasons for this. *E. coli* has a very short life cycle, is easy to culture and due to its well-known genetics can be genetically manipulated with ease. In 1982, pharmaceutical Insulin had been manufactured with *E. coli*, being the first host to produce proteins from recombinant DNA (rDNA).[61] When designing a plasmid for the genetic modification of *A. thaliana* plants, *E. coli* is preferred as a host for genetic manipulation of the T-DNA region between the LB and RB sequences (see chapter 3.2.2) and also for adding antibiotic resistance selection markers.

3.3.2 Restriction Enzymes

The first step in inserting a gene into a plasmid is the isolation of that gene which is done by restriction enzymes, also referred to as restriction endonucleases. In *E. coli* and other bacteria, restriction enzymes provide a mechanism to restrict the propagation of bacteriophages, by cutting the pathogen's DNA, essentially making the pathogen innocuous. Through the methylation of their DNA, the bacteria prevent the restriction enzymes from cutting their own genetic material. Restriction enzymes cut DNA at specific restriction sites, which are often palindromic sequences, meaning the sequence of bases is equivalent when read forwards or backwards. Two types of restriction enzymes can be differentiated based on how they cut the DNA. The first group of restriction enzymes cut DNA, such that there short overhanging single-stranded DNA sequences remain at the restriction sites. They are called sticky ends, because if you add foreign DNA that was cut with the same restriction enzyme, the sticky ends are complementary, meaning the bases overlay and form hydrogen bonds. With the help of the enzyme DNA ligase the joining of the two DNA strands can be facilitated. This is how defined fragments of DNA can be recombined into plasmids. The second group of restriction enzymes cut DNA, such that there are no overhanging single-stranded DNA sequences. They create clean cuts through both strands of the DNA and are referred to as blunt ends. Blunt ends can also be joined via the DNA ligase but are rarely used.[62]

3.3.3 Cloning Plasmids

Plasmids are small circular DNA molecules found in bacteria, which can replicate independently and are physically separate from chromosomal DNA. They are a powerful tool for genetic engineering as it is easy to isolate them and introduce foreign genetic material. Plasmids have an origin of replication, which allows plasmid replication independent of bacterial chromosomal DNA. To introduce a specific gene into a plasmid, restriction enzymes are used, which cut at restriction sites, which appear in the plasmid only once. Therefore, the plasmid is only opened at one specific location. The gene of interest (goi) is isolated with the same restriction enzyme and in vitro is introduced to the cut plasmid. The sticky ends overlay, and DNA ligase joins the DNA strands together, forming the recombinant plasmid.[63]

In addition to the goi, one or several selection marker genes encoding for either an antibiotic resistance gene or a gene encoding for a fluorescent marker are incorporated into the plasmid for the selection process. To obtain a high concentration of the desired plasmid, it has to be replicated in a suitable vector such as *E. coli*. The cloning of plasmids is the process of replicating a plasmid in a vector. The replication of the plasmid only occurs if the uptake in the bacteria was successful. To select for transformant *E. coli* cells, all the bacteria is plated on a selection media. Each surviving colony on that plate represents a transformation event and holds the plasmid. These colonies can be propagated in liquid growth media.

Once the desired level of transformed *E. coli* is reached, the bacteria is killed, and its macromolecules are separated from the plasmids in a procedure termed miniprep, short for minipreparation. The plasmids can now be sequenced to verify if the goi and other vital components are present. The plasmids are then inserted into *A. tumefaciens* via electroporation. T-DNA binary vector plasmids used for floral dip have at least two different selection marker genes, a number which can increase with increasing complexity of the plasmid. The first marker selects for the *A. tumefaciens* transformants after electroporation. After electroporation the transformed *A. tumefaciens* strain is left to propagate until the desired number of bacteria is reached, after which the inflorescences of *A. thaliana* plants can be dipped into a solution containing the *A. tumefaciens* cells. This will lead to transformed *A. thaliana* seeds, which are selected by a second marker. The term binary transformation system for transforming *A. thaliana* refer to the two vectors being used in succession, namely *E. coli* and *A. tumefaciens* (see chapter 3.4). As plasmid replication occurs in both vectors, the origin of replication of the plasmid must be compatible with both bacteria strains or different origins of replication have to be present (see figure 14, chapter 4.1 for a vector map depicting pBR322, the plasmid's origin of replication compatible with *E. coli* and pVS1, the plasmid origin for *A. tumefaciens*).

3.4 Binary Transformation System

Agrobacterium-mediated genetic transformation has been a vital tool in creating transgenic plants. The transfection mechanism of the Ti-plasmid, particularly the T-DNA region, has been taken advantage of for inserting desired genes into plant lineages. Binary transformation systems are comprised of two different plasmids, where the *vir* and T-DNA regions are split onto two different replicons. The development of binary transformation systems has allowed transformants to be selected systematically with the insertions of antibiotic resistant genes, herbicide resistant genes or genes encoding fluorescent proteins. Furthermore, the oncogenes were removed such that the formation of crown gall disease could be avoided entirely.

The function of a binary transformation system is to insert a goi into a plant, circumventing the development of crown gall disease. Ideally the goi is integrated into the T-DNA for gene transfer. Three main components are required for such a transfer: Firstly, the *vir* genes located in the *vir* region secondly, the RB and LB repeat sequences (25bp) flanking the T-DNA, which marks the region that is processed by the *vir* genes and inserted into the host's genome, and thirdly various genes located on the bacterial chromosome.[64]

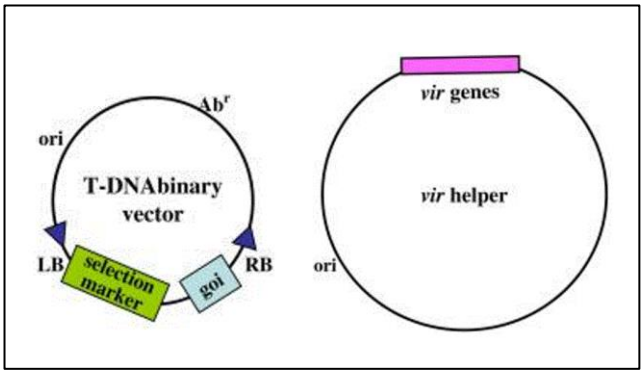

Figure 12: Schematic representation of a T-DNA binary transformation system. The gene of interest (goi) and the respective selection marker are maintained within the T-DNA region of the T-DNA binary vector. The selection marker is used as an indicator for successful transformation. The *vir* genes encoding for the *vir* proteins are on a separate replicon (*vir* helper), which mediates the T-DNA transfer from the bacterium to the host cell. ori, origin of replication; Ab^r, antibiotic-resistance gene utilised to select for the presence of the T-DNA binary vector in *E. coli* or *A. tumefaciens*. [27]

The wild-type Ti-plasmid contains a T-DNA region, which is generally large and does not have sites for endonuclease restriction suited for cloning genes of interest. To remedy this the *vir* and T-DNA regions are split into two separate plasmids (see figure 12) and artificial multiple cloning sites (MCS) are introduced in the T-DNA region. MCS are short segments of DNA, which contain up to 20 restriction sites.[i] Thus, the T-DNA region has many restriction sites and the flexibility in terms of choosing which restriction enzyme to utilise is established. The proteins encoded by the *vir* genes can still fulfil their functionality in processing and export of the T-DNA, so long both plasmids are located in the same *A. tumefaciens* cell.[65, 66] Furthermore, to circumvent tumour induction in the plant, the wild-type oncogenes and opine synthase genes are deleted. This does not affect the transfer of T-DNA, as the cutting by the endonuclease occurs between the nucleotides 3 of the RB repeat sequence and 4 of the LB repeat sequence during the T-DNA processing.[67] Thus, wild-type T-DNA genes can be replaced with genes encoding selectable markers such as an antibiotic resistance and genes of interest.[68]

The modified T-DNA is located on the T-DNA binary vector plasmid, which contains the goi and a gene encoding a selection marker. The non-T-DNA region of this particular vector contains origins of replications, which work both in *E. coli* and *A. tumefaciens* and together with the antibiotic-resistance genes used to select for the presence of the T-DNA binary vector in bacteria are termed the vector backbone sequences. The compatibility of this vector for *E. coli* is crucial as it is the favoured host for in vitro plasmid manipulation. Once a plasmid has been cloned in *E. coli*, the mobilisation of the genetic material to an appropriate *A. tumefaciens* strain via bacterial conjugation or transformation follows suit. Such strains would contain the other plasmid known as the *vir* helper, which contains the *vir* genes. This vector usually also has a selectable marker, which is different, from that included in the T-DNA binary vector plasmid. This allows the *A. tumefaciens* cells, which only hold the *vir* helper plasmid to be distinguished from those, which hold both plasmids. Selection occurs with a selection plate, containing both antibiotics.

In summary, the two main regions of the Ti-plasmid are split into two separate plasmids to enable easier genetic manipulation and cloning. A crucial part in this, is making the plasmids compatible with *E. coli*. Furthermore, there are no genes in the T-DNA region apart from the RB and LB repeat sequences, which are essential for T-DNA transfer. Thus, the oncogenes can be replaced with a goi, eliminating the tumour-inducing nature of the T-DNA, and repurposing it as a tool for genetic engineering.[27]

3.5 Floral Dip

Before the development of the floral dip transformation methods, transformation methods to obtain transgenic plants typically involved the preparation of competent plant cells or tissues, a delivery of the foreign genetic material via *A. tumefaciens* or biolistic bombardment, selection of stably transformed cells and regeneration of the transformed cells into transgenic plants. Such methods involved tissue cultures and a regeneration process, which was painstakingly time-consuming and often resulted in unwanted DNA modifications during the processes of dedifferentiation and differentiation, a consequence of the stress the plant cells underwent in the in vitro cell culture. A new method, termed "the floral dip method" circumvented the need for plant regeneration. To allow the uptake of the *A. tumefaciens* cells into the female gametes the *A. thaliana* flower buds were dipped in an *A. tumefaciens* cell suspension containing 5% sucrose, 0.05% Silwet L-77.[69, 70] The use of the surfactant Silwet L-77 helps the *A. tumefaciens* cells to better adhere to the surface of the plant inflorescences. The plants are then left to mature until they produce seeds, which are then plated on a selective medium to screen for transformants.[71] The floral dip method has several advantages (listed below) over the cumbersome traditional transformation methods, which require plant regeneration.[72, 73]

(i) The floral dip method does not require intensive labour, expensive equipment, or specialised reagents.

(ii) It is easily scalable and therefore a large number of transgenic *A. thaliana* lines can be produced in a short period. Transgenic *A. thaliana* plants can be obtained in approximately three months. As *A. thaliana* produces a vast number of seeds sufficient events of transformation occur, even if the transformation efficiency typically lies between 1 and 3%.

(iii) With the floral dip method, the genome of transgenic *A. thaliana* plants remains stable, unlike tissue culture-based transformation methods, where plants undergo stresses causing genetic mutations.

3.6 Genes of Interest

3.6.1 RWP-RK Domain Containing Gene Family

A screen for egg cell identity genes in wheat have revealed a class of transcription factors, which share a highly conserved RWP-RK domain.[74, 75, 76] The RWP-RK domain has been named according to a molecular motif consisting of invariant amino acids at the carboxyl-terminus, the end of an amino acid chain. The amino-acid residues occur in equivalent positions in the polypeptide chains for all the proteins this gene family encodes.[75, 77, j] The genomes of green algae, vascular plants and amoebozoa have been found to include this gene family, whilst it has not been identified in animals. The RWP-RK factors can be divided into two subcategories based on protein size and domain sequence: The nodule inception-like (NIN) proteins and the RKD transcription factors. Whilst the NIN factors have been characterized in several species, the role of the genes thought to encode RKD transcription factors are far from understood but are somehow involved in controlling egg cell functions.[77, 78, 79] Several studies in wheat, maize, *M. polymorpha* and *A. thaliana* have focused on elucidating their functions.[4, 74, 76, 80, 81, 82] The knockdown of the single RKD gene in the egg cells of *M. polymorpha* has been reported to induce a form of parthenogenesis. Parthenogenesis describes the spontaneous development of egg cells into embryos without fertilisation and is a component of apomixis, the process of asexual seed formation bypassing meiosis and fertilisation and generation of maternal clones.[4, 5] Engineering apomixis in crop plants is expected to have a profound impact on seed breeding and agricultural food production as clonal seed production will considerably lower the costs of maintaining consistent yields of high-quality seeds.[6, 7] Before this can happen however, the mechanisms regulating reproductive development in plants need to be better understood. RKD transcription factors are only one of several components known to play a role in egg activation and the initiation of embryogenesis, of which the molecular basis remains unknown. Investigating what role RKD transcription factors play in *A. thaliana*, an angiosperm, is a crucial step in the development of apomixis technology in crop plants. The following chapter will provide further insight into the RKD transcription factors in *A. thaliana* (see chapter 3.6.2).[3]

3.6.2 RKD Transcription Factors in *Arabidopsis thaliana*

14 RWP-RK genes have been identified in the genome of *A. thaliana*, of which five belong to the RKD subfamily.[75] The 5 RKD transcription factors have the two initial letters “At” denoting the initial of *A. thaliana* and are numbered one through five. *AtRKD1* has primarily been found in the reproductive tissue, more precisely in the egg cell and the synergid cells (see figure 4, chapter 3.1.3).[76] *AtRKD2* mainly expresses in the egg cell during the later stages of female gametophyte development.[83] The ectopic expression of *AtRKD1* and *AtRKD2* causes additional egg-cell-like structures to emerge, suggesting that these transcription factors play an intricate role in gamete formation.[81] *AtRKD3* and *AtRKD4* are present both in early and later phases of embryo sac development, however their levels were the highest during later stages .[81, 97] *AtRKD5* has been found to be expressed in a more ubiquitous fashion as the transcript levels remain constant through ovule

development.[4, 76, 81] Tedeschi *et al.* (2017) characterized single and double knockdowns of RKD genes in *A. thaliana*.[81] Single mutants did not show any severe phenotypes, possibly due to functional redundancy between the five RKD genes in *A. thaliana*. In the double mutants rkd1-1 rkd2-2 and rkd1-2 rkd5-2 low numbers of seeds have been reported.[81] Certain mutants like rkd5-2 rkd2-2 and rkd4-1 rkd2-2 show an early arrest phenotype during the functional megaspore stage (see chapter 3.1.3). These mutants were created using a downregulation method. Thus, the functions of the RKD genes during later stages of ovule development remain unknown. In Prof. Grossniklaus' lab plasmids were constructed (pNSE8 and pNSE9, see chapter 4.1) to determine the role of RKD transcription factors in mature female gametophytes. These plasmids encode for artificial microRNAs (amiRNA) (see chapter 3.6.3), which cause a knockdown of several RKD genes. To ensure that the female gametophyte reaches maturity before the transcription factors are inhibited, a late stage-specific promoter was utilised.

3.6.3 Artificial miRNA Knockdown Strategy

RNA interference (RNAi) is a method of gene knockdown, which works on the basis of mRNA degradation and is a widely used technique for genetic functional analyses. RNAi was first discovered in the nematode worm *Caenorhabditis elegans*.[84, k] It is a cellular pathway, which utilises so-called small non-coding microRNAs (miRNAs), which oversee posttranscriptional gene regulation. MiRNAs are derived from miRNA precursors, hairpin-like structures, which get cleaved by Dicer-like (DCL) enzymes. The resulting product is a short double-stranded (ds) RNA with some mismatches and a 3' overhang sequence, usually measuring 21-23 nucleotides. One strand is the mature miRNA (antisense RNA).[l] The mature miRNA is processed by the RNA-induced silencing complex (RISC) and act as a template to target complementary mRNA transcripts for degradation or repression of translation (see figure 13). Gene knockdown efficiency utilising a RNAi strategy has been documented to be up to roughly 90%.[85]

The use of amiRNAs lets specific genes be silenced even if there are no naturally occurring miRNAs, which regulate the genes of interest. Furthermore, amiRNAs are particularly useful for a multiple knockdown of closely related genes, which is why it is suited for studying the RKD genes. Similarities between closely related genes can be exploited and knocked down with a suitable amiRNA sequence present in all the genes. A functional redundancy between the five RKD genes is possible, meaning that knocking down one of the genes might not show much insight into its functions as the other four genes might be able to take over the knocked down gene's functions. Therefore, a multiple knockdown can give greater insights into their genetic functions. In this study plasmids were used to study the roles of the RKD transcription factors in the late stages of female gametophyte development. To ensure that the amiRNAs are only expressed after the female gametophyte has reached maturity an egg cell specific promoter called EC1.1 was used, which drives the expression of the amiRNAs at the fully mature stage.[87, m]

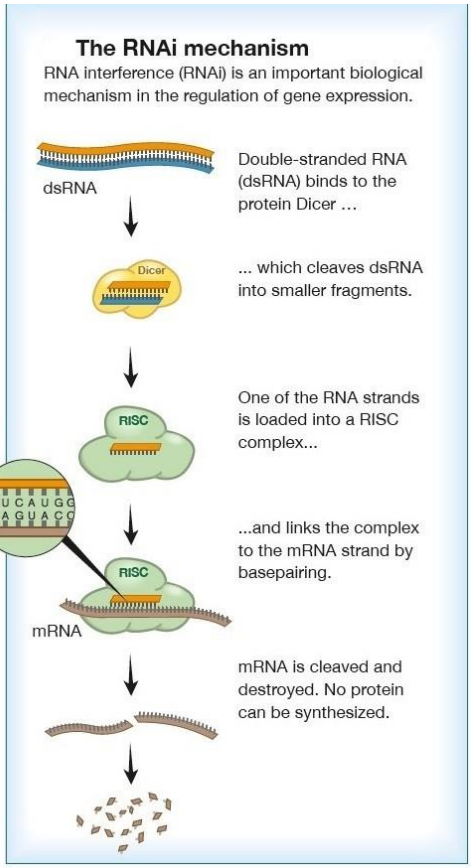

Figure 13: Graphical representation of the mechanism of RNA interference. The dsRNA is cleaved by the Dicer enzyme (in plants; dicer-like enzymes) into smaller fragments, usually measuring 21-23 nucleotides.[87, k] The dsRNA binds on to the RISC and the two RNA strands are separated, leaving the mature miRNA (antisense RNA) strand bound to the RISC. The sense strand dissociates.[87] The antisense strand serves as a template for the complex to link to a corresponding mRNA. The target mRNA is cleaved, leading to mRNA degradation and in turn the suppression of protein synthesis.[87]

