Exploring Non-transgenic CRISPR-Cas9 gRNA Delivery Using Transactivation in *Nicotiana benthamiana*

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

Abstract: Exploring Non-transgenic CRISPR-Cas9 gRNA Delivery Using Transactivation in *Nicotiana benthamiana*

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Gene editing via CRISPR-Cas9 (Clustered, Regularly Interspaced, Short Palindromic Repeats – CRISPR-associated protein 9) is a powerful tool in biotechnology. The method involves an endonuclease Cas9 forming a complex with a guide RNA (gRNA) that matches a distinct DNA target sequence, causing double-stranded breaks in the DNA site. The break induces imperfect DNA repair resulting in insertions or deletions that render the target gene non-functional. It is also possible to mutate the Cas9 protein so that it loses its cutting function, but can still sit on the target DNA when in a complex with gRNA. This deactivated Cas9 (dCas9) can be fused with transcriptional activators or repressors to adjust the expression of a target gene.

While CRISPR-Cas9 is already widely used in plants, there are limitations to the system's delivery and efficiency. The aims of the research in this thesis were to generate a dCas9-activator-reporter system and to use the system to explore the possibility of two different non-transgenic methods of delivering gRNA.

The system consists of two plant expression plasmids. One encodes a constitutively expressed dCas9 fused to a transcriptional activator and a reporter gene driven by a minimal promoter. The second contains the gRNA, designed to target the minimal promoter. When the constructs are co-expressed, the gRNA-bound dCas9-activator sits on the minimal promoter and drives increased reporter expression.

Transient assays in *N. benthamiana* established the functionality of the system, showing increased reporter levels when the two constructs were co-expressed compared to the activator-reporter construct alone. Stable transgenic *N. benthamiana* lines of the separate constructs were generated and taken to the T2 generation.

To explore non-transgenic gRNA introduction methods, I tested viral delivery and grafting. Tobacco Rattle Virus (TRV) engineered to contain gRNA for the system was able to activate the reporter. However, the results suggest that viral recombination of the gRNA insert may cause the effect to be lost over time.

The positive readout transactivation system developed in this thesis will be a valuable tool for future CRISPR development in plants. In addition, the data reported present opportunities for further exploration on potential delivery methods, spatial specificity of CRISPR, and the mobility characteristics of synthetic gRNA compared to various endogenous RNAs.

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List of Abbreviations

4-MUG	4-Methylumbelliferyl-β-D-glucuronide
ATP	adenosine triphosphate
BeYDV	bean yellow dwarf virus
BMV	Brome mosaic virus
BNYVV	*Beet necrotic yellow vein virus*
bp	base pair
BSMV	barley stripe mosaic virus
CaLCV	Cabbage Leaf Curl Virus
CaMV	Cauliflower mosaic virus
Cas9	CRISPR-associated protein 9
CC	companion cell
cDNA	complementary deoxyribonucleic acid
CmPP16	*Cucurbita maxima* PHLOEM PROTEIN 16
CmPSRP1	*C. maxima* PHLOEM SMALL-RNA BINDING PROTEIN 1
CmRBP50	*C. maxima* RNA-BINDING PROTEIN 50
CNT	carbon nanotubes
CP	coat protein
CRISPR	Clustered, Regularly Spaced, Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
DARPA	United States Defense Advanced Research Projects Agency
dCas9	deactivated Cas9
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphate
dpi	days post infection
DSB	double-stranded break
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein

GMO	genetically modified organism
gRNA	guide RNA
GUS	β-glucuronidase
HDR	homology directed repair
HR	homologous recombination
kB	kilobase
LS	less symptomatic
MCS	multiple cloning site
miRNA	microRNA
MP	movement protein
mRNA	messenger RNA
MU	4-methyl-umbelliferone
NHEJ	non-homologous end joining
OD	optical density
ORF	open reading frame
PAM	protospacer adjacent motif
PAMP	pathogen-associated molecular pattern
pco-dCas9	plant codon optimised deactivated Cas9
PCR	polymerase chain reaction
PD	plasmodesmata
PEBV	Pea Early Browning Virus
Pol II	DNA-dependent RNA polymerase II
Pol III	DNA-dependent RNA polymerase III
PP2	PHLOEM PROTEIN 2
PSTVd	*Potato spindle tuber viroid*
qPCR	quantitative RT-PCR
RBP	RNA binding protein
RdRP	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
RPM	revolutions per minute
RT	reverse transcription

RT-PCR	reverse transcriptase PCR
SAM	synergistic activation mediator
scRNA	scaffold RNA
SDS	sodium dodecylsulfate
SE	sieve element
sgRNA	subgenomic RNA
SIGS	spray-induced gene silencing
siRNA	small interfering RNA
sRNA	small RNA
SSN	sequence-specific nuclease
T-DNA	transferred DNA
TAD	TAL Activation Domain
TALEN	transcription activator-like effector nucleases
TBE	Tris/Borate/EDTA buffer
TMV	Tobacco mosaic virus
tracrRNA	transactivating CRISPR RNA
TRBO	TMV RNA-based overexpression
TRV	Tobacco Rattle Virus
TSS	transcription start site
VIGE	Virus Induced Gene Editing
VIGS	virus induced gene silencing
VP16	viral protein 16
VP64	Four repeats of VP16
VRC	viral replication complex
VS	very symptomatic
WDV	wheat dwarf virus
wt	wild type
ZFN	zinc-finger nuclease

Chapter 1: Introduction

Developing sustainable agricultural practices is an urgent global priority due to the effects of climate change, pest migration, and population increase (FAO 2016). In addition to being threatened by these phenomena, current agricultural practices are also contributing to the problem by causing a substantial amount of global greenhouse gas emissions as well as harm to land and water resources (Foley *et al.* 2011). The challenge of sustainability is to maintain high enough agricultural yield to support humans while also preserving the planet.

Plant science has responded to these issues by prioritizing the development of sustainable traits such as plants engineered to require fewer external nutrient inputs or to be resistant to drought and flood (Jez *et al.* 2016). Cutting-edge biotechnologies are allowing an unprecedented rate of discovery in these areas.

In recent years, a main focus of plant biotechnology has been gene editing. From disease resistance to yield improvement, nutritional value to fruit ripening, gene editing specific traits in plants is at the forefront of technology that has the capability to transform the crops grown around the world. The potential of gene editing has been catalysed by the discovery of the CRISPR/Cas9 system (Jinek *et al.* 2012, Khatodia *et al.* 2016). While it is already widely used in plant research and industry, CRISPR in plants is still limited compared to its use in animal systems (Cunningham *et al.* 2018).

In this introduction, I will first describe CRISPR/Cas9 gene editing and the challenges to its use in plants. I will then discuss systemic movement of infectious and endogenous RNAs in plants and how their mobility has been manipulated for biotechnology. Bringing these concepts together, I will introduce my project, which focuses on using mobile RNA for improvement of CRISPR/Cas9 gene editing in plants.

1.1 What is gene editing?

Gene editing is the precise modification of target DNA sequences. The first step in gene editing involves a nuclease creating a double-stranded break (DSB) in DNA (Figure 1.1). Once this break is created, the introduction of insertions or deletions into the DNA disrupt or alter the function of the targeted gene (Sander and Joung 2014).

There are two ways in which these broken genes can be repaired – Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). NHEJ occurs when the DNA repair mechanisms join the two ends of the break. However, before the process is complete, the DNA usually degrades or extra bases are added, causing a repair that is not perfectly

identical to the original sequence. These changes often include reading frame shifts or the introduction of stop codons, rendering the gene non-functional. This technology is valuable in research because studying the loss of a gene often reveals its function. NHEJ is also useful for applications for which it is known that the removal of a certain gene confers a desirable trait.

In contrast, HDR requires that a DNA template be artificially introduced at the same time that the gene editing occurs. In this case, the DNA repair mechanisms insert this template code in between the ends of the break site, allowing for the precise manipulation of an endogenous sequence or the addition of an engineered sequence at a specific genomic location.

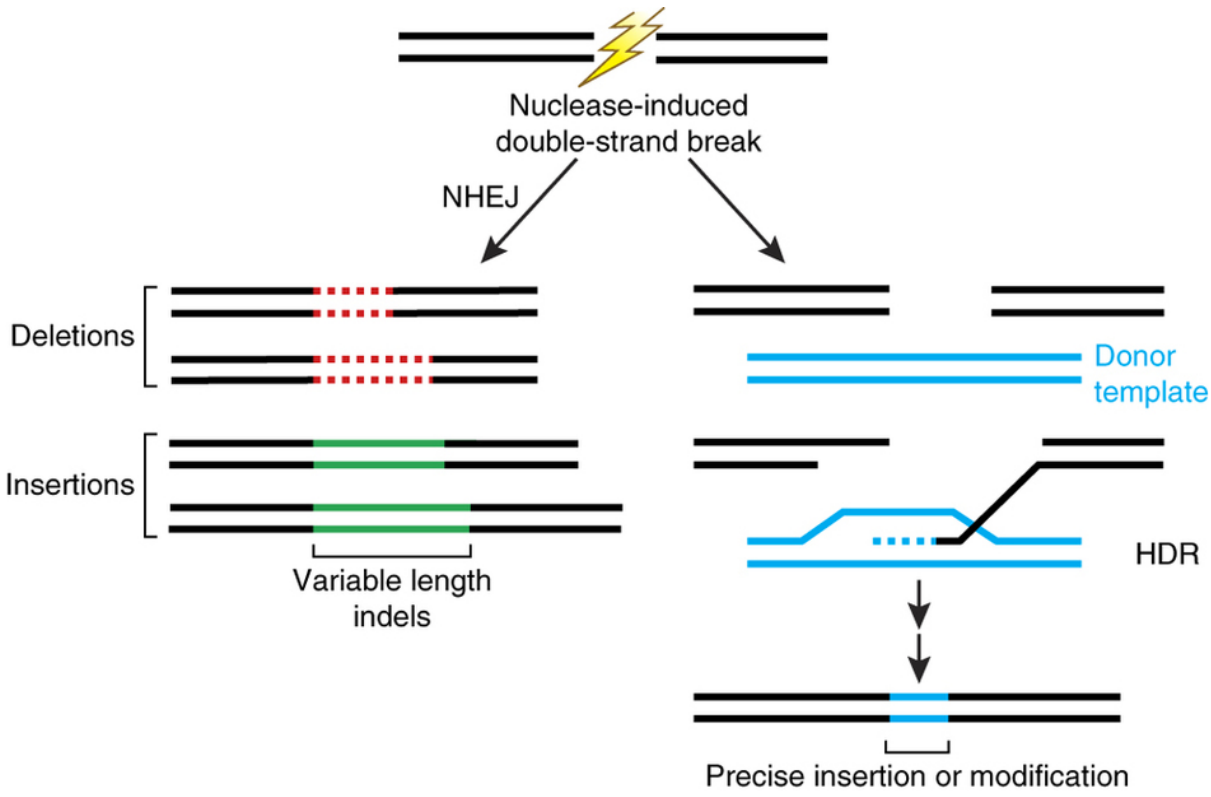


Figure 1.1 Mechanism of gene editing. Gene editing is triggered by a nuclease-induced double-stranded DNA break. Knock outs are caused via non-homologous end joining (NHEJ) in which imperfect DNA repair causes random insertions or deletions at the break site, rendering the gene non-functional. Precise insertion of engineered sequence at the break site can also be achieved via homology directed repair (HDR) in which a template DNA strand must also be provided synthetically. Image from Sander and Joung (2014).

1.2 Targeted nucleases for gene editing

Most gene editing techniques involve inducing DSBs at a defined genomic location using sequence-specific nucleases (SSNs), which recognise and cleave the target DNA (Voytas and Gao 2014).

Zinc-finger nucleases (ZFNs) were the first SSNs to be used for gene editing. It was observed that the *Fok*I type IIS restriction enzyme had a zinc-finger DNA binding domain and a cleavage domain that could be separated (Carroll 2011). Replacing the binding domain allowed targeting of different DNA sequences (Kim *et al.* 1996). ZFNs therefore consist of the non-specific DNA cleaving domain from *Fok*I fused with engineered zinc-finger proteins, which recognise the target DNA (Gaj *et al.* 2013). Individual amino acids on the zinc-finger interact with individual DNA bases (Klug 2010). Therefore, to create high specificity, multiple zinc-finger domains are usually artificially linked together in combination with the *Fok*I domains which requires complicated design and validation (Doudna and Charpentier 2014).

The next SSNs applied to gene editing were transcription activator-like effector nucleases (TALENs). Similar to ZFNs, TALENs use the *Fok*I domain for cleavage but, unlike zinc-fingers, they bind to DNA using repeated domains from TALE proteins (Gaj *et al.* 2013), which are native to the *Xanthomonas* bacteria. Each repeat domain corresponds to one DNA base pair and specificity is determined by two amino acids in the domain (Joung and Sander 2013). TALENs do not require manipulating linkage like ZFNs, however complexity is added regarding the cloning of repeat sequences (Gaj *et al.* 2013). While TALENs are faster to generate than ZFNs, they still are limited by the reliance on protein engineering for DNA target specificity (Doudna and Charpentier 2014).

1.3 Along came CRISPR

1.3.1 CRISPR as a native bacterial adaptive immune system

CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats) is the newest SSN gene editing technology and is derived from the process of forming targeted double-stranded DNA breaks during the process of natural bacterial adaptive immunity. Findings on short repeats separated by other sequences in *Escherichia coli* were first published in 1987 (Doudna and Charpentier 2014, Ishino *et al.* 1987). Twenty years later, Barrangou *et al.* (2007) showed that these repeats were involved in a mechanism for prokaryotic viral defence.

Different species of bacteria use CRISPR systems with varying characteristics. These systems are grouped in two classes; Class 1 requires multiple CRISPR-associated (Cas)

proteins to achieve nucleic acid cleavage while Class 2 only requires one (Wright *et al.* 2016). Biotechnology favours the simplicity of Class 2 systems; frequently used CRISPR nucleases Cas9, Cpf1, and C2c2 are all part of Class 2 (Xu and Qi 2018). The CRISPR-Cas9 Class 2 system of *Streptococcus pyogenes* was the first to be exploited for biotechnology (Jinek *et al.* 2012) and is still the most extensively used form of CRISPR (Wright *et al.* 2016).

The CRISPR locus in the *S. pyogenes* genome contains encodes four proteins involved in the CRISPR process: Cas9, Cas1, Cas2, and Csn2 (Wright *et al.* 2016). It also includes the sequence for a transactivating CRISPR RNA (tracrRNA) and a CRISPR array, in which short 20-50 bp repeat sequences are separated by short unique spacer sequences (Figure 1.2). When foreign DNA enters *S. pyogenes*, small pieces of it are cut and incorporated into the CRISPR array as spacers. The exact mechanism for acquiring these spacer sequences is not well understood and is a field of ongoing research (Wright et al. 2016). Current studies (Heler *et al.* 2015, Wei *et al.* 2015) suggest that Cas9 is able to recognise a protospacer-associated motif (PAM) with the sequence NGG in the invading DNA and then recruits Cas1, Cas2, and Csn2. These proteins form a complex which acquires the spacer sequence from the foreign DNA and integrates it into the CRISPR array (Wright et al. 2016).

The CRISPR array is transcribed resulting in a long precursor CRISPR RNA (pre-crRNA) (Figure 1.2). The tracrRNA, transcribed independently from the pre-crRNA, anneals to the pre-crRNA at a complementary region of the repeat sequence (Jiang and Doudna 2017). After the base pairing of the pre-crRNA and tracrRNA, endogenous RNase III cleaves the pre-crRNA in the non-annealed repeat sequence. The 5’ end of the crRNA is trimmed by an unknown nuclease to a 20nt sequence complementary to the region immediately adjacent to the PAM on the original foreign DNA (Wright *et al.* 2016).

After cleavage of the repeat and 5’ trimming, the mature CRISPR RNA (crRNA) is still annealed to the tracrRNA, and they form a complex with endonuclease Cas9. The crRNA-tracrRNA guides the Cas9 to the region of the invading DNA complementary to the 20 nt crRNA spacer sequence. Once the complex has found the target foreign DNA, each of the two nuclease domains of Cas9 (HNH and RuvC) cuts a strand of the DNA, creating a double-stranded break. This system is able to protect bacteria by adapting to the specific sequences of invasive viral or plasmid DNA.

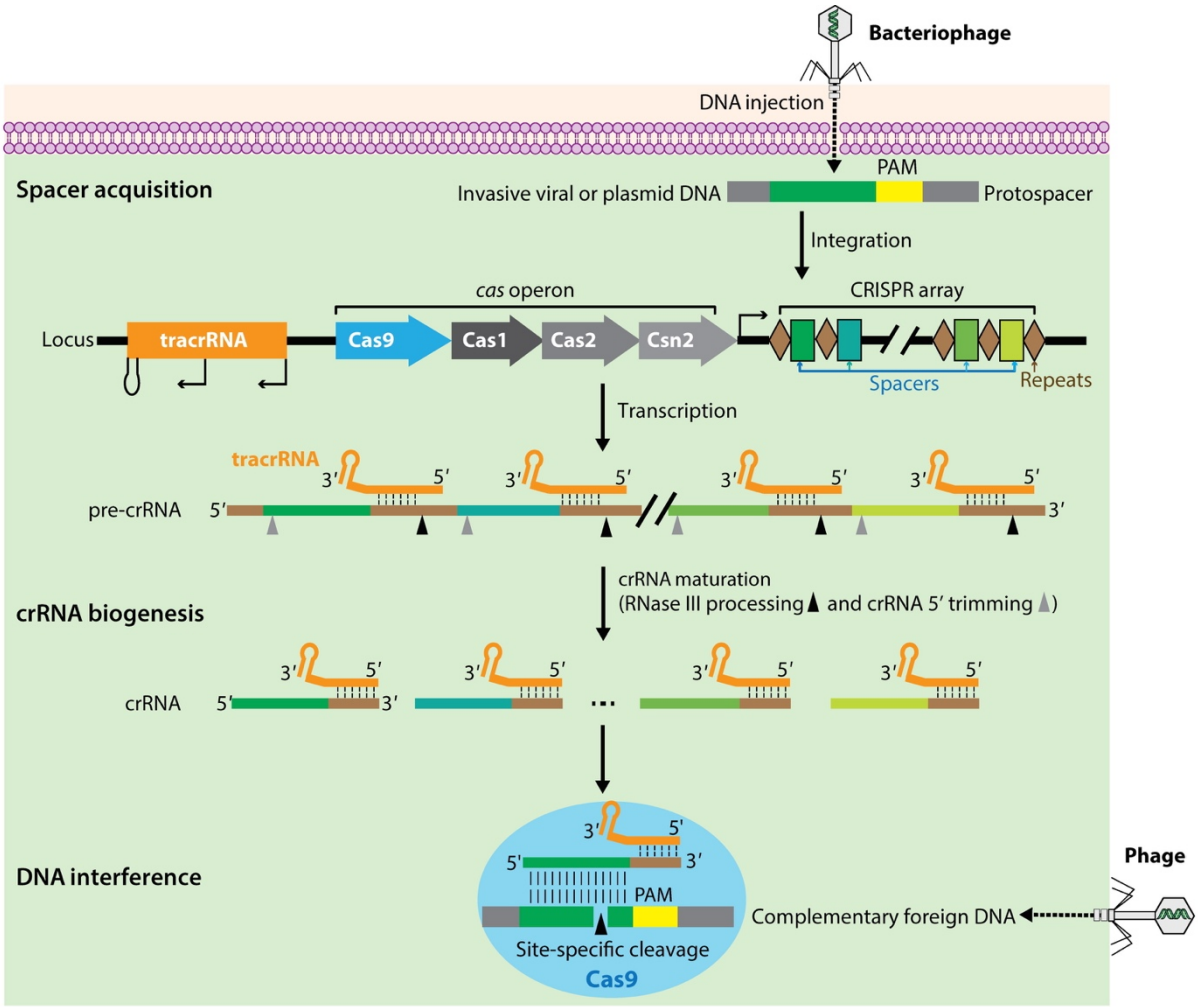


Figure 1.2 CRISPR-Cas9 as a bacterial adaptive immune system in *S. pyogenes*. The bacterial CRISPR locus encodes transactivating CRISPR RNA (tracrRNA), four CRISPR-associated (Cas) proteins, and a CRISPR array containing repeats separated by unique spacers. When foreign DNA enters the bacterial cell, small regions are incorporated into the CRISPR array as spacers, guided by a complex of Cas1, Cas2, and Csn2. The CRISPR array is transcribed into pre-CRISPR RNA (pre-crRNA) which is then annealed with tracrRNA and processed. The mature crRNA-tracrRNA forms a complex with endonuclease Cas9 and precisely cuts the invading foreign DNA at a target site adjacent to a protospacer-associated motif (PAM) in a sequence-specific manner. Image from Jiang and Doudna (2017).

1.3.2 Advantages of CRISPR for biotechnology

To simplify the system for biotechnology, Jinek *et al.* (2012) engineered the crRNA and tracrRNA into a single guide RNA (gRNA). Therefore, CRISPR gene editing has two components: the Cas9 nuclease and a gRNA complementary to the target DNA sequence (Figure 1.3). In 2013, multiple publications demonstrated that the CRISPR-Cas9 system could be used for gene editing in eukaryotes (Doudna and Charpentier 2014).

CRISPR has distinct advantages over the other SSN gene editing methods because the specificity determinant is via base pairing of an RNA rather than through a protein with more complex DNA targeting rules. Creating a gRNA is faster, easier and less expensive than protein engineering (Cunningham *et al.* 2018). In addition, multiple genes can be targeted at the same time if more than one gRNA is introduced into the system (Cunningham *et al.* 2018). This property is beneficial for studying or engineering traits that are controlled by a gene network instead of a single gene.

1.3.3 Modifications to CRISPR for transcriptional regulation

It is also possible to mutate Cas9's catalytic residues (D10A and H840A) so that it can still form a complex with the gRNA and sit on the target DNA sequence, but not cut it (Qi *et al.* 2013, Bikard *et al.* 2013). This version is called deactivated Cas9 (dCas9) (Figure 1.4). When dCas9 is bound to DNA, it can block other enzymes such as transcription factors or polymerases from reaching the DNA, therefore impairing the transcription of the target gene. This technique was the first form of CRISPR interference (CRISPRi), in which dCas9 is used for transcriptional repression (Qi *et al.* 2013, Bikard *et al.* 2013). Gilbert *et al*. (2013) improved the effect of CRISPRi by fusing dCas9 to known transcriptional repressors such as the zinc finger KRAB domain which is involved in epigenome modification (Wiznerowicz *et al.* 2007).