3.7 Operating Principles of Verification Methods

3.7.1 PCR

Polymerase chain reaction (PCR) is a method used to rapidly create a large number of copies of a single DNA molecule. It requires the sequence of interest, specially designed primers, Taq DNA polymerase, deoxyribonucleotide triphosphates (dNTPs) and a buffer. The amplification process can be broken down into three steps. The initial step is called denaturation, where the double-stranded DNA sequence of interest is heated up to 95°C, such that the hydrogen bonds are broken, and the strands separate. Next, during annealing where the temperature is usually set at 55°C, the primers bind to the flanking regions of the target DNA (the temperature can vary depending on the primer). Finally, during DNA synthesis at a temperature of 72°C the DNA polymerase extends the 3' end of each primer with the nucleotides present. These three steps are repeated in what's called a thermal cycler for 25 to 35 times, exponentially producing copies of the DNA sequence of interest. For each step a different temperature is required, which is controlled by the thermal cycler. As the temperature for denaturation is high enough to denature regular DNA polymerase, a heat-resistant enzyme isolated from thermophilic bacteria, called Taq DNA polymerase is utilised. Often multiple primers are used, to check if specific genes are present in a sequence of DNA, which can then be verified using agarose gel electrophoresis.[88, n]

3.7.2 Sanger Sequencing

Sanger sequencing is a method to sequence genetic material, which requires DNA primers, DNA polymerase, normal deoxyribonucleotide triphosphates (dNTPs) and four modified dideoxynucleotide triphosphates (ddNTPs) labelled with different fluorescent dyes for each base (A, C, G, T). Before Sanger sequencing can be initiated the DNA region of interest is often amplified with the help of PCR. To each copy of the region of interest a primer will bind, and the DNA polymerase can extend the primer by adding dNTPs, which are complementary to the DNA strand of interest. The synthesis of the DNA strand has to come to a controlled stop, which happens as soon as a ddNTP is inserted. The DNA polymerase is unable to add any further nucleotides onto this chain of DNA, as the ddNTP is missing an OH group at the 3' carbon of the ribose. It is important to note that each type of terminating ddNTP is performed in four separate reactions. At the end of the reactions, extension products of all possible lengths will have terminated with a ddNTP at the 3' end. In four lanes the ddNTP-terminated fragments are separated by capillary electrophoresis according to their molecular weight and thus also in length. At the end of the capillary a laser excites the dye, which is detected by a light sensor feeding the data into software, which then translates the sequence. The end product is a data file with an electropherogram showing the sequence of DNA.[89, o]

4 Material and Methods

In the following chapters all the necessary steps taken to get transgenic *A. thaliana* plants, which have downregulated RKD transcription factors, are described in detail. The plasmids holding the amiRNA sequences were cloned in *E. coli* via heat-shock insertion, mini-prepped and then Sanger sequencing was performed on the products of the miniprep to check if the desired plasmids were indeed cloned as intended. Next, *A. tumefaciens* bacteria were transformed with the plasmids, after which the inflorescences of two Col-0 *A. thaliana* plants were transformed via floral dip (T0, transformant generation 0). The seeds, which developed subsequently from those two plants were selected and 48 T1 (transformant generation 1) plants were transferred onto soil. After three weeks on soil DNA was extracted from the 48 plants and PCR was done on them to screen for transformants, verified with electrophoresis (see figure 7, chapter 3.2.1 for graphical representation of *Agrobacterium*-mediated transformation).

4.1 Construction of PNSE8 and PNSE9 Plasmids

The pNSE8 and pNSE9 plasmids were constructed by Nadine Eggenberger for her master thesis in plant sciences.[3] In her thesis she vaguely describes all the steps, however a complete list of the materials utilised is missing. In the following it is explained as far as it can be reconstructed how the two plasmids were designed by the work of various people who previously contributed to this project.

To insert the egg cell specific promoter EC1.1 two restriction sites (KpnI and SbfI) were added for the insertion of the promoter into a template plasmid called pMDC32. For the further propagation, sequencing and storage fragments of the promoter sequence were incorporated into a plasmid via the pGEM T-Easy Vector System, a system, which allows PCR products to be cloned with ease on a predesigned plasmid.[p] Next, by cut and paste cloning the promoter could be incorporated into pMDC32. Figure 14 shows the vector with the EC1.1 promoter.

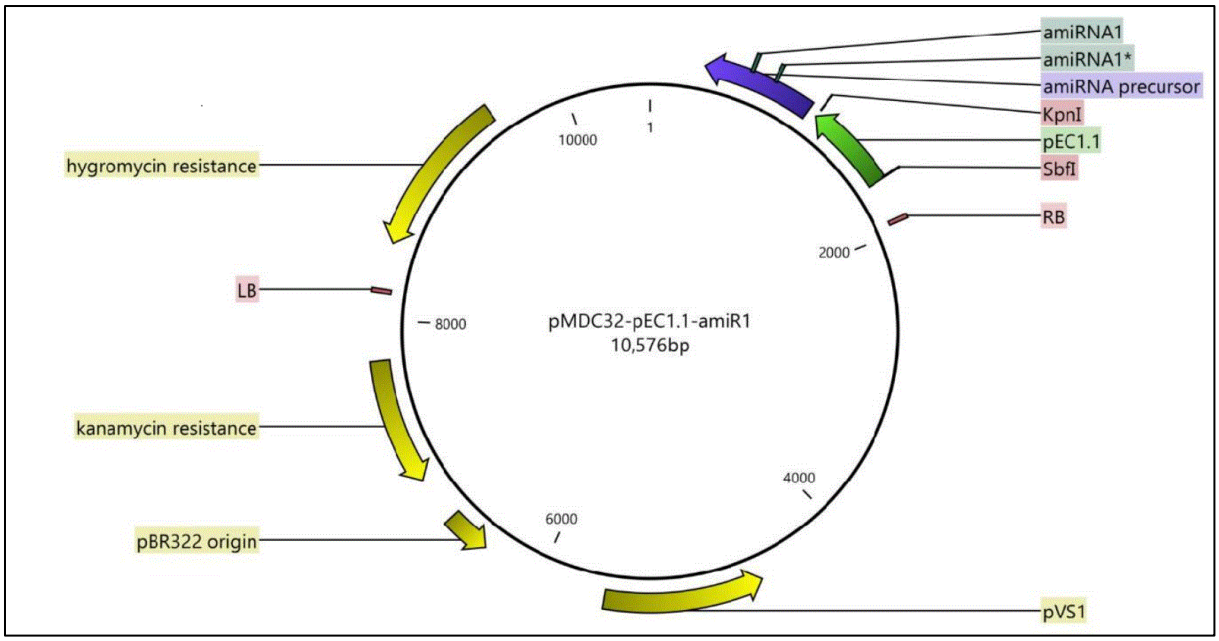

Figure 14: The vector map of the pMDC32-pEC1.1-amiRNA1 (pNSE8). The vector map with amiRNA2 (pNSE9) is not shown as it only differs in the sequence of the amiRNA/amiRNA*. Marked in blue are the amiRNA precursor, amiRNA sequence and the according complementary sequence (amiRNA*). KpnI and SbfI are the sites of restriction used to insert the EC1.1 promoter and are marked green alongside the EC1.1 promoter itself. The hygromycin resistance acts as the selection marker for plants, whilst the kanamycin resistance is the selection marker for the bacteria. pBR322 is the plasmid's origin of replication compatible with *E. coli* and pVS1 is the plasmid origin for *A. tumefaciens*. LB and RB are the left and right border, which demark the T-DNA, the sequence, which is inserted into the plant genome by *A. tumefaciens*.[3]

Next, amiRNAs were designed against the five RKD genes using the PHANTOM database (Julian Schroeder lab, UCSD).[t] No amiRNA could be designed against all five RKD genes, so instead two different amiRNAs were designed, targeting some of the RKD genes. The reason for this is simply that there are no sequences of length 21, which are equivalent in all five transcription factors, to which a single amiRNA could bind. The amiRNA strategy allows one single base mismatch but apart from that, the amiRNA knockdown strategy is restricted by the similarities among the genes encoding for the RKD transcription factors. AmiRNA1 targets RKD1, RKD3 and RKD4 whilst amiRNA2 targets RKD1, RKD2 and RKD3 (see table 1). No amiRNA could be designed to target RKD5 as no suitable primer was found.

Name	Sequence (5'-3')	Target genes	Off targets
amiRNA1	TGCTTACGGTGAGGCCGACGT	*RKD1, RKD3, RKD4*	-
amiRNA2	TGTTAAACCAATGTTAGGCGC	*RKD1, RKD2, RKD3*	-

Table 1: Sequences of amiRNA1 and amiRNA2 and the corresponding RKD genes they target.[3]

After the amiRNAs were designed, an amiRNA precursor sequence had to be generated. The product of the precursor sequence is cleaved by the Dicer enzyme resulting in the dsRNA (see chapter 3.6.3). The precursor sequence was generated by using overlap extension PCR in accordance with a protocol developed by Rebecca Schwab (WMD3, Detlef Weigel Lab, Tübingen).[u] Overlap extension PCR is a method to combine DNA fragments utilising specially designed primers at the ends of the fragments,

which are to be joined. These primers are constructed such that around 20 nucleotides match with the 3' end and have a 5' tail, which matches the sequence of the fragment to be fused. The complementary ends of the fragments act as priming sites for DNA polymerase to splice the fragments together.[90] The primers necessary for the protocol were designed by the WMD3 website.[v] Through Gateway™ cloning the expression vectors pNSE8-pMDC32-pEC1.1-amiRNA1 and pNSE9-pMDC32-pEC1.1-amiRNA2 were obtained, those being the full names of the plasmids with which the *A. thaliana* plants were transformed in this project. The expression vectors were then sequenced to confirm that the promoter and amiRNA sequences were orientated correctly as well as to check for the correct amiRNA sequence. Figure 13 shows the map of pNSE8.[3]

4.2 Insertion of pNSE8 and pNSE9 via Heat-Shock into *E. coli*

PNSE8 and pNSE9 were transformed into *E. coli* cells, which act as a vessel for plasmid cloning.

Material:

- Competent *E. coli* bacteria
- Bucket with ice
- Plasmids (pNSE8, pNSE9)
- Test tubes
- Deionized sterile water
- Heat block
- LB solution (liquid broth solution: 10 g Bacto-Tryptone, 5g Bacto Yeast Extract, 10g NaCl, 950 ml of deionized water)
- Laminar flow hood
- Kanamycin (kan)
- Incubator
- Pipettes with tips (20-200 µl)
- LB plates with kanamycin antibiotics

Step 1: Transformation of *E. coli* via heat shock

Competent *E. coli* bacteria were taken out of a -80°C freezer and placed in a bucket of ice for the bacteria cells to come out of their dormant phase. The pNSE8 and pNSE9 plasmids both encode for a kanamycin resistance (for bacteria selection process) and a hygromycin resistance (for seed selection process) and they each contain T-DNA sequences with the corresponding goi on them. Both plasmid solutions were diluted in a ratio of 1:10, since the plasmids were too highly concentrated. Into two different test tubes 200 µl of *E. coli* was added plus 50 ng of the respective plasmids (diluted in ratio 1:10). The test tubes were left to incubate on ice for 20 min. After incubation, the test tubes were placed in a heat block for 1 min, which was set at 42°C. The *E. coli* become stressed under this condition and integrate the plasmids into their interior. Next, 200 µl of LB solution was added to each test tube and they were then incubated in the heat block again for 60 min with the temperature set at 37°C.

Step 2: Selection of transformants on LB plates

In a laminar flow hood, a workbench where a constant flow of air is generated to create sterile conditions, 100 µl of each of the bacteria-plasmid-LB-solutions was pipetted onto a corresponding LB plate, which contained the selection antibiotic kanamycin. This is meant to select for the transformed *E. coli*, which were resistant to the antibiotic after incorporating the plasmid into their cells, whereas their non-transformed counterparts died. Finally, the LB plates were placed in an incubator at 37°C, where they remained for 18h-long incubation period.

Step 3: *E. coli* liquid cultures

Into two test tubes 5 ml of LB solution was pipetted. Additionally, kanamycin was added to the test tubes (50 µg/ml) to kill off any non-transformed bacteria, which might have survived on the LB plates. After the 18 h incubation period the bacteria, which had incorporated the plasmid encoding for the kanamycin resistance survived and formed small colonies on the LB plates. With pipette tips two individual bacteria colonies were picked from the LB plates and placed in the test tubes containing the LB/kanamycin solution. The test tubes were then incubated in a heat block at 37°C, where they remained overnight.

4.3 Miniprep of *E. coli*

The plasmids, which were cloned with *E. coli* were isolated from the bacteria and purified from other macromolecules.

Material

- GenElute™ HP Plasmid Miniprep Kit
- Pipettes with tips (20-200 µl, 100-1000 µl)
- Centrifuge
- *E. coli* liquid cultures
- NanoDrop spectrophotometer

Step 1: Harvest and Lyse Bacteria:

The 5ml of the each of the overnight *E. coli* liquid cultures were pelleted at 12000 x g for 1 min in the centrifuge. The supernatant was then removed, and the cells were resuspended in 200 µl of resuspension solution. Next, 200 µl of lysis buffer was added and the test tubes were inverted 8 times to mix. The solution was allowed to clear for 5 mins. The lysis buffer should destroy the *E. coli* cells, which then release the desired plasmid constructs but also different macromolecules, which have to be removed.

Step 2: Prepare Cleared Lysate

350 µl of neutralisation/binding buffer was added to the test tubes, which were then inverted several times to mix. The cell debris was then pelleted at 12000 x g for 10 mins in the centrifuge. The cleared lysate contains the plasmids, whilst the pellet at the bottom of the test tube contains cell debris.

Step 3: Prepare Column

The DNA was intended to bind to the miniprep binding columns, however these had to be prepared first. The miniprep Binding columns were inserted into the microcentrifuge tubes provided by the miniprep kit. Then, 500 µl of the column preparation solution was added to the miniprep binding column and the microcentrifuge tubes were centrifuged at 12000 x g for 30 s. Finally, the flow-through liquid was discarded

Step 4: Bind Plasmid DNA to Column

The cleared lysate from step 2 was transferred to the prepared miniprep columns and centrifuged at ≥ 12000 x g for 30 s. The flow-through liquid was discarded. The plasmid DNA was now bound to the column, which still had a few contaminants that needed to be washed away.

Step 5: Wash to Remove Contaminants

For the first washing cycle 500 µl of wash solution 1 was added to the binding columns. The columns were then centrifuged at 12000 x g for 30 s and the flow-through liquid was removed. For the second washing cycle 750 µl of wash solution 2 was added to the binding columns, followed up by a centrifugation at 12000 x g for 30 s and the discarding of the flow-through liquid. The columns were then centrifuged for an additional minute at 12000 x g to remove any excess ethanol.

Step 6: Elute Purified Plasmid DNA

After the two washing cycles the binding columns were transferred to fresh collection tubes. To each column 100 µl of elution solution was added, followed by a centrifugation at 12000 3 g for 1 min. The collection tube now contained the desired plasmids, separated from *E. coli* cell debris and other macromolecules.

After these six steps the concentration of the plasmids were measured with the help of a NanoDrop spectrophotometer. 1 µl of each plasmid was pipetted onto the spectrophotometer. Finally, the plasmids were placed into storage in a freezer at -80°C

4.4 Sanger Sequencing of Miniprep

The genetic integrity of the plasmids after miniprep were checked with Sanger sequencing to ensure that the amiRNAs function as intended.

Material:

- Eppendorf tubes
- Pipettes with tips (0.2-2 µl, 2-20 µl)
- Mini-prepped plasmids (pNSE8, pNSE9)
- NSE27 primer
- NSE28_correct primer
- Ice
- Sequencing Buffer (80 mM Tris-HCl, pH 9.0, 2mM $MgCl_2$)
- Deionized sterile water

Design of NSE27 and NSE28_correct Primers:

The two plasmids pNSE8 and pNSE9 were constructed with the same template plasmid called pMDC32 and only differ in which amiRNA they carry. PNSE8 carries amiRNA1 alongside a corresponding amiRNA precursor sequence, whilst pNSE9 carries amiRNA2 also with a corresponding precursor sequence (see table 1, chapter 4.1). The precursor sequences have a length of around 700 bp.

Sanger sequencing generally supports the generation of nucleic acid sequences up to 1000 bp, after which the polymerase separates from the DNA. The central limitations are the quality of the first 40-50 bp read and the readability of longer sequences (anywhere upwards of 800-900 bp). Due to primer binding the sequences of the first few bp are low in quality The sequences with longer segments cannot be read properly as single bp become increasingly difficult to distinguish with increasing length of the sequence in agarose gel.[91] Therefore, the ideal length of a sequence with good data after the Sanger sequence reaction is maximally 900 bp long.