Similarly, CRISPR activation (CRISPRa) using dCas9 fused to a transcriptional activator to increase target gene expression has also been established (Gilbert *et al.* 2013, Maeder *et al.* 2013, Perez-Pinera *et al.* 2013). These studies used transcriptional activator VP64, a tetrameric repeat of the Viral Protein 16 (VP16) activation domain from Herpes Simplex Virus (Beerli *et al.* 1998). Other activator domains have also been shown to function for CRISPRa (Gilbert et al. 2013, Piatek *et al.* 2015, Li *et al.* 2017), but most advancement of CRISPRa has continued to use dCas9-VP64 as the main transcriptional activator fusion component (Dominguez *et al.* 2016). Only recently has CRISPRa been applied for epigenetic modification, and these studies are discussed in Chapter 6.

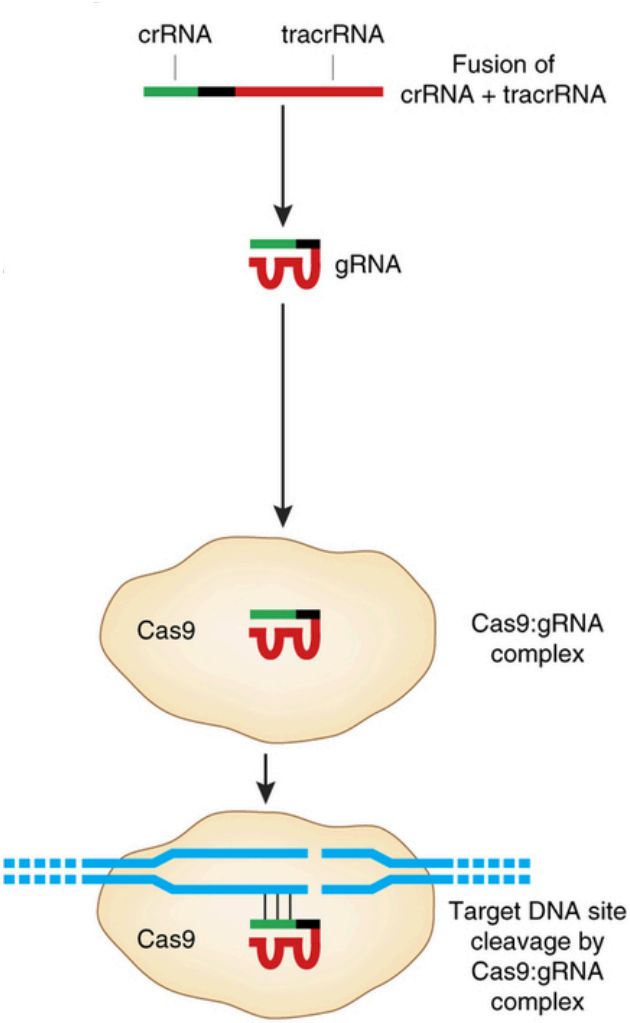


Figure 1.3 CRISPR system for biotechnology. Instead of a separate crRNA and tracrRNA, they have been fused into a single guide RNA (gRNA) that combines with Cas9 to target a specific sequence. This simplification allows for a two-component gene editing system. Image from Sander and Joung (2014).

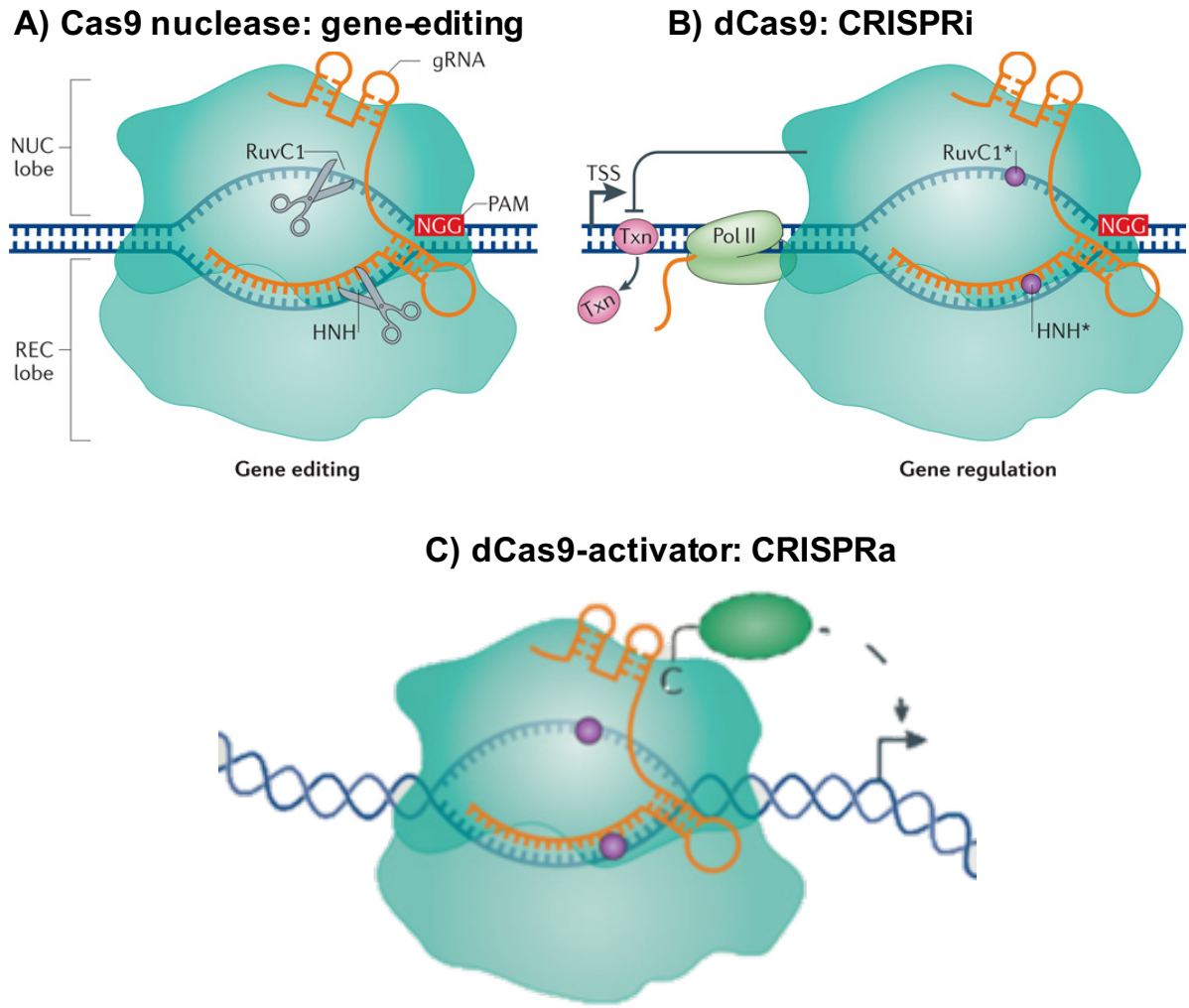


Figure 1.4 Modifications to Cas9 for CRISPRi and CRISPRa. A) A functional Cas9 endonuclease interacting with gRNA to cause a double-stranded DNA break at a target site. B) deactivated Cas9 (dCas9) can sit on target DNA matching the gRNA sequence, but is unable to cut. The dCas9 can block proper transcription by RNA polymerases (Pol II shown in green) or inhibit the binding of essential transcription factors (Txn). This is a method of transcriptional repression and is called CRISPR interference (CRISPRi). C) Shows how fusion of a transcriptional activator to dCas9 can turn the system into CRISPR activation (CRISPRa) in which the expression of a gene downstream of the gRNA target site is increased. Figures adapted from Dominguez *et al.* (2016).

1.3.4 Current limitations to CRISPR in plants

In order to achieve the goal of using CRISPR gene editing to develop crops for sustainable agriculture, it is essential that the process works efficiently in plants. Unfortunately, there are still considerable technical obstacles to effective implementation (Altpeter *et al.* 2016).

Most CRISPR applications in plants include stably transforming the genes encoding Cas9 and gRNA into the plant in order to induce the gene editing process (Figure 1.5). Methods to integrate these genes include callus bombardment, protoplast transformation, and transformation with *Agrobacterium tumefaciens* (Belhaj *et al.* 2015). The transgenic plants regenerated from these methods express the Cas9 and gRNA, allowing the CRISPR mechanism to target the desired gene. The plants are then backcrossed and progeny are selected in which the transgenes are absent, but the targeted CRISPR mutation remains.

The transformation methods involved in the CRISPR process all rely on tissue culture, whether it be a protoplast, callus, or explant. Tissue culture is known to be associated with unanticipated genetic and epigenetic mutation, causing trait variability which then needs to be further selected (Stroud *et al.* 2013). This side effect of tissue culture is detrimental to the goal of the precision CRISPR gene editing.

In addition to the unpredictability of tissue culture, it is a long process. It can take more than six months to create the desired plant with a CRISPR mutation. With current methods, every time there is a new editing target, the whole process must be repeated.

It has been identified that tissue culture steps are a bottleneck for CRISPR gene editing in plants, especially crop species (Altpeter *et al.* 2016). New delivery mechanisms are needed to reduce reliance on tissue culture and decrease time required for the process in order for CRISPR to be a transformative tool in plant biotechnology.

A considerable step toward overcoming this limitation would be to introduce the gRNA and Cas9 separately. Whenever a new target is selected the only variable component is the gRNA. If the gRNA can be delivered in trans, then only one tissue culture step would be necessary to stably transform the Cas9 or dCas9. The same Cas9 or dCas9 transgenic line could be used for every application while only changing the gRNA delivered in trans, reducing the effects caused by repeated tissue culture. In addition, using a line hemizygous for the Cas9/dCas9 would allow easy segregation in the progeny, generating transgene-free mutants.

The next sections focus on harnessing the capabilities of natural biological processes to generate new methods of introducing gRNA for CRISPR in plants.