The sequence of interest has a length of 1263 bp, which is comprised of the EC1.1 promoter measuring 562 bp and the amiRNA precursor sequence with 701 bp. To circumvent the limitation of Sanger sequencing, the T-DNA was sequenced from both directions. As there is an overlap between the sequences generated by both primers, a sequence with good quality reads can be gained with more than 1000 bp (see table 2). NSE27 was used to sequence in the forward direction flanking the promoter, whilst NSE28_correct sequenced in the opposite direction flanking the amiRNA precursor sequence (see figure 15 and figure 16). The initial NSE28 primer was mistakenly designed in such a way that it sequenced in the same direction as NSE27, which would not have yielded a useful sequence. Therefore, the NSE28_correct primer was used, which sequenced in the opposite direction of NSE27.

	NSE27 total bp	NSE28_correct total bp	Theoretical overlap bp	NSE27 good bp	NSE28_correct good bp	overlap good bp
pNSE8	1129	1015	595	800	800	51
pNSE9	1095	1082	628	800	800	51

Table 2: Lengths and overlap of the sequences of pNSE8 and pNSE9 for the Sanger sequencing reactions. In all four Sanger sequencing reactions 1000-1100 bp were generated. There is a theoretical overlap between the sequences generated by both primers for pNSE8 and pNSE9 respectively, which however does not mirror the true length of the overlap sequence, which is usable due to the limitations of Sanger sequencing. Visual inspection of the chromatograms of the sequencing results revealed that for each sequence about 800 bp were of good quality. Therefore, there is an overlap sequence measuring 51 bp for both plasmids. These sequences could be aligned with template sequences to check if the T-DNA on the plasmids was cloned correctly.

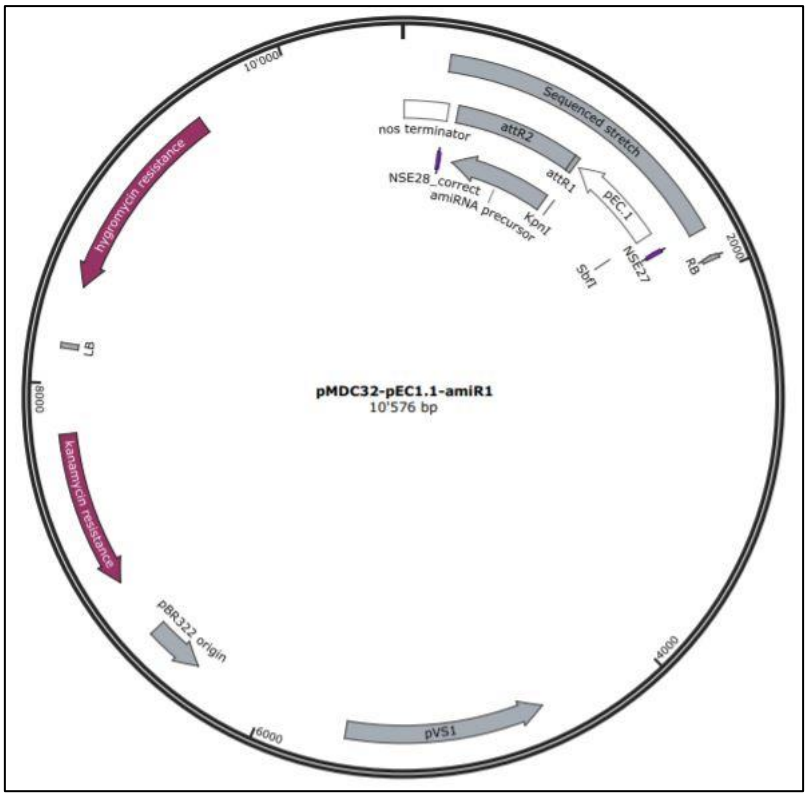

Figure 15: The vector map of the pMDC32-pEC1.1-amiRNA1 (pNSE8), including primers. The vector map with amiRNA2 (pNSE9) is not shown as it only differs in the sequence of the amiRNA/amiRNA*. The sequenced stretch is labelled. The NSE27 primer flanks the EC1.1 promoter and sequences in the forward direction (anticlockwise in the map). The NSE28_correct primer flanks the amiRNA precursor and sequences in the opposite direction (clockwise in the map). LB; left border, RB; right border.

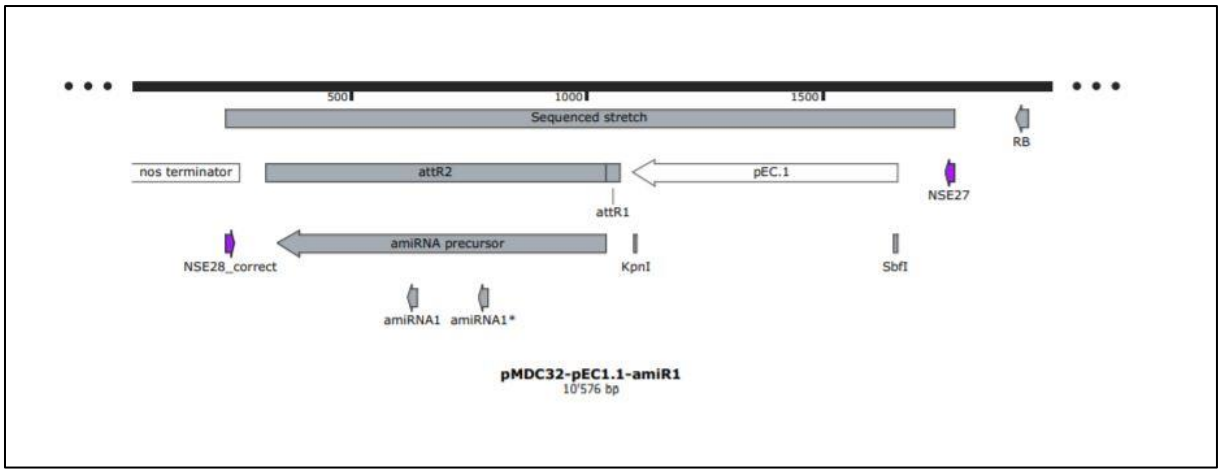

Figure 16: Schematic representation of the sequenced stretch of the pNSE8 vector map. Compare with figure 15.

The pNSE8 and pNSE9 plasmids had concentrations of 351.6 ng/µl and 309.6 ng/µl respectively. 1.5 µl of each plasmid were to be separately added to 4 Eppendorf tubes, corresponding to the two primer reactions per plasmid (forward direction and backward direction. The optimal concentration of the plasmids for sequencing was between 67 ng/µl and 120 ng/µl. Thus, the plasmids were diluted with deionized sterile water in a 1:4 ratio. The resulting concentration of pNSE8 was 87.75 ng/µl and the concentration of pNSE9 was 77.25 ng/µl. Next four Eppendorf tubes were labelled in the following way:

Sus1: 1.5 µl pNSE8 + 0.5 µl NSE27
Sus2: 1.5 µl pNSE8 + 0.5 µl NSE28_correct
Sus3: 1.5 µl pNSE9 + 0.5 µl NSE27
Sus4: 1.5 µl pNSE9 + 0.5 µl NSE28_correct

The corresponding voluminal were pipetted into the tubes, resulting in 4 different samples to be sequenced. The 4 tubes were left on ice before sequencing could start. Before Sanger sequencing could begin 0.8 µl of dye 2 µl of sequencing buffer and 5.2 µl of deionized sterile water was added to each tube such that the final volume in each tube was 10 µl. The plasmids were then sequenced in-house by the members of the Department of Plant and Microbial Biology of the University of Zurich.

4.5 Insertion of pNSE8 and pNSE9 via Electroporation into *A. tumefaciens*

A. tumefaciens cells were transformed with pNSE8 and pNSE9 for transformation mediation of the plasmids into *A. thaliana* plants.

Material:

- Eppendorf tubes
- Competent GV3101 *A. tumefaciens*
- Plasmids of interest pNSE8 (Kan), pNSS9 (Kan)
- Pipettes with tips (2-20 µl, 20-200 µl, 100-1000 µl)
- LB/kanamycin (kan) plates
- Ice
- Deionized sterile water
- Electroporation cuvette
- Heat block
- Electroporator
- LB solution (liquid broth solution: 10 g Bacto-Tryptone, 5g Bacto Yeast Extract, 10g NaCl, 950 ml of deionized water)
- Gentamicin (gen)
- Rifampicin (rif)
- Kanamycin (kan)
- Laminar flow hood

Step 1: Preparation of Electroporation Cuvettes on Ice

Both the plasmids and the competent GV3101 *A. tumefaciens* strain were taken out of the -80°C freezer and placed on ice. In addition, two electroporation cuvettes, which are made of glass and had two conducting metal plates on the side through which the current could flow in the electroporator were placed on ice. The plasmids were then diluted in a ratio of 1:100 µl with deionized sterile water. Next, 2 µl of the diluted plasmids were mixed with 50 µl of GV3101 *A. tumefaciens* in two separate test tubes. Both mixtures were then pipetted into the central cavities of the electroporation cuvettes, such that the mixtures ran the entire length of the canal. To help with this the cuvettes were tapped on the table surface for the liquid to redistribute in between gaps. The cuvettes were then sealed with a plastic cap.

Step 2: Electroporation

Before placing the cuvettes into the electroporator any condensate water was wiped off from the sides of the cuvette for ideal conduction. A cuvette was then placed in the designated slot in the electroporator such that the electrodes were touching the metal plates on both sides of the cuvette. The bacteria were then electroporated at 2500 V, which helped facilitate the incorporation of the respective plasmid into the bacteria. This was done with both cuvettes.

Step 3: Preparation of *A. tumefaciens* LB/kan plate cultures

The electroporated cuvettes were taken out of the electroporator and incubated in a heat block at 28°C for 1 h, which helps stabilise the bacteria after undergoing immense stress caused by electric shock. Next at the laminar flow hood, 50 µl of each of the electroporated mixtures was plated onto LB/kan plates for selection. The LB/kan plates were then left to incubate at 28°C for 48 h, the time period necessary for colonies to form.

Step 4: Preparation of Starter Liquid Cultures

After the 48-h incubation period, individual colonies of the transformed *A. tumefaciens* were taken from the LB/kan plates and transferred into test tubes with the help of pipette tips. The cultures were then inoculated in 5 ml of LB solution. Finally, three different antibiotics were added. Rifampicin selected for *Agrobacterium* genomic DNA to ensure only the correct strain (GV3101) was growing, gentamicin to ensure the presence of the correct helper plasmid, and kanamycin to confirm that the transformed the transformed plasmids pNSE8/pNSE9 were present. Gentamicin was added at a concentration of 30 µg/ml, whilst rifampicin and kanamycin were added at a concentration of 50 µg/ml.

4.6 Floral Dip of *A. thaliana*

The inflorescences of *A. thaliana* plants were dipped into the *A. tumefaciens* solution to transform seeds with the desired constructs. These seeds can later by selected on herbicide resistance plates as transformants integrated the T-DNA, which holds an herbicide resistance gene (resistance to hygromycin), into their genome.

Material:

- Centrifuge
- 6-week-old Col-0 *A. thaliana* plants
- Starter liquid cultures
- Scale
- Measuring cup
- Bottles for centrifugation
- Deionized sterile water
- 1 l flask
- Sucrose
- Silwet L-77
- Pipette with tips (100-1000 µl)
- LB solution (liquid broth solution: 10 g Bacto-Tryptone, 5g Bacto Yeast Extract, 10g NaCl, 950 ml of deionized water)
- Erlenmeyer flasks (500 ml)

Step 1: Preparation of Large Liquid Cultures for Transformation

1 ml of each of the starter liquid cultures (see point 4 in chapter 4.5) was extracted and inoculated in 200 ml of LB solution in an Erlenmeyer flask, after which it is termed the final liquid culture.

The final liquid cultures were set in an incubation shaker at 37°C for 24 h, which allowed the transformed *A. tumefaciens* bacteria to proliferate.

Step 2: Preparation of Dipping Solution

Next, the final liquid culture was transferred into plastic centrifugation bottles and centrifuged at 4000 rpm for 20 mins at roughly 20°C. This was done to separate the bacteria from the LB solution, which was subsequently poured away. The pellet of *A. tumefaciens* was resuspended with 600 ml of 5% sucrose water solution (30 g for 600 ml), which also contained 0.03% Silwet L-77 (200 µl for 600 ml), a surfactant which helps the *A. tumefaciens* cells adhere to the inflorescence of the *A. thaliana* plants by lowering the surface tension of the solution. Usually, 0.05% (300 µl for 600ml) is used, however a lower concentration was used. Silwet L-77 can damage the plant if it is too highly concentrated. The solution is now termed the dipping solution.

Step 3: Floral Dip

Before dipping the *A. thaliana* plants in the dipping solution, the siliques were cut as they contained wild-type seeds. The inflorescences were the desired part of the plant to be transformed, as these would produce new seeds, which are potentially transgenic. Whilst dipping the plants, it was important to ensure that the inflorescences were completely submerged.

Step 4: Drying plants

After dipping, the plants were placed under a cover for 24 h to maintain high humidity. After that the plants were watered normally until the seeds started to mature. The plants were then left to dry with paper bags tied around them to catch all the siliques before the seeds could be harvested for selection.

It is important to note that the methods described in chapters 4.2, 4.3, 4.5 and 4.6 were conducted twice on the same plants. The *A. thaliana* plants were dipped twice. This enhances the transformation efficiency and results in a greater yield of transgenic seeds.

4.7 Selection of Seeds

Transformed seeds were selected on Murashige and Skoog medium (MS) plates containing hygromycin. Around three weeks later, transformant plants were then transferred onto soil for further experimentation.

Material:

- Dried *A. thaliana* plants
- Sieves
- Precision scale
- Paper
- Test tubes (20ml)
- 70% Ethanol
- 100% Ethanol
- Vortex machine
- Filters
- Pipette with tips (100-1000 µl)
- Col-0 *A. thaliana* seeds

- MS/hygromycin (hyg) plates
- Aluminium foil
- Laminar flow hood
- Round cellulose filters

The dried *A. thaliana* plants were taken out of their drying bags and placed onto a sheet of paper. In addition, the contents of the bags were emptied onto the paper. It is important to note that the seeds were harvested separately from each other, and plants transformed with different plasmids were not mixed. With two sieves the seeds were filtered from the siliques and other big parts of the plants like leaves and stalks, which were not desired. Next, the mass of both batches of seeds was measured with a precision scale, with which the number of total seeds could be determined in an acceptable error range. Subsequently, the seeds were put into test tubes with 70% Ethanol and then vortexed for roughly 2 mins. In parallel a control group with unaltered Columbia seeds was also prepared, which is vital to check if the antibiotics have the desired effect of eliminating all non-transformants. The 70% Ethanol was emptied from all three test tubes and 100% Ethanol was added. The liquid was poured out, and 100% Ethanol was added a second time to wash away the 70% Ethanol as best as possible. In the laminar flow hood the seeds were placed on round cellulose filters and left to dry. After the filters had dried, the transgenic seeds were distributed onto MS/hyg plates, whilst the control was distributed onto a normal MS plate with no hygromycin. As a gene on the integrated T-DNA encodes for a hygromycin resistance the transformants can be selected from the non-transformants. The plates were wrapped in aluminium foil to prevent light exposure. This helps with stratification, the process of breaking seed dormancy and initiating germination. The wrapped plates were then placed in a fridge at 4°C for 60 h. After the 60-h stratification period the plants were unwrapped and stored at room temperature for 12 h under sufficient light exposure. Next, the plants were wrapped in aluminium again for 24 h. The sudden exposure to light and the subsequent period spent in darkness, helps speed up the growth process by forcing the plants to undergo etiolation, rapid plant growth in the absence of light. Hygromycin does not immediately kill seedlings, but severely stunts their development. The transformed seedlings are resistant to this stunting and are easily distinguishable from arrested seedlings. After roughly three weeks, 24 pNSE8 transformants and 24 pNSE9 transformants were transferred onto soil.

4.8 DNA Extraction

DNA of the 48 plants was extracted for subsequent PCR to check if the plants, which had survived the herbicide selection did indeed carry the T-DNA. For PCR to work smoothly, the DNA had to be isolated from all other macromolecules, which required a handful of washing processes.

Material:

- 24 pNSE8 plants (3 weeks old)
- 24 pNSE9 plants (3 weeks old)
- Labels
- Pen
- Cluster tubes with caps
- Cluster tube tray
- Glass beads
- Ice box
- Forceps
- Liquid nitrogen
- Metal plates for Geno Grinder
- Geno Grinder
- Proteinase K
- RNase A (100mg/ml)
- 2% CTAB buffer
- Pipettes with tips (2-20 µl, 20-200 µl, 100-1000 µl)
- 50% EtOH
- Multipipette (8 tubes at once) with tips
- Multipipette (1 big tube, which holds a lot of volume and has electronic dispensing mechanism) with tips
- Thermomixer
- Plates with 96 wells
- Centrifuge
- Cell lysis buffer
- Reliaprep resin
- Isopropanol
- Spacer rack
- Magnetic separation device, including holder and comb
- Elution plate
- Sterile water
- NanoDrop spectrophotometer

MM1 (Master Mix 1):

- 2% CTAB (cetyltrimethylammonium bromide) Buffer: Detergent used for extraction and isolation of DNA from other macromolecules (7.05ml)
- Proteinase K: destroys DNase in cytoplasm, which could destroy DNA in nucleus since cytoplasmic and nuclear material will come in contact after grinding (300ul)
- RNase A: destroys RNA, since RNA could also be extracted as it is also comprised of nucleotides. However only the DNA is desired. (6ul)

MM2:

- Cell lysis buffer: Breaks open cells for DNA extraction. Dissolves phospholipids of the cell membrane. (2.75ml)
- Reliaprep resin: Magnetic beads to which the DNA will bind (275ul)
- Isopropanol: To precipitate DNA, meaning aggregation of DNA (5.45 ml)

MM3 (Wash Buffer 1):

- 50% EtOH: Ethanol solution for washing impurities(1.25ml)
- Wash Buffer: An additional Ethanol solution also for washing impurities (9.75ml)

Wash Buffer 2:

- 50% EtOH

Step 1: Preparation of Plant Samples

In the first step all plants were labelled with two numbers, the first corresponding to the plasmid and the second number to the order of the plants in their respective trays, which each held 24 plants (e.g., 9_1 or 8_24). This was done to keep a better oversight of all plants. Next, 48 cluster tubes were placed in a cluster tube tray and to each tube roughly six to seven small glass beads were added, which is indispensable for grinding the plant material. The tray was then placed on ice. Each tube corresponded to a plant, thus a leaf sample was taken from each plant and added to the respective tubes. These samples contain the DNA, which was to be extracted. The tubes were then covered with caps to ensure the plant samples are contained during the grinding process. Next, the entire tray with the 48 cluster tubes containing the plant samples were placed in liquid nitrogen to freeze the tissue. The grinding process works best when the tissue is brittle. Furthermore, freezing prevents the degradation of DNA as it no longer resides in the nucleus and is exposed to the cell's DNases. Two metal plates, which enclose the tray in the Geno Grinder, a machine which oscillates at high frequencies and facilitates the grinding process, were also placed in liquid nitrogen. Their function is to keep the tray cool during the grinding process and to avoid an explosion in the machine due to thermal expansion. The frozen tray along with the two metal plates and a counterweight tray were fitted into the Geno Grinder and the samples were shaken at 1750 rpm for 1 min.