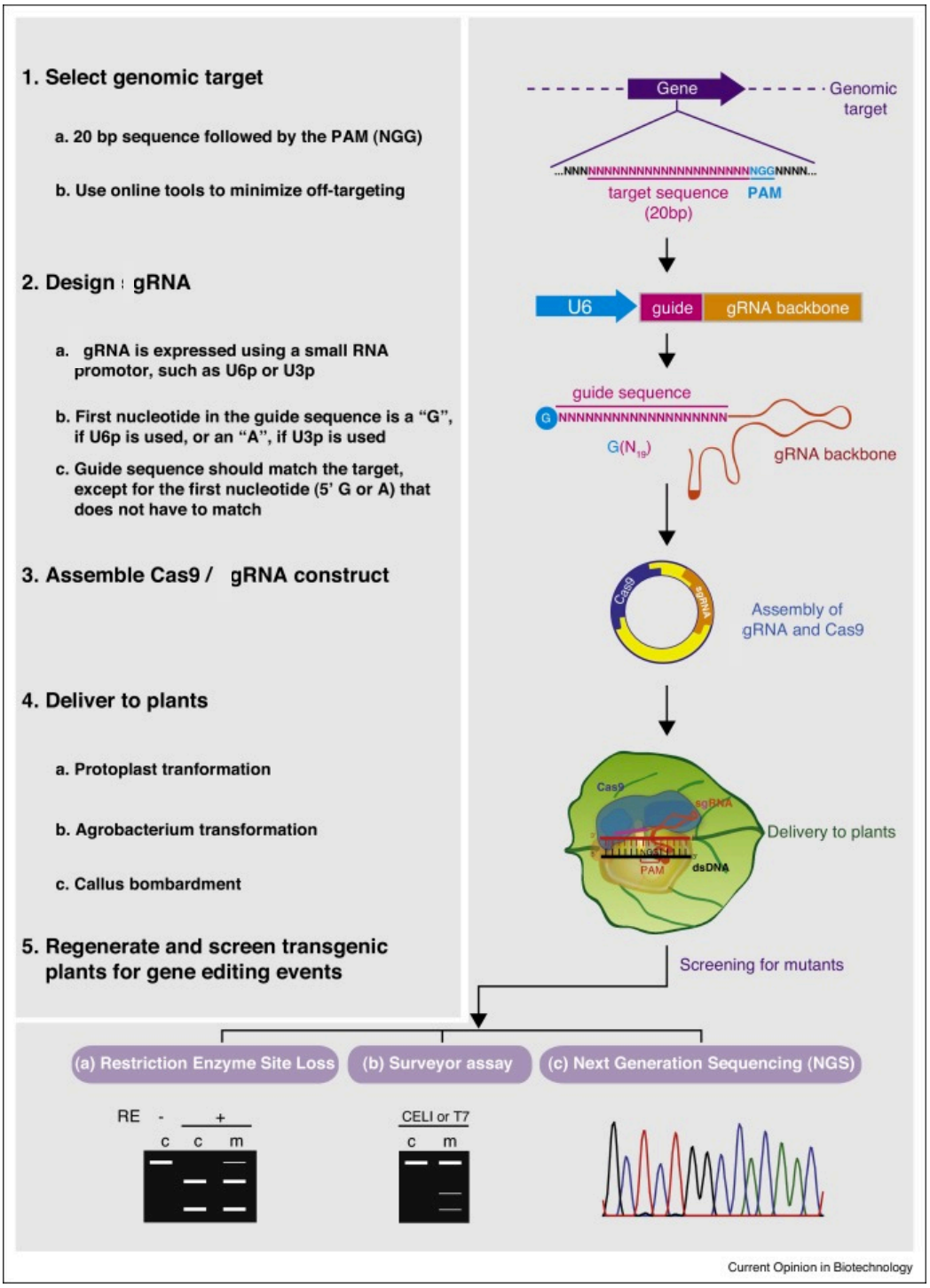


Figure 1.5 Current CRISPR method in plants. Once the gene target has been selected and gRNA designed, the constructs must be delivered to the plant. Current methods all conclude with a form of tissue culture followed by screening for mutations in the target sequence. Figure from Belhaj *et al.* (2015).

1.4 RNA mobility in plants: natural systems and use in biotechnology

In order to deliver the gRNA in trans and still cause heritable mutations, the gRNA would either need to be delivered directly to pre-germline cells expressing the nuclease or be produced in other cells and trafficked into pre-germline cells. With the aim of reducing tissue culture, the latter is a more feasible approach and would require exploiting natural systems for RNA mobility in plants to transport the gRNA.

1.4.1 Phloem transport

Classes of both endogenous and pathogenic RNAs are mobile in plants, and move long distances through the phloem vasculature (Ham and Lucas 2017). The phloem is responsible for transporting nutrients from photosynthetically productive leaves (source tissues) into developing tissues and roots (sink tissues). Phloem's unique structure consists of companion cells (CC) which have full cellular function connected via plasmodesmata (PD) to specially differentiated enucleate sieve elements (SE) (Figure 1.6). Cell files of SEs are joined to each other by open sieve plate pores (SPP) instead of plasmodesmata, allowing a continuous flow throughout the plant. Molecules for transport are loaded from the companion cells into the sieve element cell and then move with the phloem pressure gradient to sink tissues (Melnyk *et al.* 2011, Ham and Lucas 2017).

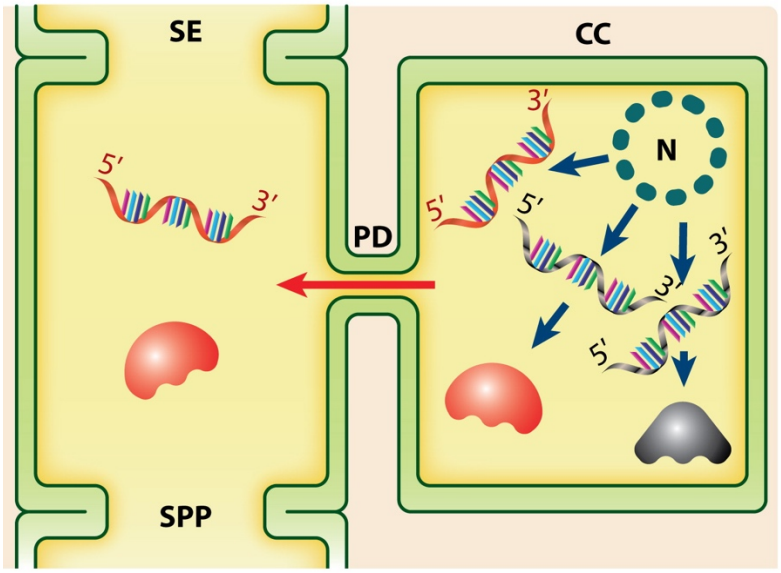


Figure 1.6. Phloem structure schematic. RNA and proteins generated in companion cells (CC) that have a nucleus (N) move into the enucleate phloem sieve elements (SE) via expandable channels called plasmodesmata (PD). Specially differentiated sieve plate pores (SPP) allow for flow through the phloem tube. Figure from Ham and Lucas (2017).

1.4.2 RNA movement for infection and use of viral vectors

1.4.2.1 Viroids

Viroids are perhaps the most mysterious plant pathogen. They are single-stranded, circular, noncoding RNAs ranging from 239-401nt in length and their origins are unknown (Kovalskaya and Hammond 2014, Takeda and Ding 2009). They exploit long-distance systemic transport via the phloem for infection. Viroids do not encode any proteins of their own, so they have to rely entirely on host machinery (Kovalskaya and Hammond 2014). The secondary structure of viroid RNA, comprised of helices and loops, is essential to the interaction with the proteins of host plants. Site-directed mutagenesis in specific structural domains in *Potato spindle tuber viroid* (PSTVd) showed that various sequence motifs played important roles in viroid replication, movement, and infection (Takeda and Ding 2009, Kovalskaya and Hammond 2014).

Various RNA-binding proteins (RBPs) in plants have been identified which interact with viroids to enable systemic movement. For example, PHLOEM PROTEIN 2 (PP2) has been shown to be a viroid RBP, shuttling viroid RNA through the phloem in a grafted system (Kehr and Kragler 2018, Ham and Lucas 2017, Kovalskaya and Hammond 2014). Since it is sequence non-specific and functions by increasing the plasmodesmata size exclusion limit, it is likely that PP2 and other viroid RBPs are also RBPs for many categories of RNA (Ham and Lucas 2017).

1.4.2.2 Plant viruses

Plant viruses also move through the phloem for systemic spread (Heinlein 2015). They rely on viral movement proteins (MP) encoded in their genomes which expand the plasmodesmata to allow cell-to-cell passage. These MPs are sequence independent, and there is therefore ongoing research on how the MP associates specifically with viral nucleic acid (Heinlein 2015). Current work suggests that the MPs associate with viral replication complexes (VRCs) which would cause the MP to only bind to the viral genome. It seems some VRCs unload viral RNA-coat protein complexes through plasmodesmata, while other viruses may move as intact VRCs (Heinlein 2015).

Biotechnology has benefitted from the use of viruses as vectors to deliver sequences of interest to plants for applications such as expressing recombinant proteins or inducing post-transcriptional gene silencing. These techniques became especially widespread after the discovery that viral genomes could be expressed as in a T-DNA binary vector for agroinfiltration (Grimsley *et al.* 1986) instead of previous methods which involved mechanical

inoculation (Peyret and Lomonossoff 2015). Different families of plant viruses are beneficial for different applications.

The genus *Tobravirus* contains an RNA genome that can be easily manipulated for delivery of RNA sequences in trans (Macfarlane 2010). A member of the genus, Tobacco Rattle Virus (TRV), has become especially popular for Virus Induced Gene Silencing (VIGS), a technique of triggering the plant's RNA silencing-based immune system against an endogenous gene by using the virus to introduce a bit of RNA that matches the target sequence (Figure 1.7). During the process of viral replication, the plant's RNA silencing machinery recognises the double-stranded RNA, and DICER-like proteins cut it into small dsRNA fragments which become small interfering (siRNAs). The siRNAs generated from the sequence inserted in the viral vector target the endogenous gene and cause post-transcriptional gene silencing (Becker and Lange 2009).

TRV vectors have also been used for gene editing. Meristem invasion capability (Martin-Hernandez and Baulcombe 2008) and the ability to infect a wide variety of plant species are traits that make TRV a desirable vector choice for genetic engineering. Marton *et al.* (2010) used TRV to deliver the coding sequence of a ZFN to *Nicotiana benthamiana*, *Nicotiana tabacum*, and *Petunia hybrida*. The infected plants were edited, and ZFN-induced mutations were detected in the next generation.

At the beginning of my PhD, there were three examples of Virus Induced Gene Editing (VIGE) for the CRISPR system, but none were able to reliably demonstrate inheritance of the induced mutation. Ali *et al.* (2015) showed editing in infected plants using a TRV vector to introduce gRNA to transgenic Cas9 *N. benthamiana* and claimed two lines of mutant progeny. However, the observed inheritance was disputed due to the minimal reported mutation rate compared to expected inheritance calculations (Yin *et al.* 2015).

Geminiviruses have also been used for VIGE. The geminivirus Cabbage Leaf Curl Virus (CaLCV) was employed by Yin *et al.* (2015) to deliver gRNA to transgenic Cas9 expressing *N. benthamiana*. The infected plant exhibited targeted mutations. Unlike TRV, CaLCV does not move to the germline (Yin *et al.* 2015). The study's proposed inheritance pathway involves regenerating edited plants from infected systemic tissues. Geminiviruses are excluded from the plant during the regeneration process (Yin *et al.* 2015), so the cultured plants would be virus-free.

Baltes *et al.* (2014) also chose a geminivirus vector for gene editing. Their deconstructed bean yellow dwarf virus (BeYDV) vector, engineered to overcome insert size limits by removing the coat and movement proteins, was non-mobile but able to deliver the

Cas9 protein and gRNA simultaneously in *N. tabacum*. The local expression of this system does not lend itself to inheritance in the progeny and would also require tissue culture to generate the desired plant. However, the strong benefit of this system is that no transgenes are required for editing since all components are included in the viral vector.

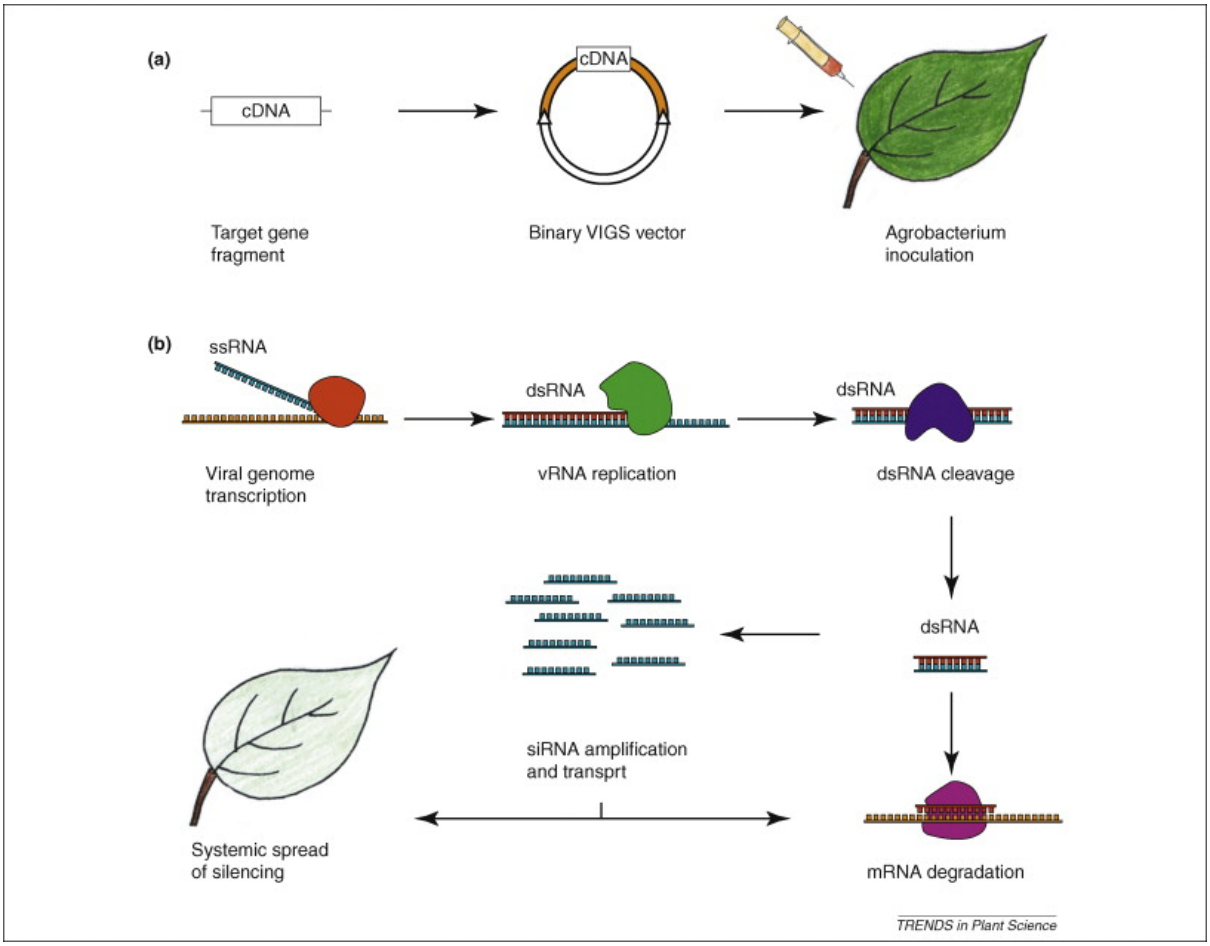


Figure 1.7 Schematic of Virus Induced Gene Silencing (VIGS). A) Shows how the designed RNA is inserted into a vector containing viral genome cDNA and is inoculated to the plant via agroinfiltration. B) Illustrates the process of post-transcriptional gene silencing caused by the siRNA encoded by the viral construct. Figure from Becker and Lange (2010).

1.4.3 Endogenous RNA movement and use of grafting

1.4.3.1 Classes of mobile endogenous RNA

Analyses of phloem sap from various plant species revealed that most categories of endogenous RNAs exist in the phloem, including messenger RNA (mRNA), small interfering RNA (siRNA), and microRNA (miRNA) (Ham and Lucas 2017). Since the sieve element cells do not have nuclei, the RNA must be produced elsewhere and trafficked into the phloem. Grafting assays allow for deeper study of which RNAs are mobile by spatially separating the production of RNAs from the destination sink tissue.

For example, Thieme *et al.* (2015) grafted together different ecotypes of *Arabidopsis thaliana* with distinct transcript variation and used sequencing data to distinguish mRNAs mobile across a graft junction. They found that mRNA moved both from scion to stock and also from root to shoot, especially to the flowers. The selection of which mRNAs enter phloem transport is contested; notably Calderwood *et al.* (2016) suggest that abundance of a certain mRNA in the companion cells is the major factor in determining whether it enters the phloem, not its function. No matter how they are chosen for long distance movement, it has been shown using grafting that mRNA in the phloem can have considerable phenotypic effects on the destination tissue, like leaf shape in tomato (Haywood et al. 2005, Kim et al. 2001) (Figure 1.8a).

Both microRNAs (miRNA) and small interfering RNAs (siRNA) are found in phloem sap (Yoo *et al.* 2004, Buhtz *et al.* 2008). The long distance mobility and function of both kinds of these small RNAs (sRNAs) have also been demonstrated in grafting assays, showing silencing phenotypes in destination sink tissues (Figure 1.8b) (Molnar *et al.* 2010, Lewsey *et al.* 2016, Pant *et al.* 2008, Dunoyer *et al.* 2010:retracted).

1.4.3.2 Mechanisms of movement

There is a limit to the size of molecules that can pass freely through plasmodesmata, but under specific signalling, the plasmodesmata can expand in order to let larger molecules through (Melnyk *et al.* 2011). There is evidence that endogenous RNAs combine with RNA-binding proteins (RBPs) which act similarly to viral movement proteins, binding RNA in a non-sequence-specific manner and increasing the size exclusion limit of the plasmodesmata in order to allow the RNA to be loaded into the phloem (Ham and Lucas 2017) (Figure 1.9).

The first mRNA RBP to be discovered was *Cucurbita maxima* PHLOEM PROTEIN 16 (CmPP16). It was observed to behave similarly to a viral movement protein in the way it increases plasmodesmata size exclusion limit (Xoconostle-Càzares *et al.* 1999). It also binds

RNA in a non-sequence specific manner, like viroid RBP PP2, mentioned above (Ham and Lucas 2017). *C. maxima* RNA-BINDING PROTEIN 50 (CmRBP50) was demonstrated to form a complex with *CmGAIP* mRNA, one of the long-distance mobile mRNAs previously functionally studied via grafting (Haywood *et al.* 2005). Interestingly, grafting assays showed an intact CmRBP50 ribonucleoprotein (RNP) complex in the phloem sap of destination tissue (Ham *et al.* 2009), suggesting that CmRBP50 does not just participate in loading the mRNA into the phloem, but that the complete RNP moves systemically.

sRNAs are also known to be associated with RBPs for long distance systemic movement (Ham and Lucas 2017). For example, *C. maxima* PHLOEM SMALL-RNA BINDING PROTEIN 1 (CmPSRP1) binds to single-stranded sRNA and increases plasmodesmata size exclusion limit like other known RBPs (Yoo *et al.* 2004). It does not bind mRNA or double-stranded sRNA. Interestingly, in order to function upon reaching destination tissues, sRNA would need to dissociate from a movement protein in order to bind to proteins involved in the silencing mechanism. Indeed, it was found that the CmPSRP1 RNP complex destabilised when exposed to sink tissue phloem sap, but not source tissue phloem sap (Ham *et al.* 2014). This finding suggests that the single-stranded sRNA can separate from phloem movement proteins upon reaching sink tissues in order to function in its silencing capacity.

sRNA may also be able to move as a double-stranded duplex without an RBP. The phloem sap is RNAse-free, so the sRNA could be stable in its double-stranded form as it moves systemically (Melnyk *et al.* 2011). Once outside of the vasculature, one of the strands is destroyed in order for the single stranded sRNA to load into the silencing protein complex (Melnyk *et al.* 2011).

1.4.3.3 Grafting in biotechnology

Basic research has shown that RNA can provide a function in trans via a graft. This knowledge is beginning to be applied to crop improvement through transgrafting, in which a genetically engineered root stock is grafted with a wild type scion with the purpose of inducing certain traits in the commercial product (Haroldsen *et al.* 2012). This method of RNA delivery holds promise for many beneficial purposes such as increasing nutrient uptake and resistance to disease and pests (Haroldsen *et al*. 2012, Nawaz *et al.* 2016). Graft delivery of gRNA to the CRISPR system has not yet been reported.

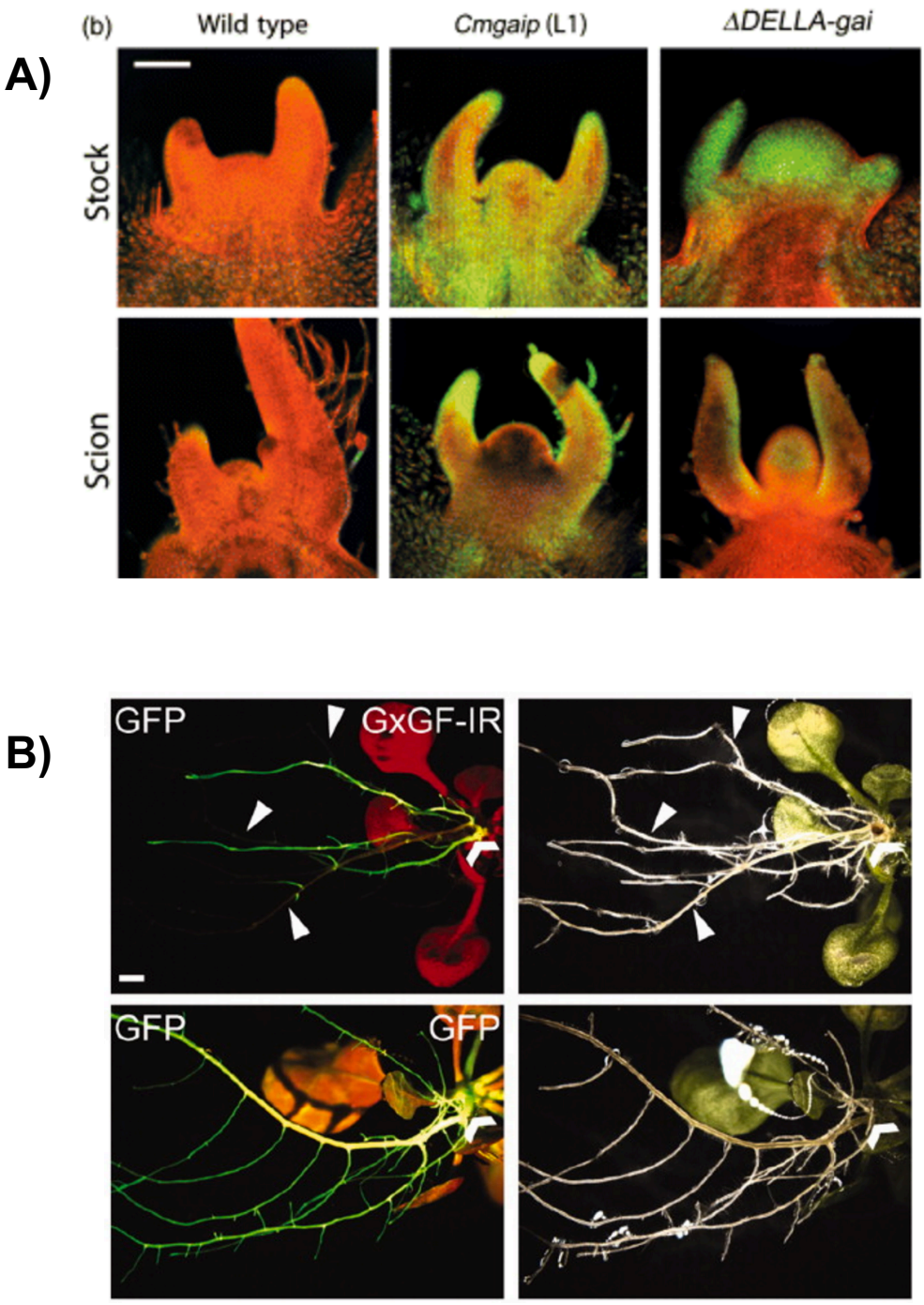


Figure 1.8 Demonstrations of function mobile RNAs. A) Haywood *et al.* 2005 showed via *in situ* RT-PCR that mutant mRNA transcripts (shown in green) accumulate in the apices of wild type scions grafted onto mutant stock. In maturity, the leaves from these scions showed a mutant phenotype, demonstrating that the mRNA was both mobile and functional upon arriving at the destination tissue. B) Molnar *et al.* 2010 demonstrated the mobility and function of siRNAs from shoot to root by grafting a GFP silencing line scion to a GFP expressing root. siRNAs moved across the graft junction and silenced GFP in the root. Figures from Haywood *et al.* (2005) and Molnar *et al.* (2010).