Step 2: DNA Extraction

50xMM1 (50 portions of Master Mix 1) was mixed and 150 µl was added to each sample. The two extra portions were mixed to circumvent pipette inaccuracy and to ensure that the 48 samples could be processed. This holds true for all other subsequent master mixes. The samples were then placed in a thermomixer for 3 mins at 40°C, taken out and shaken by hand such that the plant material resided at the bottom of the tubes, this was to prevent leakage when adding subsequent master mixes. Then, the samples were incubated at 70°C in the thermomixer for 30 mins. Higher temperatures allow the enzymes to carry out their functions more efficiently. Next 50xMM2 was prepared and 150 µl was distributed with a multipipette into each well of a new plate. The sample plate (with MM1) was taken out of the thermomixer, spun down at 1000 rpm for 20 mins in a centrifuge and 100 µl of the supernatant of each sample was added to a well of the plate containing MM2. During centrifugation the heavier molecules get spun down to the bottom of the tubes, whilst the DNA remains more at the top in the supernatant. When transferring the supernatant, the liquid was pipetted up and down several times to mix MM2 with the genetic material.

Subsequently the plate containing MM2 was shaken at 1200 rpm at room temperature, which allowed the DNA to bind to the resin beads. Next 50xMM3 was prepared and 220 µl was pipetted into each well of a new plate. To transfer the DNA from the MM2 plate a magnetic separation device was used, which consists of an assortment of long tubes with magnets at their tips and a plastic comb enclosing these tubes to prevent the sample from coming in contact with the device. The magnetic separation device plus the shielding comb were placed into the MM2 rack and the lysate was left to clear. Cleared lysate indicates that the magnetic resin beads to which the DNA binds are bound to the magnetic holder and can thus be moved. The magnetic holder and comb could then be transferred to the MM3 plate. The magnetic holder was released, and the comb was moved up and down several times to mix the MM3 with the DNA. After a couple of minutes, the magnetic holder was placed again into the MM3 plate and the wash buffer was allowed to clear for 2 mins, allowing the beads to bind to the magnetic holder. Next, to complete the washing process, 50X Wash Buffer 2 was prepared and 200 µl was pipetted into each well of a new plate. The magnetic holder was transferred into this new plate and then released. The comb was moved up and down several time to wash the beads. The solution was left to rest for several minutes. Next, the magnetic holder was placed into the comb in the plate containing Wash Buffer 2 and the liquid was left to clear for 2 mins. The magnetic holder and comb were then moved to the drying station (holder) for the ethanol to evaporate. Whilst the samples were left to dry, 60 µl of water preheated at 75°C was distributed onto an elution plate. The magnetic holder and comb were moved to the elution plate after the samples had dried and the magnetic holder was released. The comb was once again moved up and down and then shaken at 400 rpm at room temperature for 15 mins. This transfer to the elution plate is meant to separate the DNA from the resin beads. The resin beads were then removed from the elution plate with the magnetic holder and comb. Next, 1 µl of each sample was placed onto a spectrophotometer to measure the nucleotide concentration of each sample. Finally, the eluted DNA was transferred to a new plate via pipetting for storage in the fridge at 4°C.

4.9 Genotyping PCR and Electrophoresis

A region of the amiRNA precursor sequence measuring roughly 500 bp, which also includes the amiRNA, was amplified in both plasmids. The amplification of the region would only work if the plant had the T-DNA as the primers bind to sequences found on the T-DNA. This was intended as a secondary control to check if the herbicide selected plants were indeed transgenic.

Material:

- DNA extracted from pNSE8, pNSE9 plants
- 5x GoTaq Buffer (green dye, which separates into blue and yellow bands, 7.5 mM $MgCl_2$)
- dNTPs (deoxyribonucleotide triphosphates, 2.5 mM)
- NSE28_correct primer (19.2 nM)
- NSE Rev primer (25.9 nM)
- Sample shaker
- Taq polymerase
- Sterile water
- pNSE8 plasmid
- pNSE9 plasmid
- Eppendorf tubes
- Cluster tubes with caps
- pipettes with tips (0.2-2 µl, 2-20 µl)
- Multipipette (8 tubes at once) with tips
- Multipipette (1 big tube, which holds a lot of volume and has an electronic dispensing mechanism) with tips
- Centrifuge
- Thermocycler (BIO-RAD C1000 Touch™ Thermal Cycler)
- Agarose
- 1x TBE Buffer (12.1g Tris base, 6.2g boric acid, 0.74g disodium salt, 1L water)
- 20x gel electrophoresis combs
- Midori Green Xtra colouring
- GeneRuler 1kb DNA ladder
- Electrophoresis box
- Anode, cathode, and voltage supply
- Gel electrophoresis imaging device

PCR Master mix:

- 5 µl 5x GoTaq Buffer
- 1 µl dNTPs (2.5 mM)
- 1 µl NSE28_correct primer (10 µM)
- 1 µl NSE Rev (10 µM)
- 0.05 µl Taq polymerase
- 10.95 µl sterile water

Step 1: Preparation of PCR Master Mix:

Both the NSE28_correct and NSE Rev (reverse) primers were diluted to 100 µM and then shaken for 15 mins to resuspend the pellets in the liquid. After resuspension the 10 µl of each plasmid was added to a respective Eppendorf tube along with 90 µl, such that they were diluted in a ratio 1:10, thus having a molarity of 10 µM. As there was one sample for each plant (48 DNA samples for 48 plants) along with one negative control (water) and 2 positive controls (pNSE8 plasmid and pNSE9 plasmid) 55 portions of the PCR master mix were prepared. The additional four portions were prepared to circumvent pipette inaccuracy. The master mix was distributed à 19 µl into 51 cluster tubes with a digital multipipette. Next, with an analogue 8-tube multipipette 6 µl of each of the 48 DNA sample was pipetted into its own cluster tube. For the control samples 6 µl of water was added to each of the three tubes. To the positive controls, 0.5 µl of pNSE8 and pNSE9 respectively were added in addition. Less volume is required for the plasmids as the sequence of interest is more highly concentrated than in the actual DNA samples, which contain copies of the entire *A. thaliana* genome.

Step 2: PCR:

The cluster tubes were closed off with caps and spun down in a centrifuge, such that all the liquid was set at the bottom of the tubes. The cluster tubes were then placed in a thermocycler, a device which can heat up at pre-set temperatures for specific durations of time in cycles, creating the ideal conditions for the polymerase chain reaction. The following cycle protocol was used:

1. 3 mins 30 s at 95°C (denaturation)
2. 30 s at 55°C (annealing)
3. 45 s at 72°C (DNA synthesis)

The cycle was repeated 34 times, after which the thermocycler would remain at 72°C for 5 mins, followed by an indefinitely long phase at 12°C until the samples were collected. The thermocycling lasted roughly 90 mins.

Step 3: Preparation of Electrophoresis Gel:

As there were 51 samples, two electrophoresis gels had to be prepared as one can only hold 40 samples divided into two rows of 20 wells. To prepare each gel 1 g of agarose was added to 100 ml of 1xTBE buffer. Next, 6 µl of Midori Green Xtra colouring was added to the agarose solution, which colours the DNA, which is necessary for the imaging system to capture the DNA bands. Subsequently, the solution was microwaved until the agarose was completely dissolved, meaning that the liquid was clear. The liquid was poured into electrophoresis mould with 20x electrophoresis gel moulds, which created wells in the gel for the DNA samples. The moulds were set after roughly 30 mins.

Step 4: Electrophoresis

After the thermocycler had gone through all the cycles and the electrophoresis gel was set, electrophoresis could be conducted on the DNA samples. The gels were placed into electrophoresis boxes, which were connected to an anode and a cathode. The potential difference between them creates a voltage across which the negatively charged DNA can flow through in the gel. Next the 51 samples alongside DNA ladders were loaded onto the two gels. The voltage supplier connected to the electrodes was set at 100 V and 0.06 A. After roughly 45 mins the DNA samples had flown through the gels, and they were taken out of the electrophoresis boxes to be placed in an imaging device. The device captured the DNA bands.

4.10 Seed Set Analysis

To see if transformed plants showed a defect in seed development the siliques were screened for non-wild-type phenotypes under a dissecting microscope.

Material:

- dissecting microscope
- forceps
- syringe with tips
- 15 transgenic *A. thaliana* plants (confirmed with PCR and electrophoresis)
- double-sided tape

With the help of PCR and gel electrophoresis 15 plants were identified to be successfully transformed with the pNSE8 and pNSE9 constructs respectively. From each of these plants a silique from the main inflorescence, the tallest shoot, was removed with forceps and cut open with the sharp tip of a syringe. The siliques were screened under a dissecting microscope for high frequency of seed set abortion, which causes the seeds to not develop and remain as small shrivelled white embryo sacs. If a distinct phenotype was observed, an additional four siliques of the same plant were dissected and screened. In such siliques, the number of wild-type seeds, which underwent normal development, and the number of underdeveloped seeds was counted.

4.11 Gametophyte Development Analysis

From the preliminary seed set screening of the 15 plants, plant 9_18 was the sole transformation line identified to show seed set defect. The goal of gametophyte development analysis was to look for potential defects in mature unfertilised female gametophytes. There might be other causes for seed set defect apart from the amiRNA, e.g., chromosomal translocation induced by T-DNA insertion. The differentiation between the different possible causes was not possible during seed set analysis and therefore gametophyte development analysis was necessary.

Material:

- forceps
- Transformed *A. thaliana* plant of interest (9_18)
- *A. thaliana* plant from same line with wild-type phenotype (9_1is the control)
- Acetic acid
- 100% Ethanol
- 70% Ethanol
- Chloral hydrate solution (24:9:3, chloral hydrate: water: glycerol)
- Glass slide
- Cover glass
- Dissecting microscope
- Differential interference contrast (DIC) microscope
- Water
- Pipette with tips (2-20 µl, 20-200 µl)

The pollen grains of the 9_18 plant as well as of the 9_1 plant were screened under a dissecting microscope. With forceps the anthers were removed from the flower to prevent fertilisation of the egg cells. This process is termed emasculation. The emasculated inflorescences were placed in a 1:9 acetic acid ethanol mixture for 4 h at 23°C. Next, the inflorescences were transferred to a 70% ethanol solution and were left in it overnight (12 h). Following that the inflorescences were transferred to chloral hydrate solution and were also left in it overnight. These three steps are vital for clearing the inflorescences such that they are translucent, which makes the analysis under the DIC microscope easier. Finally, the pistils containing the ovules were dissected under a microscope to help isolate individual female gametophytes and then analysed under a DIC microscope.

5 Results

5.1 Results of Miniprep

These NanoDrop results (see table 3) show the concentrations and the purity of pNSE8 and pNSE9 after they were mini-prepped out of *E. coli* (see chapter 4.3 for procedure). The concentration has to be looked at in respect to the total volume of the samples. Usually, a yield upwards of 5 µg is sufficient to continue with the transformation of *A. tumefaciens*. For both samples there were 100 µl, therefore a yield of roughly 30 µg or 35 µg respectively was achieved. The absorbance measurement made on the spectrophotometer includes the absorbance of all molecules in the sample that absorb at the wavelength of interest. The 260/280 ratio is used to assess the purity of DNA in the sample and a ratio of ~1.8 is the accepted value for "pure" DNA. Changes in the acidity of the sample can alter the ratio by 0.2-0.3, therefore the purity of the samples are in the acceptable range. The 260/230 ratio is a secondary measure of nucleic acid purity. A value between 2.0 and 2.2 indicates a pure sample.[q] The plasmids were adequately highly concentrated with very little contaminants, confirming that the miniprep of the *E. coli* was successful.

Results Mini Prep					
Sample ID	ng/ul	A260	A280	260/280	260/230
pNSE8	351.6	7.032	3.599	1.95	2.20
pNSE9	309.6	6.192	3.169	1.95	2.20

Table 3: The results of the NanoDrop spectrophotometer of the mini-prepped plasmids.

5.2 Results of Sanger Sequencing of Miniprep

The reverse complement of the sequence, which resulted from Sanger sequencing, was compared with the template sequence of the pNSE8 and pNSE9 plasmids respectively to check if the amplification of the plasmids through *E. coli* cloning was done correctly. Specifically, the sequences encoding for the amiRNA1 and amiRNA2 were checked respectively to see if they matched up with the templates as those were crucial for the transformation experiment (see table 1, chapter 4.1). Note that these amiRNA sequences in table 16 and 17 are reverse complement to the amiRNAs shown in table 1, as their products should end up being the amiRNAs designed (display 3' to 5' from left to right). AmiRNA1 is located at bp 621 and 641 of pNSE8 and amiRNA2 is located between bp 622 and 642 of pNSE9. These sequences aligned well, meaning that the plasmids were cloned correctly and could be transformed into *A. tumefaciens* for further experiments (see Figure 17 and 18). The quality of the sequence towards the beginning and end are not that high, evident by the frequent misalignments (see figures 28 and 29 in appendix A for complete alignment).

```
pNSE8_template      caagaaaattggaatacaaaagagagaacgtcggcctcaccgtaagcatcaaagagaatc
pNSE8_SangerS       CAAGAAAATTGGAATACAAAAGAGAGAACGTCGGCCTCACCGTAAGCATCAAAGAGAATC
amiR1               ----------------------------ACGTCGGCCTCACCGTAAGCA------------
                                                 ********************
```

Figure 17: pNSE8 template sequence compared with sequence generated by Sanger sequence reaction. The top line is the template sequence of the plasmid. The second line is the reverse complement sequence to the sequenced sequence. The third row is the complement sequence to amiRNA1. The amiRNA1 sequence is marked in green. An asterisk stands for a correct alignment between the three sequences.

```
pNSE9_template      caagaaaattggaatacaaaagagagagcgcctaacattggtttaacatcaaagagaatc
pNSE9_SangerS       CAAGAAAATTGGAATACAAAAGAGAGAGCGCCTAACATTGGTTTAACATCAAAGAGAATC
amiR2               ----------------------------GCGCCTAACATTGGTTTAACA------------
                                                 ********************
```

Figure 18: pNSE9 template compared with sequence generated by Sanger sequence reaction. The top line is the template sequence of the plasmid. The second line is the reverse complement sequence to the sequenced sequence. The third row is the complement sequence to amiRNA2. The amiRNA2 sequence is marked in green. An asterisk stands for a correct alignment between the three sequences.

5.3 Results of Selection of Seeds

After the drying process the T0 *A. thaliana* plants, which were dipped twice, yielded a lot of seeds as expected. The mass of the seeds was measured to gain an estimate of the number of seeds (see table 4). To roughly deduce how many seeds each plant generated, the average mass of a single seed was estimated at (19.5 ± 2.5) µg, adapted from Jofuku *et al*.[92] The number of pNSE8 seeds was calculated to be 7882 ± 1010, whilst the number of pNSE9 seeds was 16312 ± 2091. The seeds were plated on hygromycin plates to select for transformants. The surviving plants could be categorized into two different phenotypes, which differ amongst each other but are distinct from the other plants, which were stunted in growth. One set of the surviving plants were considerably smaller than the other set of plants and sometimes had a slight form of discolouration, indicating that developmentally the surviving plants were in different stages. These somewhat "delayed plants" were discernible from their healthy counterparts, which had grown to a considerable size, but all of them survived the selection process (see figure 19). In figure 20 all transformation lines are shown immediately after the transfer onto soil and 19 days later. Some developmental variation can be observed. The developmental discrepancy becomes clearly visible in figure 21, which shows two transformation lines of each plasmid. Some of the transformation lines had clearly defined primary and secondary inflorescences, whilst other transformation lines lacked a primary inflorescence. The transformation rate of pNSE8 was calculated to be 0.61% ± 0.08% and that of pNSE9 was calculated to be 0.46% ± 0.06% (see table 4). Both transformation rates are below average. According to Zhang *et al*. a minimal transformation rate of one percent is to be expected.[72] The wild-type Col-0 seeds plated on a normal MS plate, which acted as a control for the effects of hygromycin, grew normally without any stunting as expected.