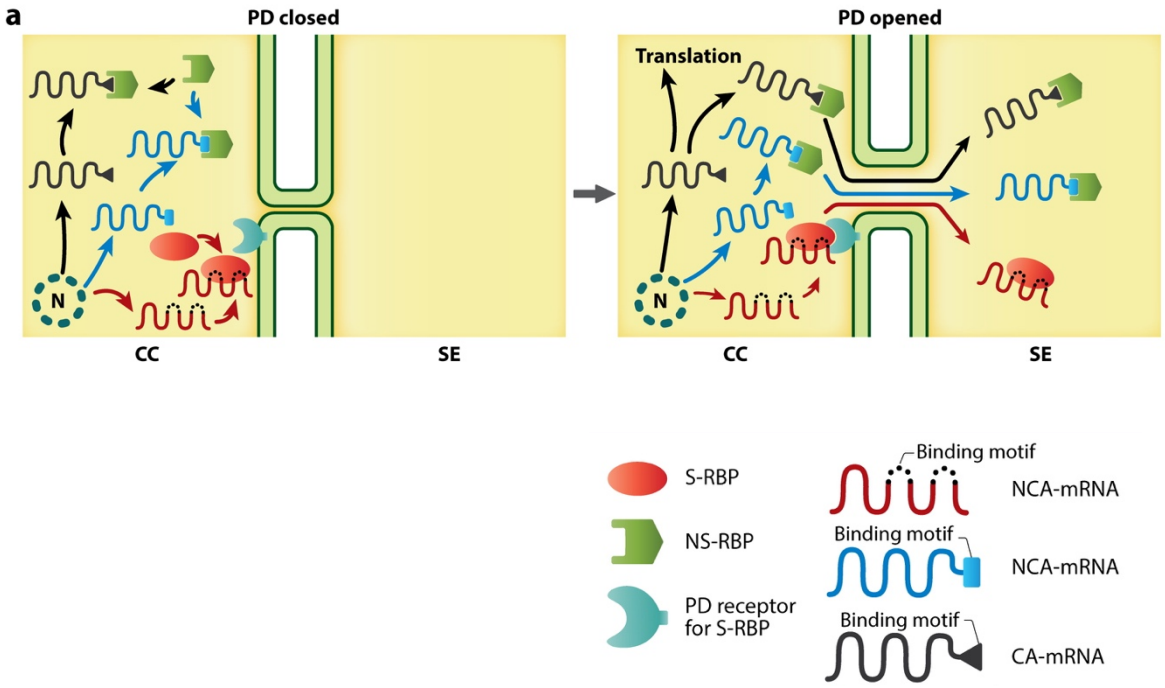


Figure 1.9 Schematic mobile mRNA trafficking into the phloem. When plasmodesmata (PD) are closed, it is difficult for RNA molecules to move from the companion cells (CC) to the sieve elements (SE) of the phloem. RNA binding proteins (RBPs) can increase the size exclusion limit of the PD and allow RNAs into the phloem. In the figure, non-cell autonomous (NCA) mRNAs shown in red and blue either bind with sequence specific RBPs (S-RBP) or non-specific RBP (NS-RBP) to get channeled into the phloem. The mRNA shown in black is cell autonomous (CA) and represents how sheer abundance of certain mRNAs in CCs may cause them to be trafficked into the phloem and do not actually have a reason to be mobile, based on the reporting of Calderwood *et al.* (2016). Figure modified from Ham and Lucas (2017).

1.5 Project goals

This work in this thesis focused on addressing the challenges of using CRISPR gene editing in plants by exploring non-transgenic gRNA delivery methods based on natural systems of mobile RNA. The project had three main objectives:

Objective 1: Generate a CRISPRa system in plants optimised for testing gRNA delivery in trans.

I chose to work with CRISPRa transcriptional activation in order to have a positive readout assay that could be easily analysed. At the time of starting this project, there were only two demonstrations of CRISPRa in plants (Lowder *et al.* 2015, Piatek *et al.* 2015). These systems were designed either only for transient expression or stable transformation of all components of the system in the same construct. Therefore, my first goal was to create a CRISPRa system in plants designed specifically for exploring various methods of gRNA delivery.

Objective 2: Test viral delivery of gRNA to transactivate the CRISPRa system.

Next, I set up experiments to test the efficiency of delivering gRNA in trans via a viral vector. While there had already been attempts at Viral Induced Gene Editing (VIGE) when this project began (Ali *et al.* 2015, Yin *et al.* 2015, Baltes *et al.* 2014), none were able to reliably achieve heredity of the CRISPR mutation to the next generation. My transactivation system was designed to be able to analyse where the flaw was occurring, in order to improve VIGE as a potential useful method in the future.

Objective 3: Explore graft delivery of gRNA to the CRISPRa system.

Since grafting is already a commonly used technique for commercial crop production, graft delivery of gRNA for CRISPR would be a useful technical advance. In addition, there is evidence for most classes of endogenous RNAs to be mobile in the phloem, but there has been no study to date on whether gRNA can move systemically in plants. Exploration of gRNA movement would add to the ongoing body of work on the mobility of different classes of RNAs by testing a synthetic RNA that may not be designed to interact with host proteins.

1.5.1 Model plant species selection

Nicotiana benthamiana is an advantageous model organism for all three objectives of this project. First, it is frequently used for its ability to express transgenes transiently upon agroinfiltration (Bally e*t al.* 2018, recently used for CRISPRa by Piatek *et al.* 2015, Lowder *et*

al. 2015) and was therefore a logical choice for testing the constructs of my CRISPRa system developed under Objective 1.

N. benthamiana has also become the standard species for plant-virus studies such as VIGS and, more recently, VIGE. A mutation in a gene directly involved in plant immune response (RNA-dependent RNA polymerase 1 *Rdr1*) causes *N. benthamiana* to be highly susceptible to infection (Yang *et al.* 2004, Bally *et al.* 2018). This characteristic made it a suitable model plant for Objective 2 of my project. In addition, there are many examples of successful grafting of *N. benthamiana* which is beneficial for the grafting work included in Objective 3.

Finally, since it is a member of the family *Solanaceae*, which also includes important crop plants such as tomato, pepper, potato, and aubergine, the findings of gene editing assays in *N. benthamiana* could be easily translated to crop applications in the future.

Chapter 2: Materials and Methods

2.1 Bacteria

2.1.1Bacterial strains used:

Strain	Antibiotics	Competency/source
E. coli dH5α	N/A	Chemical/Lab stock
E. coli One Shot Top10	N/A	Chemical/Invitrogen
E. coli One Shot *ccdB* Survival 2 $T1^R$	N/A	Chemical/Invitrogen
A.tumefaciens C58C1(pSoup)	Rif, Tet, Gent	Electro/Lab stock
A. tumefaciens GV3101 (pSoup)	Rif, Tet, Gent	Electro/Lab stock

Lab stocks were prepared by James Barlow.

2.1.2 Chemical Transformations (*E. coli*)

For dH5α prepared in the lab, 50μL of cells were thawed on ice until just thawed. 2-4μL of plasmid was added to the cells, gently flicked to mix, and then kept ice for 20 minutes before a heat shock at 42°C for 60 seconds and immediately returning to ice for 2 minutes. 950μL of room temperature SOC medium was added to the cells and mixed by inverting. The mixture was incubated at 37°C with shaking for an hour and then spread on LB-agar plates with the appropriate antibiotics.

For transformation of chemically competent One Shot Top10 and One Shot *ccdB* Survival 2 $T1^R$, the manufacturer's protocol was followed.

2.1.3 Electroporation (*A. tumefaciens*)

First, sterile water, SOC media, and 1mm cuvettes were chilled on ice. Pre-prepared 50μL aliquots of electro-competent *A. tumefaciens* were thawed on ice. In a sterile hood, the competent cells were diluted using 200μL of cold sterile water for every 50μL tube of cells and mixed. The cell dilutions were divided into 50μL aliquots. 1μL of purified plasmid was added to the 50μL cell dilution. The mixtures were transferred into labelled cuvettes, taking care to not cause any air bubbles. Each cuvette was inserted into the Bio-Rad GenePulser Xcell and electroporated with the pre-set *A. tumefaciens* programme (voltage 2400V, capacitance 25 μF, resistance 200Ω, 1mm cuvette). 950μL cold SOC medium was quickly added to the cuvette and mixed with the electroporated cells before transferring back into the corresponding

Eppendorf tubes on ice. The tubes were shaken at 28°C for 2-4 hours to allow the cells to recover before spreading 50-100μL of the culture on plates with appropriate antibiotics. Colonies could be observed 48 hours after transformation.

2.1.4 Glycerol stocks

Glycerol stocks were made by mixing 800μL of the overnight bacterial culture with 600μL autoclaved 100% glycerol in a Nunc CryoTube Vial (Thermo Scientific). Stocks were stored at -80°C.

2.1.5 Bacterial plates and liquid growth conditions

Plates for all bacterial applications were made by mixing heated 2X LB broth and 2% (w/v) Agar, adding appropriate selection antibiotics, and pouring into 90mm round petri plates.

E. coli from glycerol stocks were grown in LB broth with appropriate antibiotics at 37°C with shaking overnight.

A. tumefaciens from glycerol stocks were grown in LB broth with appropriate antibiotics at 28°C for 48 hours.

LB broth, agar, and antibiotics were all prepared by lab technician James Barlow.

2.2 *N. benthamiana*

2.2.1 Stable transformations

A. tumefaciens C58C1 (pSoup) was cultured in 10mL LB broth with appropriate antibiotics for 48 hours, then pelleted and re-suspended in infiltration medium to an OD of 0.4 at 600nm. Using a needle-less 1mL syringe, leaves of 4-week old *N. benthamiana* were completely infiltrated with the culture. 3 days later, the leaves were harvested and cut into squares after large veins had been removed. The squares were placed in stainless steel mesh infusion balls and surface sterilise in 70% (v/v) ethanol for 5 minutes. Next, the infusion balls were immersed in 20% (v/v) sodium hypochlorite and Tween for 10 minutes. They were then rinsed four times in in sterile water, 10 minutes per rinse. Flame-sterilised curved forceps were used to place individual leaf squares onto shooting medium plates with selection antibiotics adaxial side up, leaving space between the squares. The plates were sealed with a double layer of of micropore tape and placed in a growth chamber at 24°C with 16h days.

Explants were transferred to fresh selection plates as needed, and shoots began to appear around 4 weeks later. Shoots were cut with a flame-sterilsed scalpel, and transferred into rooting medium with selection antibiotics. Around 2 weeks later, roots began to appear and plantlets were transferred to sterilised peat blocks. When the plantlets seemed stable, they were moved to non-sterile soil and kept under propagation lids for the first few days before gradual decrease in humidity.

Agroinfiltration medium

Ingredient Stock	Amount added for 10 mL	Final Concentration
100 mM $MgCl_2$/100 mM MES pH 5.6	1 mL	10 mM/10 mM
H_2O	9 mL	
150 mM acetosyringone (Sigma-Aldrich)	10 µL	150 µM

Shooting Medium

Ingredient Stock	Amount added for 1 L	Final Concentration
Murashige and Skoog with Gamborg B5 vitamins	2.2 g	2.2 g/L
Sucrose	10 g	1% (w/v)
Agargel	5 g	0.5% (w/v)
pH 5.7		
After autoclaving and while still molten, add the following:		
BAP at 1 g/L	2 mL	2 mg/L
NAA at 0.1 g/L	0.5 mL	0.05 mg/L
Cefotaxime at 500 mg/L	2 mL	1 mg/L
Timentin at 320 mg/L	1 mL	0.32 mg/L
Selection: Kanamycin at 100 mg/L	2 mL	0.2 mg/L

Rooting Medium

Ingredient Stock	Amount added for 1 L	Final Concentration
Murashige-Skoog basal salt mixture	2.15 g	2.15 g/L
Sucrose	5 g	0.5% (w/v)
Gelrite/Gelzan	2.5 g	0.25% (w/v)
pH 5.8		
After autoclaving and while still molten, add the following:		
NAA at 0.1 g/L	0.5 mL	0.05 mg/L
Cefotaxime at 500 mg/L	2 mL	1 mg/L
Timentin at 320 mg/L	1 mL	0.32 mg/L
Selection: Kanamycin at 100 mg/L	2 mL	0.2 mg/L

Protocol from Temur Yenusov, The Sainsbury Lab Cambridge University.

2.2.2 Crossing

It was important to ensure that flowers had not yet been self-pollinated before crossing. Flowers that had not yet bloomed and were still slightly green were opened using forceps and the immature anthers removed. The petals were cut so that the stigma was just exposed. Pollen from mature open flowers was harvested into the cap of a 2mL tube. The pollen in the cap was then pressed onto the exposed stigma. The pollinated flower was marked with string to denote it was the product of a cross.

2.2.3 Grafting

The technique used for full plant grafting of *N. benthamiana* was based on the conditions of available growth chambers. First, *N. benthamiania* seeds were sown in 24-pot trays which allowed the plants to shoot up competing for the light instead of staying close to the soil. Five weeks after sowing, the plants were moved to larger individual pots to prepare for grafting. Plants were grafted using a V-cut at six weeks post sowing and held together with a grafting clip. Before the actual grafting, apical meristems and all leaves except two were removed from the plants in order to encourage healing (Figure 2.1). The newly grafted plants were placed in a tray layered with a heating pad set to 22°C, watertight plastic, capillary mat, and plastic with holes (Figure 2.2). The capillary mat extended past the ends of the tray into reservoirs of water on either end, to ensure it stayed wet. Once the grafts were in the tray, they were covered with propagation lids and white plastic to keep moisture in and protect them from the light. Two weeks after grafting, the clips were removed and plants were kept under the white plastic for partial humidity 2-3 days before moving them into full light with no covering. Two weeks after moving them to full light, tissue was sampled and frozen.
The steps of the grafting procedure are summarised in Figure 2.1.

2.2.4 Growth conditions

Wild type *N. benthamiana* for transient expression assays were provided by the lab of Sebastian Schornack and grown in a glasshouse environment.

Transgenic *N. benthamiana* for transient expression and viral infection were grown in controlled chambers at 20°C and 60% humidity.

Transgenic and wild type *N. benthamiana* for grafting were grown in a controlled chamber with 16h day length, 22°C day, 18°C night, 70% humidity.

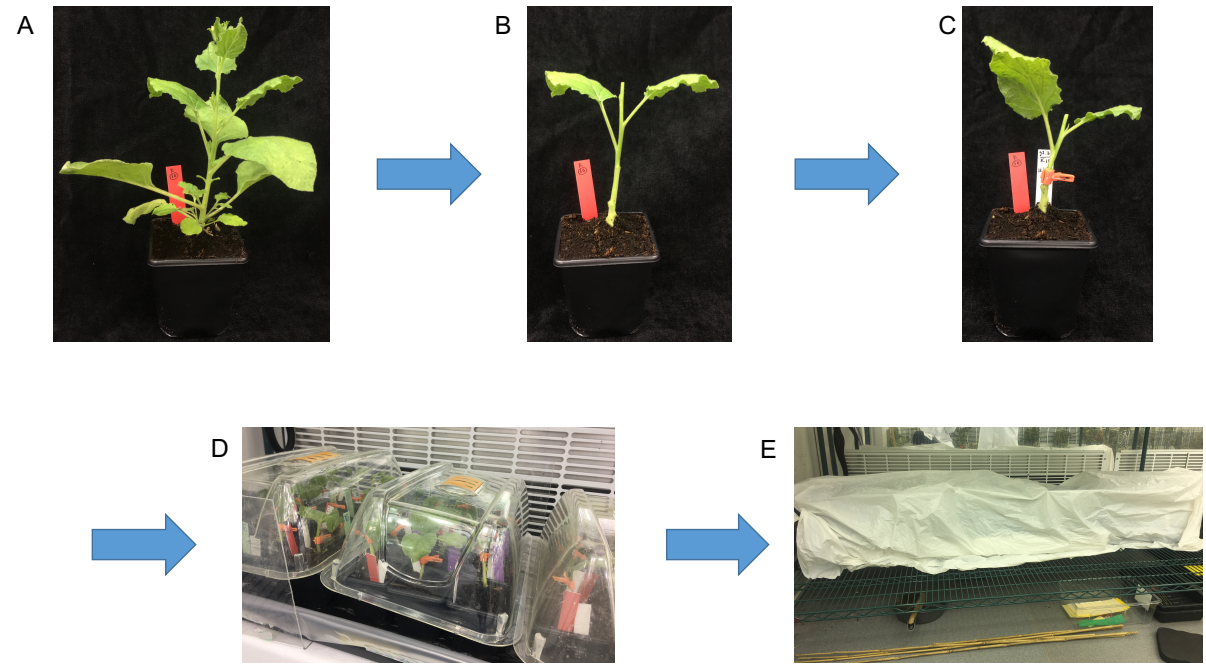


Figure 2.1. Grafting procedure. Six week old *N. benthamiana* (A) have apical meristems removed and all leaves cut off except for two (B). The stems are cut , V-grafted to a different plant, and held together with a grafting clip (C). The new grafts are placed in the grafting tray under propogation lids (D) and the whole system is covered by white plastic to protect the plants from harsh light (E).

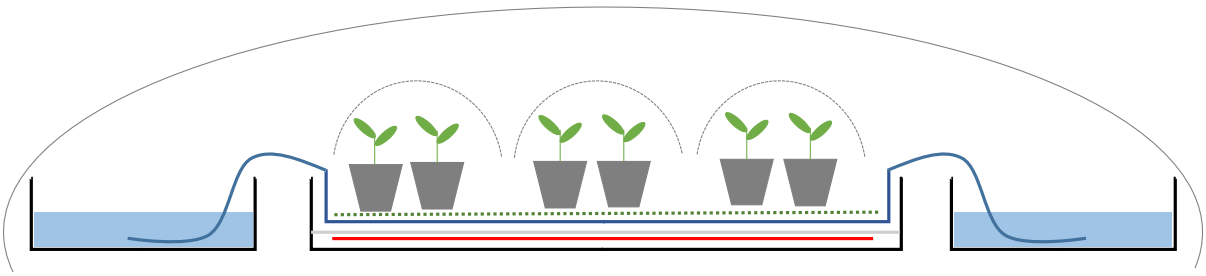


Figure 2.2. Grafting tray set up. Grafted N. benthamiana were placed in a humid, low light environment to heal. To created the necessary conditions, a heating pad set to 22ºC was placed in a plant growth tray and covered with a layer of impermeable plastic. On top of it, a moist capillary mat lined the tray and extended into reservoirs of water on either side to keep it continually saturated. Thin perforated plastic laid on top of the capillary mat. Once the plants were in the tray, propogation lids were placed on top to keep in the humidity. Finally, white plastic covered the whole enclosure to protect the plants from too much light.

2.3 Agroinfiltrations for transient expression and virus assays

LB broth, Mg_2SO_4 1M, and Acetosyringone were prepared by Lab Technician James Barlow. 0.5M MES pH 5.6 was prepared by me.

2.3.1 Infiltration medium recipe

Ingredient Stock	Amount added for 100 mL	Final Concentration
Mg_2SO_4 1M	1 mL	10 mM
MES 0.5M pH 5.6	2 mL	10 mM
H_2O	97 mL	
Acetosyringone 0.1M	150 µL	150 µM

2.3.2 *A. tumefaciens* for Transient expression

To prepare for transient expression assays, *A. tumefaciens* GV3101 glycerol stocks were grown in LB broth for 48 hours in 28°C with shaking. The liquid cultures were pelleted and re-suspended in Infiltration Medium to an OD of 0.4 measured at wavelength 600nm. The re-suspended *A. tumefaciens* were mixed to the desired combination for the assay and then recovered in 28°C with shaking for 2-4 hours.

3-4 week old *N. benthamiana* leaves were infiltrated with the cultures in localised spots using a needle-less syringe, avoiding major veins. Tissue from infiltrated leaves was sampled 3dpi.

2.3.3 *A. tumefaciens* for viral infection

To prepare for viral assays, *A. tumefaciens* GV3101 glycerol stocks were grown in LB broth for 48 hours in 28°C with shaking. The liquid cultures were pelleted and re-suspended in Infiltration Medium to an OD of 1.0 measured at wavelength 600nm. The re-suspended *A. tumefaciens* were mixed to the desired combination for the assay and then recovered in 28°C with shaking for 2-4 hours.

3-4 week old *N. benthamiana* leaves were entirely infiltrated with the cultures using a needle-less syringe, two leaves per plant. Tissue from infiltrated leaves was sampled 3 dpi and from systemic leaves at 14 dpi.

2.4 Nucleic acid purification

2.4.1 Plasmid purification

Plasmid DNA minipreps were performed using Qiagen QIAprep® Spin Miniprep Kit following the manufacturer's instructions. DNA was eluted in DNase/RNase-free water heated to 50°C.

2.4.2 Genomic DNA extraction

Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. DNA was eluted in DNase/RNase-free water.

2.4.3 RNA purification

Tissue was ground using a mortar and pestle with liquid nitrogen or in a tissue lyser using 3mm glass beads. RNA purification was then performed using either the Direct-Zol RNA MiniPrep kit (Zymo Research) following manufacturer's instructions, or using TRIzol and ethanol precipitation (method below).