Results Selection of Seeds					
Sample ID	mass of seeds [g]	est. # seeds	# healthy transformants	total # transformants	transformation rate
pNSE8	0.1537	7882 ± 1010	29	48	0.61%±0.08%
pNSE9	0.3181	16312 ± 2091	45	75	0.46%±0.06%

Table 4: The results of the selection of seeds. The number of the seeds was calculated by adopting the mass values of Columbia wild-type seeds determined by Jofuku *et al*.[92] For the estimate of the number of seed, the average mass of a single seed was estimated at (19.5±2.5) µg. The deviation for the estimation of number of seeds and the transformation rates are proportional to the deviation in the mass value of a single seed. Est; estimated, #; number of.

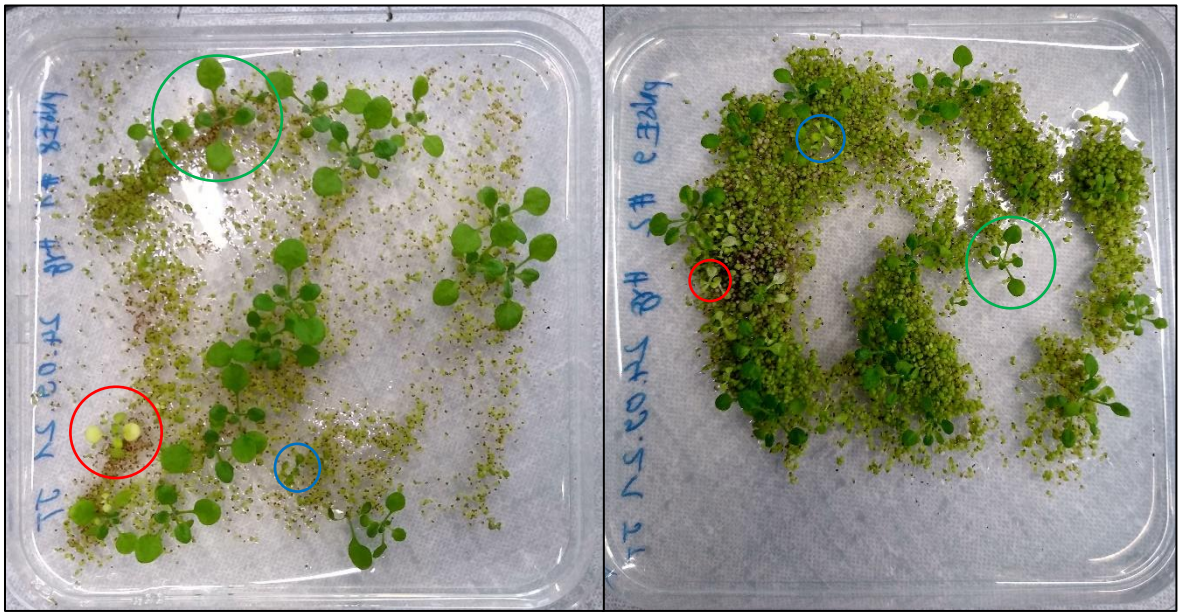

Figure 19: (Left) Picture of one of the two selection plates containing the plants transformed with pNSE8 19 days after they were plated. (Right) Picture of one of the two selection plates containing the plants transformed with pNSE9 19 days after they were plated. Circled in red are examples of what a discoloured transformant looks like. Circled in blue are examples of transformants, which are slightly stunted in growth. Circled in green are examples of transformants considered to be healthy as they show considerable growth.

Figure 20: (Top left) Picture of 24 pNSE8 plants transferred from selection plate onto soil (Day 0). (Bottom left) Picture of the same 24 pNSE8 plants 19 Days after the transfer onto soil. (Top right) Picture of 24 pNSE9 plants transferred from selection plate onto soil (Day 0). (Bottom right) Picture of the same 24 pNSE9 plants 19 Days after the transfer onto soil.

Figure 21: (Left) Both plants were transformed with pNSE8, however the plant on the right is in a later developmental stage, the primary inflorescence alongside the two secondary inflorescences are clearly developed. The main inflorescence of the plant on the left has not grown yet. These two plants are representative of the varying growth stages of the pNSE8 plants. (Right) Similarly to the pNSE8 plants, the pNSE9 plants vary in growth stages. The plant on the left is severally stunted in growth compared to the plant on the right. The plant on the right shows the distinct primary inflorescence alongside the two secondary inflorescences, whilst the main inflorescence of the plant on the left has just barely started growing.

5.4 Results of DNA Extraction

The 48 T1 *A. thaliana* plants that were transferred on to soil were left to grow for three weeks, after which a leaf of each plant was taken for DNA extraction (see chapter 4.7). The concentrations of the DNA samples were measured with a NanoDrop spectrophotometer and showed a varying degree of success of the DNA extraction (see table 5). DNA concentration under 30 ng/µl is considered to be poor in quality and everything above is in the acceptable range. The value of the 260/280 ratio measures nucleotide purity and lies at ~ 1.8 for pure DNA. The value of the 260/230 ratio is a secondary measure for nucleotide purity and lies between 2.0 and 2.2 for pure DNA.[q]

Sample ID	Conc.[ng/µl]	A260	A280	260/280	260/230
8_1	8.654	0.173	0.105	1.65	0.83
8_2	35.23	0.705	0.343	2.06	1.51
8_3	77.1	1.542	0.741	2.08	1.84
8_4	13.02	0.26	0.148	1.75	0.97
8_5	61.06	1.221	0.667	1.83	0.93
8_6	151.7	3.033	1.415	2.14	2.02
8_7	11.66	0.233	0.144	1.62	0.86
8_8	34.67	0.693	0.355	1.95	1.6
8_9	78.29	1.566	0.776	2.02	1.96
8_10	12.57	0.251	0.13	1.94	0.72
8_11	8.499	0.17	0.097	1.76	0.65
8_12	74.01	1.48	0.719	2.06	1.45
8_13	15.25	0.305	0.185	1.65	0.88
8_14	24.78	0.496	0.284	1.74	0.98
8_15	63.1	1.262	0.614	2.06	1.81
8_16	21.91	0.438	0.236	1.85	1.01
8_17	21.83	0.437	0.231	1.89	1.15
8_18	150.9	3.019	1.401	2.16	2.03
8_19	14.14	0.283	0.194	1.46	0.77
8_20	17.63	0.353	0.219	1.61	1.08
8_21	159.6	3.192	1.534	2.08	2.06
8_22	7.678	0.154	0.115	1.33	0.64
8_23	20.08	0.402	0.242	1.66	1.02
8_24	180.5	3.61	1.712	2.11	1.94

Sample ID	Conc.[ng/µl]	A260	A280	260/280	260/230
9_1	69.56	1.391	0.667	2.09	1.91
9_2	109.9	2.199	1.01	2.18	2.17
9_3	141.4	2.828	1.354	2.09	1.88
9_4	109.8	2.196	1.037	2.12	1.99
9_5	98.38	1.968	0.95	2.07	1.95
9_6	77.74	1.555	0.749	2.08	1.92
9_7	112.9	2.258	1.095	2.06	2.07
9_8	181.6	3.632	1.728	2.1	2.09
9_9	59.67	1.193	0.655	1.82	0.9
9_10	111.8	2.236	1.046	2.14	1.97
9_11	136.6	2.733	1.261	2.17	2.08
9_12	37.2	0.744	0.346	2.15	1.68
9_13	154	3.081	1.456	2.12	2.05
9_14	81.43	1.629	0.815	2	1.49
9_15	98.84	1.977	0.947	2.09	2.07
9_16	209.8	4.195	1.923	2.18	2.13
9_17	7.196	0.144	0.085	1.69	0.67
9_18	203	4.059	1.875	2.16	2.14
9_19	175.2	3.505	1.681	2.09	2.06
9_20	100.7	2.014	1.01	1.99	1.98
9_21	181.1	3.621	1.728	2.1	2.19
9_22	9.256	0.185	0.133	1.39	0.52
9_23	117.2	2.344	1.145	2.05	1.97
9_24	8.824	0.176	0.117	1.51	0.61

Table 5: (Left) The DNA samples of the 24 pNSE8 plants are listed, with their corresponding concentrations in ng/µl and various absorption values at various wave lengths. (Right) Ditto, apart from the samples being listed, coming from the 24 pNSE9 plants. DNA concentration under 30 ng/µl is considered to be poor in quality and everything above is in the acceptable range.

5.5 Results of PCR and Electrophoresis

The PCR and subsequent electrophoresis of the DNA samples of the 48 *A. thaliana* plants showed that at least 15 plants have been successfully transformed, indicated by a band at around 500 bp (see figures 22 and 23). The sequence between the primers utilised for PCR measured either 511 or 512 bp for the pNSE8 and pNSE9 plasmids respectively. The primers were designed to bind to sequences of the T-DNA, which is only present in genomes of plants, which have successfully been transformed. In the first gel a DNA ladder was added in the first well alongside the first 19 pNSE8 DNA samples. In the first row of the second gel the remaining five pNSE8 DNA samples, another DNA ladder, and 14 pNSE9 DNA samples were added. In the second row of the second gel the remaining ten pNSE9 DNA samples, a third DNA ladder and the three control samples were added. There were seven confirmed pNSE8 transformants (four with weak signal and three with rather strong signal) and eight confirmed pNSE9 transformants (four with weak signal and four with strong signal).

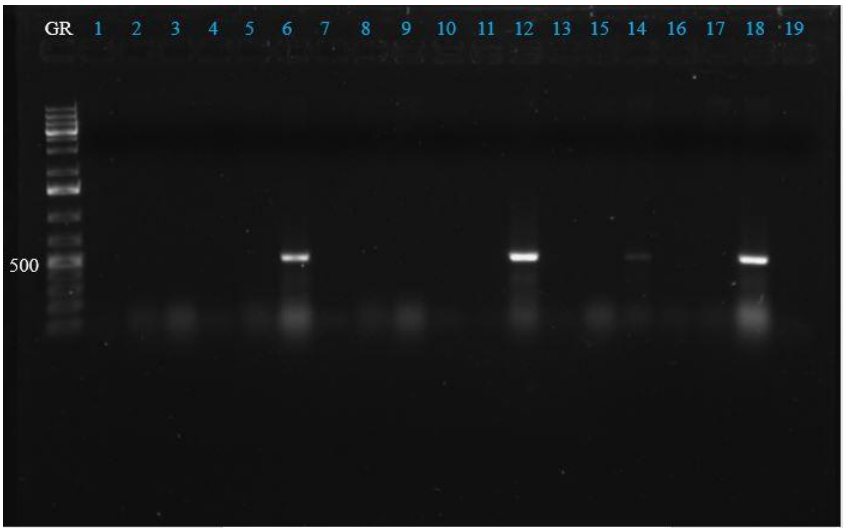

Figure 22: Picture of electrophoresis gel with the first 19 DNA samples of the pNSE8 *A. thaliana* plants (blue colour denotes pNSE8, instead of the notation from 5.4, e.g., 8_1). On the left most column is the Gene Ruler, which serves as reference for the lengths of the DNA fragments of the different bands. The band indicating 500 bp is indicated, the crucial point of reference as the sequence between the primers utilised for PCR had a length of 511 bp. The 14th and 15th sample were switched accidentally, when pipetting the DNA samples into the wells of the gel. GR; Gene Ruler

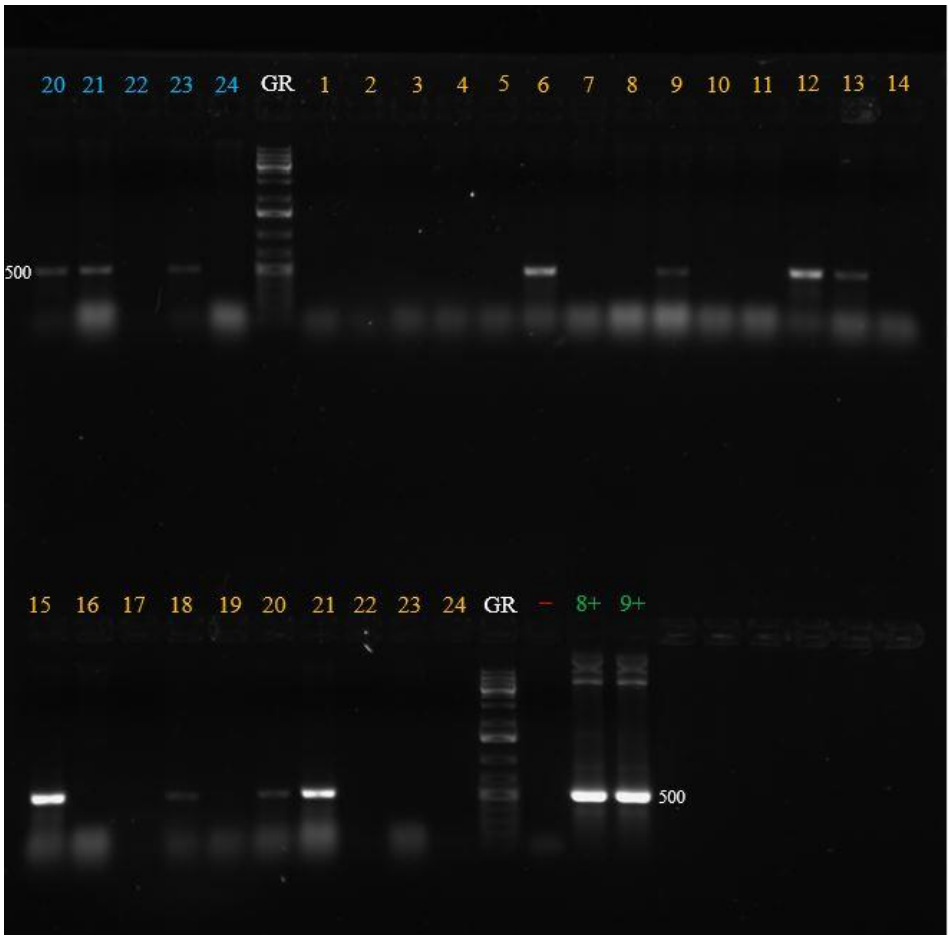

Figure 23: Picture of electrophoresis gel with the five remaining DNA samples of the pNSE8 *A. thaliana* plants (blue numbers) and all 24 DNA samples of the pNSE9 *A. thaliana* plants (orange numbers) alongside the negative control (red, water) and two positive controls (green, pNSE8 plasmid and pNSE9 plasmid). Again, the 500 bp mark is indicated as the sequence between the primers measures 512 bp. GR: gene ruler, −; negative control, 8+; pNSE8 positive control, 9+; pNSE9 positive control.

5.6 Results of Seed Set Analysis

Seed set analysis was done as a preliminary screening for potential phenotypes induced by the amiRNA knockdown of the RKD transcription factors. Abnormalities in the siliques containing the seeds such as developmental arrest or low-seed count are easily recognizable under a dissection microscope. Out of the 15 transformants confirmed by PCR and electrophoresis only one individual plant showed a different seed set phenotype from wild-type plants. None of the pNSE8 plants showed any substantial amount of seed abortion and were indistinguishable from the wild-type phenotype (see figure 24). In the siliques of the 18th plant transformed with pNSE9 (plant 9_18) a high frequency of underdeveloped seeds of roughly 50% was observed (see table 6). As mentioned before (see chapter 4.10) the underdeveloped seeds are small and white (see figure 25). The remaining pNSE9 plants, which were screened showed the wild-type phenotype. The control silique was taken from plant 9_1. No underdeveloped seeds were observed there.

Figure 24: Picture of a silique taken from a pNSE8 plant. All the seeds are of roughly equal size and no aborted seeds are present. This is equivalent to the phenotype for siliques in wild-type plants.

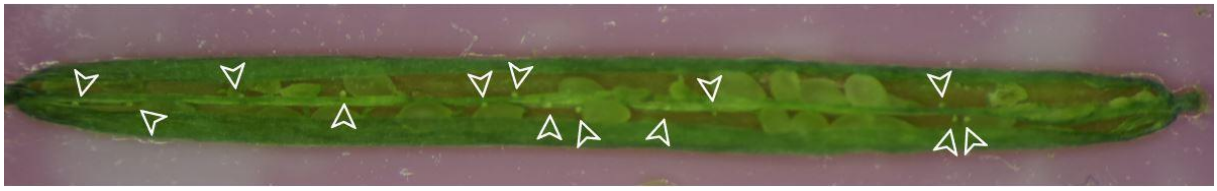

Figure 25: Picture of a silique taken from the plant 9_18 (transformed with pNSE9). There are gaps in the siliques, which are not filled. As roughly half of all seeds did not develop properly and remained as small white embryo sacs (marked with white arrow heads). The space defined by the gaps is not utilised. This is a phenotype for siliques, which is distinct from that of wild-type plants.

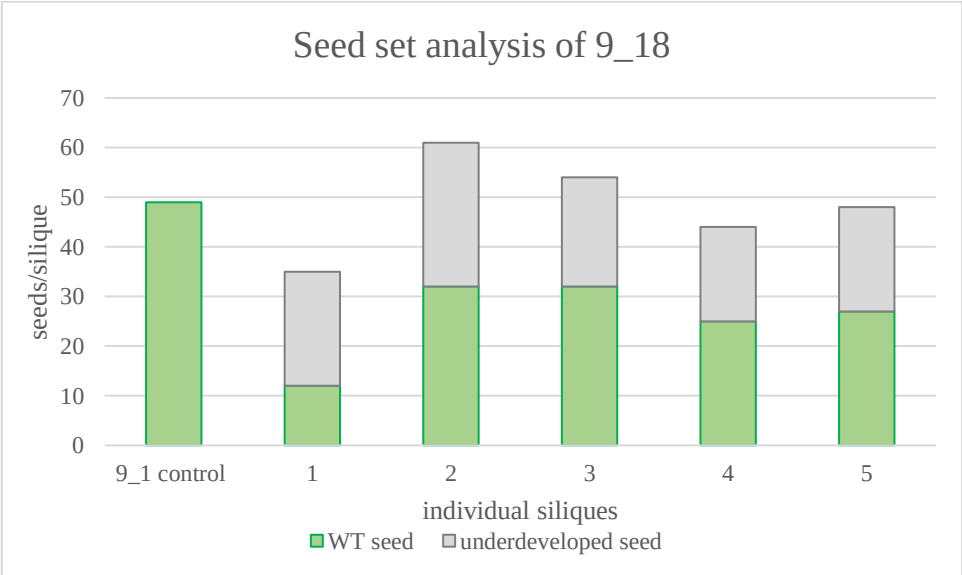

Table 6: Seed set analysis of five siliques taken from the plant 9_18. and one from plant 9_1. WT; wild-type

5.7 Results of Gametophyte Analysis

During the initial screening of the 15 transformation lines one outlier (plant 9_18) was identified. Around half of all the seeds in the siliques were developmentally arrested. To further investigate the cause of the underdeveloped seeds, pollen grains of the plant were isolated and analysed under a dissection microscope. If the phenotype was caused by the amiRNA the pollen should be unaffected. The pollen and the mature female gametophytes of the plant 9_18 showed abnormalities in size and developmental growth. Roughly 50 percent of all the pollen grains were aborted, meaning development had ceased, evident in their diminished size and distinct discolouration. These pollen grains are not viable and are unable to fertilise egg cells. In the plant 9_1, another independent transformant line acting as the control, no such phenotype distinct from the wild-type phenotype was observed (see figure 26). The abortion of pollen grains of plant 9_18 points towards issues during meiosis being the cause of the phenotype. In a next step, the female gametophytes were cleared and checked for abnormalities under a DIC microscope. Abnormalities in the embryo sacs would further support the idea of complications during meiosis being the root of the underdeveloped seeds. The chloral hydrate solution used to clear the female gametophytes was too potent, resulting in low contrast images, when analysed under a DIC microscope. The individual cells of the embryo sac are not distinguishable. Nevertheless, in a handful of female gametophytes of plant 9_18, underdeveloped embryo sacs were observed, recognizable by their diminished size relative to healthy embryo sacs of female gametophytes (see figure 27).

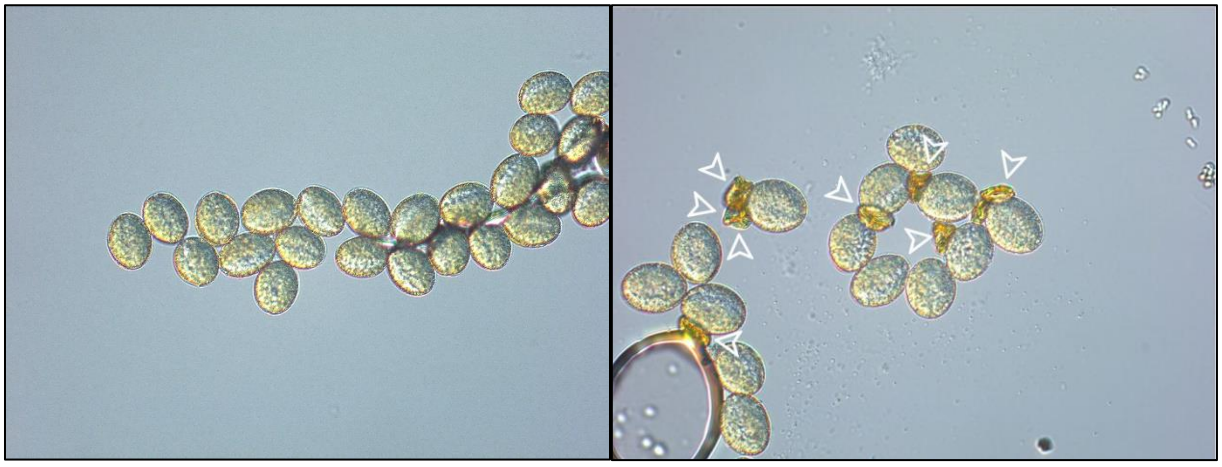

Figure 26: (Left) Pollen grains from plant 9_1 (acting as a control), which is phenotypically not distinct from the wild-type phenotype. All the pollen grains are of equal size and show no discolouration. (Right) Pollen grains from plant 9_18, of which the aborted pollen grains are clearly visible (marked with white arrow heads) and distinct to viable pollen grains due to their smaller size and yellow discolouration.

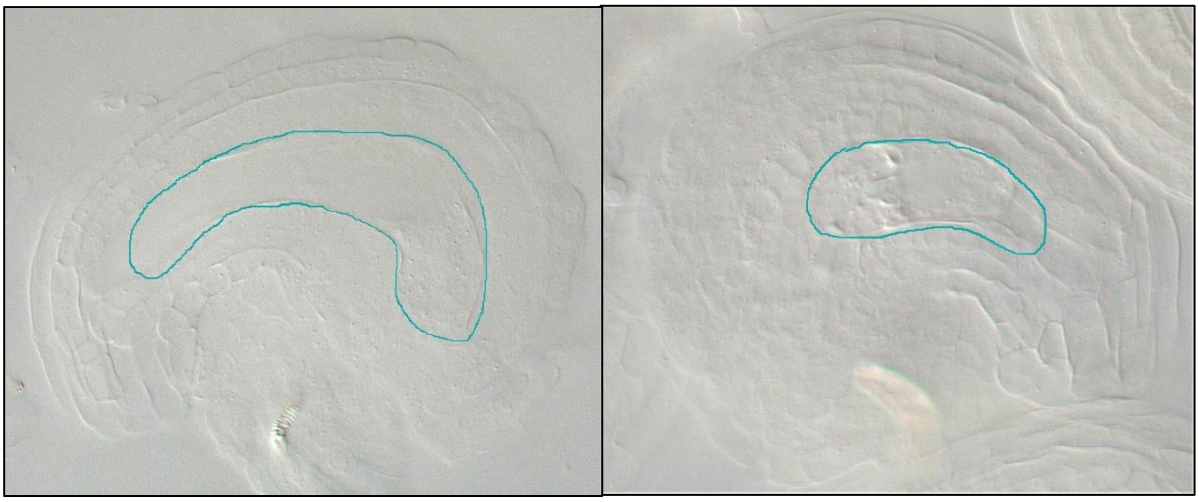

Figure 27: (Left) Cleared female gametophyte of plant 9_1. None of the cells in the embryo sac are clearly recognizable, however the general shape of the embryo sac resembles the wild-type phenotype, which is fairly elongated (outline of embryo sac is marked). (Right) Cleared female gametophytes of plant 9_18. Similarly, the individual cells are not distinguishable, however the general shape of the embryo sac can be observed. The outline of the embryo sac of an aborted female gametophyte is marked, which is not elongated and much smaller in size than the wild-type phenotype.

6 Discussion

6.1 Discussion of Miniprep

The concentration of the mini-prepped DNA for both the pNSE8 and pNSE9 plasmids were considerably high, both above 300 ng/µl. Anything above 30 ng/µl is a high enough yield to continue working with the DNA, however the optimal concentration value can sway depending on how big the plasmid is. Therefore, looking at the concentration in respect to the total volume of the samples gives a better idea of how successful a miniprep was. Anything above 5 µg is sufficient to continue with the transformation of *A. tumefaciens*. For both samples there were 100 µl, therefore a yield of roughly 30 µg or 35 µg respectively was achieved. Both the high concentration and above average mass of genetic material indicate that the uptake of the plasmid in the *E. coli* via heat-shock was successful and the cloning of the plasmid occurred at a frequent rate.

6.2 Discussion of Sanger Sequencing

The amiRNA sequences, the genes of interest in the pNSE8 and pNSE9 plasmids, were confirmed to be correct with Sanger sequencing, meaning that the cloning of the plasmids via *E. coli* had worked successfully. Sanger sequencing is an important tool to check the sequence of a genetic construct, however it has certain limitations, which have to be considered, when analysing results. The quality of the first 40-50 bp is low as is the quality of the data after 800-900 bp. Therefore, realistically a sequence of 800-850 bp is to be expected for a normal Sanger sequencing reaction using one primer. Using two primers, which overlap in 51 bp, sequences could be generated that are roughly 1500 bp long by manually aligning the two sequences, which covers the sequence of interest measuring 1263 bp. The lowered quality of the sequence read towards the beginning and the end was observed (see appendix figure 28 and figure 29 for complete alignment). Due to primer binding the sequences of the

first few bp are low in quality. The sequences with longer segments cannot be read properly as single bp become increasingly difficult to distinguish with increasing length of the sequence in agarose gel.[91]

6.3 Discussion of Transformation Rate of *A. thaliana* Plants

The transformation rate of the seeds is rather low for both constructs. Usually, at least one percent of all the seeds are transformed.[72] The ecotype of *A. thaliana* alongside the strain of *A. tumefaciens* play a key role in how high the transformation rate is. The Col-0 ecotype was used, which is known to show significant variability in transformation rates from plant to plant across biological repeats.[93] This would explain why the transformation rates for the pNSE8 and pNSE9 plants are inconsistent with the minimal value of one percent. No biological repeats were included in the experiments, where discrepancies between the transformation rates would be observable. An analysis of the transformation rates of pNSE8 and pNSE9 could be done in a future study.

Amongst the plants, which survived the selection process two main phenotypes were distinguishable. Over half of all the surviving plants had multiple leaves and were substantially bigger than the remaining group of plants. These “healthy” plants had a successful uptake of the T-DNA, which contains a hygromycin resistance gene, allowing the plants to metabolise the herbicide and survive. The remainder of the surviving plants were slightly smaller in size and occasionally showed slight discolouration. This group of slightly stunted plants tangibly demonstrate how *A. tumefaciens* inserts the T-DNA into its host’s genome at random loci. The T-DNA might have inserted in between a sequence, which is crucial for normal plant development, essentially disabling genes. An alternate explanation for this phenotype might be that a too high number of transformation events could have occurred in a single plant, which can stress it and lead to developmental stunting. A possible but less likely third explanation might be that these plants are not transformants at all but survived due to the actually transformed plants metabolising and neutralising enough of the herbicide for non-transformants to survive. This occurs when seeds are plated too densely on the selection media, which leads to clumping of the seeds. In summary, the selection via plating on herbicide gives a good indication for potential transformants but no individual plant can be labelled as transgenic with absolute certainty, which is why PCR genotyping was done in addition.

6.4. Discussion of DNA Extraction

Two main issues were observed during DNA extraction. For a small amount of the samples the grinding with the glass beads did not work properly, as the tissue remained intact after vigorous shaking. This was due to the glass beads getting stuck in the cluster tubes. For the remaining samples the low concentration of DNA can be traced back to the step were the MM2 plate was shaken at 1200 rpm (see chapter 4.8). The lid covering the plate was not properly sealed, which resulted in a loss of sample liquid containing DNA. Nevertheless, for 32 of the samples the DNA extraction went smoothly. The suboptimal execution of the steps mentioned above, had an influence on the PCR and

electrophoresis of the DNA samples. This could have been resolved by redoing the DNA extraction, but due to time constraints this was not possible.

6.5 Discussion of PCR and Electrophoresis

The PCR and subsequent gel electrophoresis confirmed that 15 out of the 48 plants, which survived the herbicide selection process carry one of the respective constructs (pNSE8 or pNSE9). If a dipped *A. thaliana* seed can grow normally on a selection media, containing herbicide, it indicates that the T-DNA was integrated and the herbicide resistance gene is active, which helps metabolise the herbicide to ensure the plant's survival. Therefore, it is unexpected that only 15 of the 48 plants show a positive signal during gel electrophoresis. As the herbicide resistance gene was part of the T-DNA of both plasmids desired to be inserted into *A. thaliana*, all surviving plants are expected to be transformants, which would be verifiable with PCR. As mentioned in chapter 6.3, it is possible for a plant to survive on the selection media under specific conditions, even if it itself does not have an herbicide resistance gene, namely when neighbouring plants, which have the resistance gene neutralise enough of the surrounding herbicide. It is unlikely that this is the primary reason, why not all of the 48 plants show a band at 500 bp as only healthy-looking plants, which were developmentally ahead of the surrounding plants were picked to continue with during the transfer onto soil. A more conspicuous explanation is the fact that two crucial steps during DNA extraction were not done properly, resulting in the loss of genetic material (see chapter 6.4). This would explain the absence of the bands at 500 bp for the remaining 33 plants as during PCR there was no T-DNA present to be amplified, probably not because these plants were not transformants, but because the sequences containing the T-DNA were not extracted properly, indicated by the low concentrations of DNA measured by the spectrophotometer. Amongst the 15 confirmed transformants, the bands at 500 bp have varying signal strengths. A few factors play into this. Firstly, the signal is dependent on how much starting DNA material is present, i.e., the concentration of DNA before PCR. The more DNA there is to begin with, the more T-DNA is present to be amplified. Secondly, the amount of transformation events in an individual plant can increase the total potential sites for the PCR to amplify. Lastly, the quality of the gel and the distribution of the dye might impact the signal strength in a minimal way.

6.6 Discussion of Seed Set Analysis

In the siliques of the 15 transformants screened only one transformation line (9_18) showed a female gametophyte abortion phenotype. This result is not expected as some degree of female gametophyte abortion should be expected in all transformation lines if that is the phenotype induced by the loss-of-function experiments. In the double mutants rkd1-1 rkd2-2 and rkd1-2 rkd-5-2, also created using a downregulation method, low numbers of seeds have been reported.[81] Other mutants such as rkd5-2 rkd2-2 and rkd4-1 rkd2-2 show an early arrest phenotype during the functional megaspore stage. Therefore, it is highly unlikely that the amiRNAs selectively only express in one transformation line such that a phenotype is observable. The plants screened overcame both herbicide selection and PCR

to confirm that they did in fact have the T-DNA. An absence of the T-DNA and thus the goi is therefore not an explanation for the wild-type phenotype in 14 of the 15 screened transformants. Furthermore, the expected phenotype is not recessive. When the inflorescences of the T0 *A. thaliana* plant are dipped in the *A. tumefaciens* solution, transformation events occur in the unfertilised female gametophytes. Therefore, the goi resides on the maternal chromosomes of the T1 generation. However, the amiRNA should be expressed at a level sufficient to downregulate the RKD transcription factors, expressed by both parental genomes. Phenotypes induced by an amiRNA downregulation method are always dominant as the mRNAs generated from both alleles are degraded in the cytosol through the RNAi.

The lack of transformation lines showing a female gametophyte abortion phenotype indicates that the expression of the amiRNA in most of the transformation lines screened is not having the desired effect. One explanation for this could be that the downregulation of three RKD transcription factors (depending on the construct different RKD transcription factors were designed to be downregulated, see table 1, chapter 4.1) is not happening to a sufficient degree or at all. It remains unclear if the amiRNAs are even expressed. The level of downregulation of the RKD transcription factors could be checked with quantitative reveres transcription PCR (RT-qPCR), a method used for gene expression analysis. To measure the RKD transcription levels the mRNA of a transformant line would have to be isolated, then transcribed with a reverse transcriptase, which yields complementary DNA (cDNA) that can be used as a template for the qPCR reaction accompanied by appropriate primers, designed for the individual RKD transcription factors.[r] However, isolating enough tissue containing the RKD transcription factors at a detectable level for RT-qPCR will be a challenging task as the embryo sac is very small. Alternatively, the Northern blot method could be used to determine the level of expression of the amiRNAs (see chapter 6.8).

Another factor which has to be taken into consideration is that the downregulation of three RKD transcription factors might not suffice to induce a female gametophyte arrest phenotype, due to a functional redundancy amongst all five RKD transcription factors in *A. thaliana*. Complications in the formation of the RISC-complex and suboptimal base pairing between the mature miRNA and RNA, which is to be silenced might also contribute to the poor expression of the amiRNAs. Redesigning the plasmids might help solve such issues. It is also possible that the amiRNA knockdown method is not suitable to induce an egg-cell related phenotype.

In the siliques of plant 9_18 screened, roughly half of the female gametophytes were underdeveloped (see table 6, chapter 5.6). This does not confirm that the amiRNA is having the desired effect. A more in-depth discussion is only possible when looking at the results of gametophyte analysis (chapter 5.7). These results are discussed below in chapter 6.7.

6.7 Discussion of Gametophyte Analysis

The analysis of the pollen of plant 9_18 showed that roughly half of the pollen grains was aborted. Additionally, in the analysis of the cleared female gametophytes, underdeveloped embryo sacs were observed, however due to the use of a too potent chloral hydrate solution, which makes the plant tissue transparent, the microscopy imagery was low in contrast and thus the intricate structures of these smaller embryo sacs remain unclear. Furthermore, the DIC microscope used was not calibrated properly as a new camera had recently been installed. Abundant one-nucleate stage embryo sacs have been observed in a previous study using similar constructs.[3] This is a phenotype, which presumably occurs due to an arrest during the functional megaspore stage of female gametophyte development. It is unclear if the abnormal female gametophytes of 9_18 were in the one-nucleate stage, however some hints can be taken when examining both gametes and the abundance of gamete abortion. Since roughly half of all the pollen grains were aborted and likewise around half of all female gametophytes were aborted in this particular line, it points towards a possible undesired effect occurring, called reciprocal translation or chromosomal translocation. Chromosomal translocations occur during meiosis when the chromosomes are aligning and have difficulty pairing up. This phenomenon has been documented to occur quite frequently in T-DNA insertion lines.[94, 95] A reciprocal translation leads to abortion in 50% of both male and female gametes as these are missing some parts of the genome. This coincides with the observations made for the pollen and female gametophytes of plant 9_18. It is unlikely that the phenotype is caused by the amiRNAs (amiRNA1 and amiRNA2) as the promoter used (EC1.1), drives the goi during late-stage embryonic development and the arrest at the functional megaspore stage happens before the amiRNA is expressed. It is possible that the EC1.1 promoter is expressed earlier than expected due to promoter leakiness, however this specific promoter has been used in previous studies before and no such issues were reported.[76, 96] Furthermore, RKD1 and RKD2 are inherently expressed at the later stages of female gametophyte development, therefore it is improbable that an arrest at the functional megaspore stage occurs due to the downregulation of those transcription factors. On the other hand, RKD3 and especially RKD4 are expressed at early stages and a downregulation of these genes might contribute to the arrest phenotype. However, as amiRNA1 downregulates two early-stage transcription factors (RKD3, RKD4), whilst amiRNA2 only downregulates one (RKD3) a higher amount of one-nucleate stage embryo sacs would be expected in the plants transformed with pNSE8, on which amiRNA1 resided. This is not the case; not a single pNSE8 transformant line showed such a phenotype. The fact that 14 out 15 plants did not show the one-nucleate stage phenotype might mean that the amiRNA is not functioning as intended or that the downregulation of three out of the five RKD transcription factors in *A. thaliana* does not suffice to induce a phenotype. Therefore, plant 9_18 shows a side-effect phenotype probably not caused by the successful downregulation of the RKD transcription factors via the amiRNAs but by DNA translocation, a side-effect of T-DNA insertion.[95]

6.8 Outlook

The phenotypical analysis of the *A. thaliana* plants yielded the characterization of one transformant line with a possible one-nucleate immature embryo sac phenotype, which is not expected to be the result of the expression of the amiRNA, as the promoter used should allow the embryo sac to fully mature. This should not be considered as a general phenotype of *A. thaliana* plants with downregulated RKD transcription factors (RKD1, RKD3, RK4 and RKD1 RKD2, RKD3 for the respective constructs, see table 1, chapter 4.1) but as a side-effect phenotype. No phenotypical difference was observed between transformation lines transformed with pNSE8 or pNSE9 as neither of the constructs were able to induce an egg-cell related phenotype.

Both the fact that 14 confirmed transformant lines show no phenotype and the fact that one outlier transformant line shows a phenotype probably caused by the side effects of T-DNA insertion, raises the question if the amiRNA is expressed at a high enough level or expressed at all. A next step would be to check if the occurrence of the one-nucleate phenotype is caused by the downregulation of the RKD genes or is indeed a side effect as proposed.

A preliminary test could be done to see if the amiRNA precursor sequence is being expressed, which could be determined with RT-qPCR. The detection of the amiRNA sequence itself will be slightly more challenging as it is only 21 bp in length and thus roughly the size of a primer, a far too short sequence to do PCR on. Instead, the Northern Blot method could be applied to check if the amiRNA is even abundantly present in the egg cells. For this method, RNA molecules have to be isolated from the cells of the plant, a probe, which is complementary to the amiRNA and labelled with a radioactive atom or fluorescent dye for detection, has to be designed and the amiRNA has to be sufficiently concentrated. The RNA molecules are separated according to their sizes using gel electrophoresis and then transferred onto a blotting membrane.[s] The probe then binds to the amiRNA, allowing the amiRNA to be detected among the various RNA molecules on the membrane. A potential issue is that the amiRNA will never be present in a high enough concentration as the promoter driving the amiRNA is egg-cell specific and the egg cell is vastly outnumbered by other cell types. The egg cells would have to be individually isolated before further testing. It goes without saying that detecting the amiRNA will be challenge due to technical issues and not solely because of the uncertainty if the amiRNA is even expressed correctly.

The exact functions of the RKD transcription factors are still to be determined. When the single MpRKD gene in *M. polymorpha* is downregulated its egg cells proliferate without fertilisation and form disorganized embryos, which abort.[4] MpRKD regulates the gametophyte development by maintaining the quiescent state of the egg cell state until fertilisation occurs.[4] The hope is that by downregulating RKD transcription factors, egg cells will start developing and maturing into viable embryos in the absence of fertilisation. As the RKD transcription factors are only one of many

components regulating female gametophyte development engineering apomixis in plants is not that straightforward and requires further research.

The preferential stages and locations in which the five AtRKD transcription factors are expressed in *A. thaliana* have been determined in various studies.[4, 76, 81] The ectopic expression of AtRKD1 and AtRKD2 in sporophytic cells of *A. thaliana* induces severe cell proliferation. Such cells demonstrate a shift in their gene expression pattern, which is similar to that of an egg cell (expression of an egg-cell marker).[80] So far however, no phenotypes relating to the development of egg cells have been identified in loss-of-function experiments in *A. thaliana*. If the amiRNA downregulation method itself is not the issue and cause for the absence of egg-cell related phenotypes identified in this project, then the functional redundancy between the five RKD transcription factors might be the issue.

It is possible that downregulating three of the five RKD transcription factors in *A. thaliana* is not sufficient to induce a phenotype relating to the development of the female gametophyte. Therefore, transformation lines with amiRNAs knocking down four or even all five RKD transcription factors could be of interest. The pNSE8 and pNSE9 transformation lines could be crossed to produce plants with a quadruple knockdown. Furthermore, a separate construct could be designed with an amiRNA that knocks down RKD4 and RKD 5 or RKD2 and RKD5 with which a different variety of transformation lines could be generated. These lines could then be crossed with the transformation lines from this project, yielding plants, which would have all five RKD transcription factors knocked down. Alternatively, a different approach, e.g., CRISPR-Cas could be utilised to knock out the five RKD genes.

If developmental issues in the egg cells do turn out to be one of the consequences of a quintuple knockdown of the RKD genes, it might be hard to maintain such transformation lines as. It would be difficult to maintain the lines with the offspring due to their developmental defects, but the more detailed functions of the RKD transcription factors could be elucidated. There are still many challenges in regard to understanding the mechanisms regulating reproductive development in plants but overcoming these could lead to the development of apomixis technology, generating maternal clones without the need for male fertilisation.[5, 7]

7 Conclusions

The functions of the RKD transcription factors have been linked to the development of the female gametophyte. Understanding the underlying molecular processes of egg cell activation and initiation of embryogenesis might be the key to unlocking apomixis technology. This would essentially usher in a new age in the agricultural industry. However, the precise role that each of the five RKD transcription factors in *A. thaliana* play in the development of the female gametophyte still remain obscure and understanding the underlying mechanisms would be one of the requirements for the development of engineered apomixis.

A. thaliana plants were generated with amiRNAs targeting three RKD genes (RKD1, RKD3 & RKD4 and RKD1, RKD2 & RKD3 respectively) in this project to check for egg-cell related phenotypes in a three out of five RKD transcription factor knockdown. These plants were doubly verified to be transformants through herbicide selection and genotyping PCR. To gain these transformant lines many steps were necessary, which include plasmid cloning with *E. coli*, mini prep of *E. coli*, transformation of *A. tumefaciens* via electroporation, floral dip of the T0 plants, selection, and transfer onto soil of the T1 plants. These steps were executed effectively and have been documented in such a way that they can be reproduced.

Apart from a single transformation line, which showed pollen and egg cell abortion likely caused by a side effect of T-DNA insertion, no phenotypes relating to egg cell development could be identified in the transformation lines generated with the amiRNA knockdown method. Whether this is due to the method itself or due to the functional redundancy between the five RKD transcription factors of *A. thaliana* remains to be determined. These transformation lines can be potential subjects of further RKD transcription factor loss-of-function experiments, e.g., acting as parental lines for new plants with a quadruple or even a quintuple knockdown of the RKD genes.

8 Reflection

Over the course of my entire work process for this paper I have learned a lot. I have learned how to plan a big project and also schedule time to complete the various steps necessary to complete it. During my time at the Department of Plant and Microbial Biology of the University of Zurich I learned how to work in a research laboratory. This includes how to use a wide range of equipment, the accompanying methods which are employed to understand how plants function but also how to collaborate with different people. Learning not just how to read but truly understand scholarly papers goes hand-in-hand with learning these new methods. I came to the conclusion that this process requires a great amount of focus, determination, and resilience as it is laborious and time-consuming.-I also learned how to deal with frustrations regarding experiments not working out as planned. A lot of patience and mental endurance is required when working with plants as experiments can last for extended periods of time. Checking what was done between each step is crucial to avoid mistakes and having to repeat the whole process again. For quite some time it was not even clear what the subject of my project would be. I experienced some anxiety concerning that, but I believe it is normal to second-guess what you are doing occasionally, checking to see if what you are doing even makes sense. It ensures that the quality of your product satisfies you as you poured your blood, sweat and tears into it. Through writing this paper I have gained a first-hand understanding and appreciation for how scientists work, which has cemented my desire to pursue a career in science.

9 Acknowledgements

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Finally, I would like to thank my family who were instrumental in motivating me throughout the year. This paper would never have reached completion had they not been so understanding and supportive when I needed to invest time into the project.

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[u] https://genome.cshlp.org/content/early/2011/09/22/gr.123547.111.full.pdf, 29.12.21

[v] http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd, 29.12.21

10.3 Figures

Title page picture: T1 Columbia-0 *A. thaliana* plant transformed with pNSE8.
Self-made picture, 01.11.21

Figure 1: Ler WT *A. thaliana* plant and Col WT *A. thaliana* plant.
Adapted from Fernández-Nohales, P., Domenech, M. J., Martínez de Alba, A. E., Micol, J. L., Ponce, M. R., & Madueño, F. (2014). AGO1 controls arabidopsis inflorescence architecture possibly by regulating TFL1 expression. Annals of botany, 114(7), 1471–1481.
https://doi.org/10.1093/aob/mcu132

Figure 2: Life cycle of *A. thaliana*.
Adapted from Eggenberger, N., Functional characterization of the role of RKD transcription factors during the female gametophyte development in *Arabidopsis thaliana*, Master thesis in Plant Sciences, University of Zurich, Department of Plant and Microbial Biology May 2019

Figure 3: Graphical representation of the stages of monosporic female gametophyte development.
Adapted from Yadegari, R., & Drews, G. N. (2004). Female gametophyte development. The Plant cell, 16 Suppl(Suppl), p133–141.
https://doi.org/10.1105/tpc.018192

Figure 4: A schematic representation of the seven stages of Female Gametophyte (FG) development.
Adapted from Skinner, D. J., & Sundaresan, V. (2018). Recent advances in understanding female gametophyte development. F1000Research, 7, F1000 Faculty Rev-804.
https://doi.org/10.12688/f1000research.14508.1

Figure 5 (Left): Healthy blackberry plant.
Adapted from https://www.planetnatural.com/pest-problem-solver/plant-disease/crown-gall/, 12.12.21

Figure 5(Right): Blackberry plant with crown gall disease
Adapted from https://www.planetnatural.com/pest-problem-solver/plant-disease/crown-gall/, 12.12.21

Figure 6: Scanning electron microscope image of *A. tumefaciens* attached to a root of an *A. thaliana* plant.
Adapted from Petrovicheva, A., Joyner, J., & Muth, T. R. (2017). Quantification of Agrobacterium tumefaciens C58 attachment to Arabidopsis thaliana roots. FEMS microbiology letters, 364(18), 10.1093/femsle/fnx158.
https://doi.org/10.1093/femsle/fnx158

Figure 7: Graphical representation of *Agrobacterium*-mediated transformation of plants.
Adapted from https://www.brainkart.com/article/Methods-of-Gene-Transfer_38242, 01.11.21

Figure 8: A schematic representation of a Ti plasmid.
Adapted from Gordon, J. E., & Christie, P. J. (2014). The Agrobacterium Ti Plasmids. Microbiology spectrum, 2(6), 10.1128/microbiolspec.PLAS-0010-2013.
https://doi.org/10.1128/microbiolspec.PLAS-0010-2013

Figure 9: Schematic representation of bacterial conjugation.
Adapted from Clark, D.P., Pazdernik, N.J., McGehee, M. R., Molecular Biology, Third Edition (2019), Chapter 28: Bacterial Genetics
https://doi.org/10.1016/C2015-0-06229-3

Figure 10: Schematic representation of the four main steps of T-DNA transfer from and *A. tumefaciens* cell into the genome of a plant cell.
Adapted from Lacroix, B., & Citovsky, V. (2019). Pathways of DNA Transfer to Plants from Agrobacterium tumefaciens and Related Bacterial Species. Annual review of phytopathology, 57, 231–251. https://doi.org/10.1146/annurev-phyto-082718-100101

Figure 11: Schematic representation of a type IV secretion system.
Adapted from Fischer, W., Tegtmeyer, N., Stingl, K., & Backert, S. (2020). Four Chromosomal Type IV Secretion Systems in Helicobacter pylori: Composition, Structure and Function. Frontiers in microbiology, 11, 1592.
https://doi.org/10.3389/fmicb.2020.01592

Figure 12: Schematic representation of a T-DNA binary transformation system.
Adapted from Lee, L. Y., & Gelvin, S. B. (2008). T-DNA binary vectors and systems. Plant physiology, 146(2), 325–332.
https://doi.org/10.1104/pp.107.113001

Figure 13: Graphical representation of the mechanism of RNA interference.
Adapted from https://www.nobelprize.org/prizes/medicine/2006/press-release/, 12.12.21

Figure 14: The vector map of the pMDC32-pEC1-1-amiRNA1 (pNSE8).
Adapted from Eggenberger, N., Functional characterization of the role of RKD transcription factors during the female gametophyte development in *Arabidopsis thaliana*, Master thesis in Plant Sciences, University of Zurich, Department of Plant and Microbial Biology May 2019

Figure 15: The vector map of the pMDC32-pEC1-1-amiRNA1 (pNSE8), including primers.
Self-made figure, 09.12.21

Figure 16: Schematic representation of the sequenced stretch of the pNSE8 vector map.
Self-made figure, 09.12.21

Figure 17: pNSE8 template sequence compared with sequence generated by Sanger sequence reaction.
Self-made figure, 09.12.21

Figure 18: pNSE9 template compared with sequence generated by Sanger sequence reaction.
Self-made figure, 09.12.21

Figure 19 (Left): Picture of one of the two selection plates with the plants transformed with pNSE8.
Figure 19 (Right): Picture of one of the two selection plates with the plants transformed with pNSE9.
Self-made pictures, 13.10.21

Figure 20: pNSE8 and pNSE9 *A. thaliana* transformation lines immediately after transfer onto soil and 19 days later.

Self-made pictures, 13.10.21 & 01.11.21

Figure 21 (Left): Two *A. thaliana* plants transformed with pNSE8 19 days after transfer onto soil.

Figure 21 (Right): Two *A. thaliana* plants transformed with pNSE9 19 days after transfer onto soil.

Self-made pictures, 01.11.21

Figure 22: Picture of first electrophoresis gel.

Self-made picture, 12.11.21

Figure 23: Picture of second electrophoresis gel.

Self-made picture, 12.11.21

Figure 24: Picture of a silique taken from a pNSE8 plant.

Self-made picture, 19.11.21

Figure 25: Picture of a silique taken from the plant 9_18

Self-made picture, 19.11.21

Figure 26 (Left): Pollen grains from plant 9_1.

Figure 26 (Right): Pollen grains from plant 9_18.

Self-made pictures, 24.11.21

Figure 27 (Left): Cleared female gametophyte of plant 9_1.

Figure 27 (Right): Cleared female gametophytes of plant 9_18.

Self-made pictures, 26.11.21

Figure 28: Complete alignment of pNSE8 template sequence and sequence generated by Sanger sequence reaction.

Self-made figure, 11.12.21

Figure 29: Complete alignment of pNSE9 template sequence and sequence generated by Sanger sequence reaction.

Self-made figure, 11.12.21

10.4 Tables

11 Appendices

Appendix A: Complete Alignment of Sanger Sequencing

The full alignment of the plasmids with the products from the Sanger sequencing reaction is shown below. Important components of the plasmid are indicated with different colours.

```
pNSE8_template      aattcagtaacatagatgacaccgcgcgcgataatttatcctagtttgcgcgctatattt  60
PNSE8_SangerS       ------------------------------------------------------------  0

pNSE8_template      tgttttctatcgcgtattaaatgtataattgcgggactctaatcataaaaacccatctca  120
PNSE8_SangerS       ------------------------------------------------------------  0

pNSE8_template      taaataacgtcatgcattacatgttaattattacatgcttaacgtaattcaacagaaatt  180
PNSE8_SangerS       ------------------------------------------------------------  0

pNSE8_template      atatgataatcatcgcaagaccggcaacaggattcaatcttaagaaactttattgccaaa  2
PNSE8_SangerS       ------------------------------------------------------------  0

pNSE8_template      tgtttgaacgatcgggaaattcgagctccaccgcggtgggcggccgctctagaactagt  300
pNSE8_SangerS       ----------------GGGGTTGAAACCTCCCCGTCGGTGGCGGCGCTCTAGAACTAGT  43
                                  * **   *  **     * *  * ***************

pNSE8_template      taattaaggaattatcgaaccactttgtaca-agaaagctgaaccggataacaatttcac  359
pNSE8_SangerS       TAATTAAGAAT-TATCGAACCACTTTGTACAAGAAAGCTGGGTGCGGATAACAATTTCAC  102
                    ******** *  ******************   **    *   ***************

pNSE8_template      acaggaaacagctatgaccatgattacgccaagctcgaaattaaccctcactaaagggaa  419
pNSE8_SangerS       ACAGGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAA  162
                    ************************************************************

pNSE8_template      caaaagctggagctccaccgcggtggcggccgctctagaactagtggatccccccatggc  479
pNSE8_SangerS       CAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCCATGGC  222
                    ************************************************************

pNSE8_template      gatgccttaaataaagataaacccaaaatgttaattttaccagaactatatatacgaagg  539
pNSE8_SangerS       GATGCCTTAAATAAAGATAAACCCAAAATGTTAATTTTACCAGAACTATATATACGAAGG  282
                    ************************************************************

pNSE8_template      cagcatatatgtcacttagtggatcaagcatgtttttgtgcaggaaagattaatcaagaa  599
pNSE8_SangerS       CAGCATATATGTCACTTAGTGGATCAAGCATGTTTTTGTGCAGGAAAGATTAATCAAGAA  342
                    ************************************************************

pNSE8_template      aattggaatacaaaagagagaacgtcggcctcaccgtaagcatcaaagagaatcaatgat  659
pNSE8_SangerS       AATTGGAATACAAAAGAGAGAACGTCGGCCTCACCGTAAGCATCAAAGAGAATCAATGAT  402
                    ************************************************************

pNSE8_template      ccaatttgtctaccgcatcattcattcatttaacgagtctagtttgaattttggcgactc  719
pNSE8_SangerS       CCAATTTGTCTACCGCATCATTCATTCATTTAACGAGTCTAGTTTGAATTTTGGCGACTC  462
                    ************************************************************

pNSE8_template      ggtatttggatgaatgagtcggaagctaattgaatcatatcacgacctgtgaagcttacc  779
pNSE8_SangerS       GGTATTTGGATGAATGAGTCGGAAGCTAATTGAATCATATCACGACCTGTGAAGCTTACC  522
                    ************************************************************

pNSE8_template      gtgaggccgatgttctacatatatattcctaaaacatcaattcaaaacagcgagtattaa  839
pNSE8_SangerS       GTGAGGCCGATGTTCTACATATATATTCCTAAAACATCAATTCAAAACAGCGAGTATTAA  582
                    ************************************************************

pNSE8_template      gtgtatgaacatgtgtaatatgcgtccgagcgtgtgtttggggctgcaggaattcgatat  899
pNSE8_SangerS       GTGTATGAACATGTGTAATATGCGTCCGAGCGTGTGTTTGGGGCTGCAGGAATTCGATAT  642
                    ************************************************************

pNSE8_template      caagcttatcgataccgtcgacctcgaggggggg-gcccggtacccaattcgccctatagt  958
pNSE8_SangerS       CAAGCTTATCGATACCGTCGACCTCGAGGGGGGGGCCCGGTACCCAATTCGCCCTATAGT  702
                    ********************************* ************************

pNSE8_template      gagtcgtattacaattcactggc-cgtcgttttacaacgtcgtgactgggaaaaccctgg  1017
pNSE8_SangerS       GAGTCGTATTACAATTCACTGGCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGG  762
                    ********************** ***********************************
```

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pNSE8_template      cgttacccaacttaatcgccttgcaggttcagct-tttttgtacaaacttgtttgatagc  1076
pNSE8_SangerS       CGTTACCCAACTTAATCGCCTTGCAGAGCCTGCTTTTTTTGTACAAACTTGTTTGATAGC  822
                    *************************   * *** ************************

pNSE8_template      ttggcgcgcctcgaggggggggcccggtaccttctcaacagattgataaggtcgaaagaag  1136
pNSE8_SangerS       TTGGCGCGCCTCGAGGGGGGGCCCGGTACCTTCTCAACAGATTGATAAGGTCGAAAGAAG  882
                    ************************************************************

pNSE8_template      aaagagtagaagttgctttggttgatttgtttgtgatgagaattgtgtgttaactttaga  1196
pNSE8_SangerS       AAAGAGTAGAAGTTGCTTTGGTTGATTTGTTTGTGATGAGAATTGTGTGTTAACTTTAGA  942
                    ************************************************************

pNSE8_template      gagtttatatagagacagtagagacttgactgttacaaataaatggtagagtgaaagccc  1256
pNSE8_SangerS       GAGTTTATATAGAGACAGTAGAGACTTGACTGTTACAAATAAATGGTAGAGTGAAAGCCC  1002
                    ************************************************************

pNSE8_template      aagtaattacgagggaagctcattagattgtgagataattagtgcttagtggtttagtgg  1316
pNSE8_SangerS       AAGTAATTACGAGGGAAGCTCATTAGATTGTGAGATAATTAGTGCTTAGTGGTTTAGTGG  1062
                    ************************************************************

pNSE8_template      gtaaactaggtgtaattactctattaaaagctgcgtaattagtgggtctgtttaggggct  1376
pNSE8_SangerS       GTAAACTAGGTGTAATTACTCTATTAAAAGCTGCGTAATTAGTGGGTCTGTTTAGGGGCT  1122
                    ************************************************************

pNSE8_template      aatgggccggattagaatcactcagtctgaaactcgtttgatttagttcaatagaaccaa  1436
pNSE8_SangerS       AATGGGCCGGATTAGAATCACTCAGTCTGAAACTCGTTTGATTTAGTTCAATAGAACCAA  1182
                    ************************************************************

pNSE8_template      atcagctttaccatcaaaatctttgcgagaaaatagatttgggtaagtaaagtatgtaca  1496
pNSE8_SangerS       ATCAGCTTTACCATCAAAATCTTTGCGAGAAAATAGATTTGGGTAAGTAAAGTATGTACA  1242
                    ************************************************************

pNSE8_template      catctaatgagcatgtgtgagcatgaaatctataactatttgatcatcttgaaaccgaag  1556
pNSE8_SangerS       CATCTAATGAGCATGTGTGAGCATGAAATCTATAACTATTTGATCATCTTGAAACCGAAG  1302
                    ************************************************************

pNSE8_template      aaatcataaggcaactattttaaagacacagagacacctgcaatactgcaaatttgaaag  1616
pNSE8_SangerS       AAATCATAAGGCAACTATTTTAAAGACACAGAGACACCTGCAATACTGCAAATTTGAAAG  1362
                    ************************************************************

pNSE8_template      gttgctagatttagtagagctaattcatgataggcgttcctgcaggcacgccaagcttgg  1676
pNSE8_SangerS       GTTGCTAGATTTAGTAGAGCTAATTCATGATAGGCGTCCTGC-AGGCATGCAAGCTTGGC  1421
                    ************************************ *    ***** ** *   * *

pNSE8_template      cactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatc  1736
pNSE8_SangerS       ACTGGCCGTAAGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTAATCG  1481
                        * *    ******************************************** *

pNSE8_template      gccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatc  1796
pNSE8_SangerS       CCTGCCAGGAATAACCTTAGCCCCT----------------------------------  1506
                     *      * * * **      *
```

Figure 28: Complete alignment of pNSE8 template sequence and sequence generated by Sanger sequence reaction. The template sequence is shown with lowercase letters, whilst the Sanger sequence is capitalised. Asterisks signify alignment between the two sequences. The base pair positions are given on the right-hand side. Orange text; nose terminator, dark blue highlight; NSE28_correct, red text; amiRNA precursor, green highlight; amiRNA1, grey highlight NSE Rev primer, blue highlight; amiRNA1*, green text; pEC1.1, pink highlight; NSE27.

```
pNSE9_template      aattcagtaacatagatgacaccgcgcgcgataatttatcctagtttgcgcgctatattt  60
pNSE9_SangerS       ------------------------------------------------------------  0

pNSE9_template      tgttttctatcgcgtattaaatgtataattgcgggactctaatcataaaaacccatctca  120
pNSE9_SangerS       ------------------------------------------------------------  0

pNSE9_template      taaataacgtcatgcattacatgttaattattacatgcttaacgtaattcaacagaaatt  180
pNSE9_SangerS       ------------------------------------------------------------  0

pNSE9_template      atatgataatcatcgcaagaccggcaacaggattcaatcttaagaaactttattgccaaa  240
pNSE9_SangerS       ------------------------------------------------------------  0

pNSE9_template      tgtttgaacgatcggggaaattcgagctccaccgcggtgggcggccgctctagaactagt  300
pNSE9_SangerS       ------------------ATGGTTTGGCTCACGTCGGTGGCGGCGCTCTAGAGCTA-G  39
                                     * *     * *      * *  * ********* ***

pNSE9_template      taattaaggaattatcgaaccactttgtacaagaaagctgaacgcggataacaatttcac  360
pNSE9_SangerS       TTAATTAAGAATTATCGAACCACTTTGTACAAGAAAGCTGGGTGCGGATAACAATTTCAC  99
                    * * * * ********************************   *****************

pNSE9_template      acaggaaacagctatgaccatgattacgccaagctcgaaattaaccctcactaaagggaa  420
pNSE9_SangerS       ACAGGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAA  159
                    ************************************************************

pNSE9_template      caaaagctggagctccaccgcggtggcggccgctctagaactagtggatccccccatggc  480
pNSE9_SangerS       CAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCCATGGC  219
                    ************************************************************

pNSE9_template      gatgccttaaataaagataaacccaaaatgttaatttttaccagaactatatatacgaagg  540
pNSE9_SangerS       GATGCCTTAAATAAAGATAAACCCAAAATGTTAATTTTACCAGAACTATATATACGAAGG  279
                    ************************************************************

pNSE9_template      cagcatatatgtcacttagtggatcaagcatgtttttgtgcaggaaagattaatcaagaa  600
pNSE9_SangerS       CAGCATATATGTCACTTAGTGGATCAAGCATGTTTTTGTGCAGGAAAGATTAATCAAGAA  339
                    ************************************************************

pNSE9_template      aattggaatacaaaagagagagcgcctaacattggtttaacatcaaagagaatcaatgat  660
pNSE9_SangerS       AATTGGAATACAAAAGAGAGAGCGCCTAACATTGGTTTAACATCAAAGAGAATCAATGAT  399
                    ************************************************************

pNSE9_template      ccaatttgtctaccgcatcattcattcatttaacgagtctagtttgaattttggcgactc  720
pNSE9_SangerS       CCAATTTGTCTACCGCATCATTCATTCATTTAACGAGTCTAGTTTGAATTTTGGCGACTC  459
                    ************************************************************

pNSE9_template      ggtatttggatgaatgagtcggaagctaattgaatcatatcacgacctgtgaagttaaag  780
pNSE9_SangerS       GGTATTTGGATGAATGAGTCGGAAGCTAATTGAATCATATCACGACCTGTGAAGTTAAAG  519
                    ************************************************************

pNSE9_template      caatgttaggtgctctacatatatattcctaaaacatcaattcaaaacagcgagtattaa  840
pNSE9_SangerS       CAATGTTAGGTGCTCTACATATATATTCCTAAAACATCAATTCAAAACAGCGAGTATTAA  579
                    ************************************************************

pNSE9_template      gtgtatgaacatgtgtaatatgcgtccgagcgtgtgtttggggctgcaggaattcgatat  900
pNSE9_SangerS       GTGTATGAACATGTGTAATATGCGTCCGAGCGTGTGTTTGGGGCTGCAGGAATTCGATAT  639
                    ************************************************************

pNSE9_template      caagcttatcgataccgtcgacctcgaggggggggcccggtacccaattcgccctatagtg  960
pNSE9_SangerS       CAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTG  699
                    ************************************************************

pNSE9_template      agtcgtattacaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcg  1020
pNSE9_SangerS       AGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG  759
                    ************************************************************

pNSE9_template      ttacccaacttaatcgccttgcaggttcagcttttttgtacaaacttgtttgatagcttg  1080
pNSE9_SangerS       TTACCCAACTTAATCGCCTTGCAGAGCCTGCTTTTTTGTACAAACTTGTTTGATAGCTTG  819
                    ************************   * ******************************

pNSE9_template      gcgcgcctcgaggggggcccggtaccttctcaacagattgataaggtcgaaagaagaaa  1140
pNSE9_SangerS       GCGCGCCTCGAGGGGGGGCCCGGTACCTTCTCAACAGATTGATAAGGTCGAAAGAAGAAA  879
                    ************************************************************
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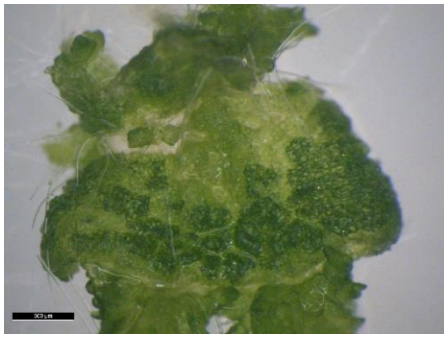
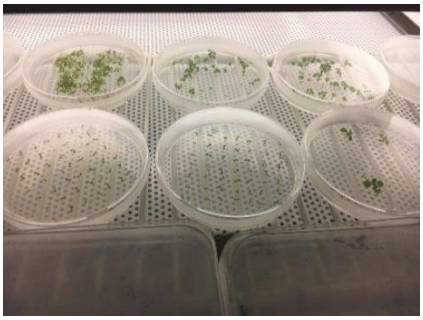
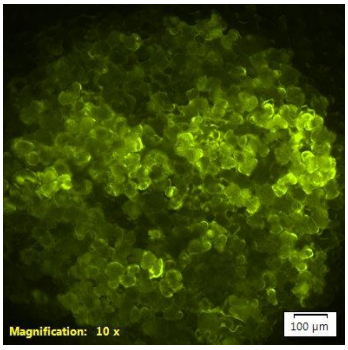
```
pNSE9_template      gagtagaagttgctttggttgatttgtttgtgatgagaattgtgtgttaactttagagag  1200
pNSE9_SangerS       GAGTAGAAGTTGCTTTGGTTGATTTGTTTGTGATGAGAATTGTGTGTTAACTTTAGAGAG  939
                    ************************************************************

pNSE9_template      tttatatagagacagtagagacttgactgttacaaataaatggtagagtgaaagcccaag  1260
pNSE9_SangerS       TTTATATAGAGACAGTAGAGACTTGACTGTTACAAATAAATGGTAGAGTGAAAGCCCAAG  999
                    ************************************************************

pNSE9_template      taattacgagggaagctcattagattgtgagataattagtgcttagtggtttagtgggta  1320
pNSE9_SangerS       TAATTACGAGGGAAGCTCATTAGATTGTGAGATAATTAGTGCTTAGTGGTTTAGTGGGTA  1059
                    ************************************************************

pNSE9_template      aactaggtgtaattactctattaaaagctgcgtaattagtgggtctgtttaggggctaat  1380
pNSE9_SangerS       AACTAGGTGTAATTACTCTATTAAAAGCTGCGTAATTAGTGGGTCTGTTTAGGGGCTAAT  1119
                    ************************************************************

pNSE9_template      gggccggattagaatcactcagtctgaaactcgtttgatttagttcaatagaaccaaatc  1440
pNSE9_SangerS       GGGCCGGATTAGAATCACTCAGTCTGAAACTCGTTTGATTTAGTTCAATAGAACCAAATC  1179
                    ************************************************************

pNSE9_template      agctttaccatcaaaatctttgcgagaaaatagatttgggtaagtaaagtatgtacacat  1500
pNSE9_SangerS       AGCTTTACCATCAAAATCTTTGCGAGAAAATAGATTTGGGTAAGTAAAGTATGTACACAT  1239
                    ************************************************************

pNSE9_template      ctaatgagcatgtgtgagcatgaaatctataactatttgatcatcttgaaaccgaagaaa  1560
pNSE9_SangerS       CTAATGAGCATGTGTGAGCATGAAATCTATAACTATTTGATCATCTTGAAACCGAAGAAA  1299
                    ************************************************************

pNSE9_template      tcataaggcaactatttaaagacacagagacacctgcaatactgcaaatttgaaaggtt  1620
pNSE9_SangerS       TCATAAGGCAACTATTTTAAAGACACAGAGACACCTGCAATACTGCAAATTTGAAAGGTT  1359
                    ************************************************************

pNSE9_template      gctagatttagtagagctaattcatgataggcgttcctgcaggcacgccaagcttggcac  1680
pNSE9_SangerS       GCTAGATTTAGTAGAGCTAATTCATGATAGGCGTCCTGCA--GGCATGCAAGCTTGGCAC  1417
                    ********************************** *      *     ************

pNSE9_template      tggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgcc  1740
pNSE9_SangerS       TGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCC  1477
                    ************************************************************

pNSE9_template      ttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcc  1800
pNSE9_SangerS       TGCAGCATAACCTTATCCCG----------------------------------------  1497
                    *        * *
```

Figure 29: Complete alignment of pNSE9 template sequence and sequence generated by Sanger sequence reaction. The template sequence is shown with lowercase letters, whilst the Sanger sequence is capitalised. Asterisks signify alignment between the two sequences. The base pair positions are given on the right-hand side. Orange text; nos terminator, dark blue highlight; NSE28_correct, red text; amiRNA precursor, green highlight; amiRNA1, grey highlight NSE Rev primer, blue highlight; amiRNA1*, green text; pEC1.1, pink highlight; NSE27.

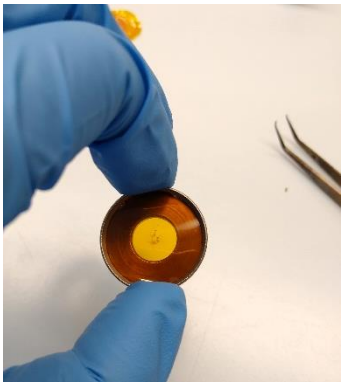
Appendix B: Highlights

An assortment of pictures taken during my stay at the Department of Plant and Microbial Biology of the University of Zurich.






12. Declaration of Authenticity (Eigenständigkeitserklärung)

Der Unterzeichnete bestätigt mit Unterschrift, dass die Arbeit selbständig verfasst und in schriftliche Form gebracht worden ist, dass sich die Mitwirkung anderer Personen auf Beratung und Korrekturlesen beschränkt hat und dass alle verwendeten Unterlagen und Gewährspersonen aufgeführt sind.

Ort, Datum

_____

Unterschrift

_____