In a chemical hood, 1.5mL of TRIzol (Ambion) was added to the frozen ground tissue sample and mixed vigorously by vortexing followed by centrifugation at 10,000RPM for 10 minutes at 4°C. The supernatant was transferred to a new tube and incubated at room temperature for 5 minutes. 300µL of chloroform was then added. The mixture was vortexed for 15 seconds and incubated at room temperature for 3 minutes before centrifugation for 15 minutes at 10,000RPM at 4°C. ~750µL of the aqueous phase was transferred to a new tube, careful not to disrupt the interphase. 750µL of isopropanol was added and the mixture was incubated at -20°C from 2 hours to overnight. The sample was then centrifuged for 10 minutes at 10,000 RPM at 4°C and supernatant discarded. The pellet was washed twice with 80% (v/v) ethanol for 5 minutes at 4°C. After the second wash, the pellet was dried for 15 minutes and then re-suspended in RNase-free H_2O. Purified RNA was stored in -20°C.

2.4.4 Nucleic acid quantification

Purified DNA and RNA was quantified using either Qubit or Nanodrop equipment according to manufacturer's instructions.

2.5 Polymerase Chain Reaction

2.5.1 DNA PCR (genomic and plasmid)

DNA PCRs were performed either using the Phire Plant Direct PCR Kit (Thermo Scientific) or using Phusion High-Fidelity DNA Polymerase (Thermo Scientific).

The Phusion mix used for one sample is as follows:

Ingredient	**Volume**
H_2O	13.4 µL
5X Phusion HF Buffer	4 µL
dNTP mix	0.4 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Phusion polymerase	0.2 µL
Template DNA	1 µL

The cycling protocol used for Phusion reactions was:

98°C, 30 sec
98°C, 10 sec
60°C, 30 sec
72°C, 15 sec
] 35 cycles
72°C, 10 min
4°C, forever

2.5.2 Colony PCR

Colonies to be screened via PCR were picked and re-streaked onto a numbered and gridded plate in order to create a stock for future applications. PCR master mix was prepared using PCRBIO Taq for 50µL reactions using the following recipe:

Ingredient	**Volume**
5x PCRBIO Reaction Buffer	10 µL
Forward primer (10 µM)	2 µL
Reverse primer (10 µM)	2 µL
PCRBIO Taq	0.5 µL
H_2O	15.5 µL

The master mix was divided into a 96 well plate, and pipette tips were used to touch the colony on the numbered gridded plate and place it in the corresponding numbered well. A multichannel pipette was used to mix the bacteria and the master mix in the well. The plate was sealed and run using the following PCR programme:

95°C, 3 min
94°C, 30 sec
57°C, 30 sec
72°C, 1 min
] 30 cycles
72°C, 5 min
4°C forever

2.5.3 cDNA synthesis and RT-PCR

One-Step RT-PCR (Qiagen) was carried out according to manufacturer's instructions and using this cycling protocol:

50°C, 30 min
95°C, 15 min
94°C, 1 min
57°C, 1 min
72°C, 1 min
] 35 cycles
72°C, 10 min
4°C, forever

For two step RT-PCR, cDNA was synthesised using SuperScript IV Reverse Transcriptase (Invitrogen) and a mixture of Oligo d(T) and random hexamer primers according to the manufacturer's protocol. The samples were incubated at 23°C for 10 min, 52°C for 10 min, and 80°C for 10 min. cDNA was then used as template in a PCR with Phusion polymerase.

2.5.4 Gel electrophoresis

1% - 2% (w/v) electrophoresis gels were prepared by mixing agarose and 1X TBE buffer. Ethidium bromide or Midori Green Advance (Nippon Genetics) dye was added to the gel before pouring. Orange DNA loading dye (New England Biolabs) was added to the PCR samples and gels were run using 80-110V charge.

1X TBE prepared by Lab Technician James Barlow.

2.6 Molecular cloning techniques

2.6.1 Restriction digestions of DNA

Typical restriction reaction for a New England Biolabs High Fidelity restriction enzyme:

Ingredient	Volume
DNA	500 ng
Cutsmart Buffer (NEB)	2.5 μL
High Fidelity Restriction Enzyme	0.5 μL
H_2O	To 25 μL

2.6.2 DNA Dephosphorylation

Dephosphorylation was performed using Antarctic Phosphatase (New England Biolabs). 3μL Antarctic Phosphatase Reaction Buffer and 2μL Antarctic Phosphatase were added to a 25μL restriction reaction and incubated for 30 minutes at 37°C before heat inactivation at 80°C.

2.6.3 Gel extraction and PCR purification

DNA fragments from excised gel bands were extracted using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. Samples were eluted in DNase/RNase-free water. QIAquick PCR Purification Kit (Qiagen) was used for PCR purification according to manufacturer's instructions. Samples were eluted in DNase/RNase-free water.

2.6.4 DNA Ligations

Ligations were performed using T4 DNA Ligase (either NEB or Thermo Fisher) following manufacturer's instructions.

2.6.5 Sanger sequencing

Sanger sequencing was carried out by Source Bioscience.

2.6.6 Gateway reactions

Gateway reactions were performed using LR Clonase II (Thermo Fisher) for a single site reaction and LR Clonase II Plus (Thermo Fisher) for a multisite reaction according to manufacturer's instructions.

2.6.7 Ligation into pGEM-T Easy vector

If a PCR amplification was performed using a polymerase that does not add an A-tail to the end of the fragment, an A-tail must be added before ligation into the linear T-tailed pGEM-T Easy Vector via the following protocol:

Ingredient	**Volume**
DNA from PCR or gel extraction	50 μL
$MgCl_2$ (25mM)	10 μL
dATP (1mM)	4 μL
PCRBIO Taq	1 μL
H_2O	15 μL
Incubate at 72℃ for 20 minutes	

After A-tail addition, the sample is cleaned via PCR purification (2.6.4). The sample is dried down until no liquid remains, and re-suspended in 6μL H_2O. 3μL of the resuspension is used for ligation into pGEM-T Easy Vector (Promega) according to manufacturer's instructions.

2.6.8 Golden Gate method

The method used for Type II restriction cloning was based on the Golden Gate "Long protocol in ligase buffer" from The Sainsbury Laboratory. The following were added to a PCR tube and brought to a total volume of 20μL with H_2O:

100-200ng of acceptor plasmid
Plasmids containing each part to be inserted (Use a 2:1 molar ratio of insert:acceptor)
1.5μL T4 Ligase Buffer (NEB)
1.5μL Bovine Serum Albumin 10x
0.5μL T4 DNA Ligase 400U/μL (NEB)
0.5μL BsaI 100U/μL (Thermo Fisher)

The mixture was then cycled:

37°C, 20 sec
37°C, 3 min] 26 cycles
16°C, 4 min
50°C, 5 min
80°C, 5 min
16°C, forever

2.7 Cloning of specific constructs

2.7.1 Cloning of pK-AR (Illustrated in Figure 3.3)

I amplified the 2x35S promoter from pYL156 (Liu *et al.* 2002) using Phusion DNA polymerase with SacI and SpeI restriction sites on the tails of the primers (Figure 3.3 Step 1). Primer sequences are in Appendix I. I excised the band from the agarose gel, extracted the DNA, and ligated the fragment into a pGEM-T Easy vector. After successful transformation shown by blue/white screening, I further confirmed the correct insert with Sanger sequencing.

Next, I performed SacI-HF and SpeI-HF double restrictions of pKGWFS7 (Karimi *et al.* 2002) and the pGEM-T Easy vector containing 2x35S promoter (Figure 3.3 Step 2). I used Antarctic Phosphatase to dephosphorylate the pKGWFS7 digest and then heat inactivated all the enzymes. To ligate together the 2x35S insert digest and the pKGWFS7 digest, I used T4 ligase overnight and transformed the ligation into One Shot ccdB survival cells that could accommodate the toxic ccdB gene. I then selected positive clones of this transformation. The final product of this step was a Gateway destination vector containing 2x35S promoter before the attR1 site, followed by the ccdB gene, the attR2 site, and finally the GFP:GUS reporter.

I PCR amplified the minimal Bs4 302bp promoter sequence from pGWB19-300 (Schornack *et al.* 2005) using Phusion DNA polymerase (Figure 3.3 Step 3). The primers, listed in Appendix I, were designed with the appropriate restriction sites and 4bp overlaps to prepare for a Type II Restriction reaction with BsaI. I excised the band from the gel and extracted the DNA fragment. I then followed the Golden Gate "Long protocol in ligase buffer" from The Sainsbury Laboratory to insert the Bs4 minimal promoter into pYPQ143 (Lowder *et al.* 2015), a Golden Gate destination and Gateway entry vector. After transformation into dH5α *E. coli*, miniprep, and Sanger sequencing, I identified a positive clone. The result of these steps (Figure 3.3 Steps 3-4) was a Gateway entry vector containing the Bs4 302bp minimal promoter followed by a Gateway cassette with attL5 and attL2 sites.

A Gateway entry vector with attL1 and attR5 sites containing the dCas9-VP64 fusion and a transcriptional terminator already existed, generated by Lowder *et al.* (2015), called pYPQ152.

The final step of the process to obtain a dCas9-activator-reporter construct was to perform a multisite Gateway including pYPQ152, pYPQ143+Bs4, and pKGWFS7+2x35S (Figure 3.3 Step 5). The reaction product was transformed into *E. coli* One Shot Top10 cells for selecting positive clones. I confirmed the correct construct via Sanger sequencing (primers in Appendix I). The full map and sequence of pK-AR can be found in Appendix II.

2.7.2 Cloning of gRNA constructs (Illustrated in Figure 3.4)

Following the protocol in Schiml *et al.* (2016), I designed oligos for the three different gRNAs with proper overlaps for insertion into pEN-Chimera (Fauser *et al.* 2014). Oligo sequences can be found in Appendix I. I digested pEN-Chimera with BbsI, following the protocol from Schiml *et al.* (2016), and PCR purified the product. I annealed the gRNA oligos and ligated them into the digested pEN-Chimera, transformed the ligations into *E. coli*, and confirmed the positive clones via colony PCR and Sanger sequencing (Figure 3.4 Steps 1-3). The resulting pEN-Chimera+gRNA vectors were Gateway entry vectors with attL1 and attL2 sites.

Next, I combined pEN-Chimera+gRNA with a binary vector that was also a Gateway destination. The final step (Figure 3.4 Step 4) was a single site Gateway reaction between pEN-Chimera+gRNA and pGWB401, pGWB401 (Nakagawa *et al.* 2007), an empty binary Gateway destination vector with Kanamycin resistance in plants. The reaction product was transformed into *E. coli* One Shot Top10 cells for selecting positive clones.

2.7.3 Cloning of pYDg2 (TRV RNA2 gRNA vector) (Illustrated in Figure 4.5)

First, I amplified sequences close to unique restriction sites (AatII and EcoRI) on either side of the deletion region using primers with overhangs that would cause the resulting fragments to have overlapping regions and gel extracted these fragments. Next, the two fragments were combined in a PCR using the outer two primers of the previous reaction, producing a combined fragment with the overlap region in the middle. The cycling method, based on Phusion Polymerase protocol and the overlap deletion method published by Lee et al. (2010) was:

98°C, 30 sec
98°C, 10 sec
60°C, 30 sec
72°C, 30 sec
] 25 cycles
72°C, 10 min
4°C, forever

I gel extracted the resulting fragment, cloned it into a pGEM-T Easy vector, and confirmed with Sanger Sequencing. Restriction of pYL156 and the pGEM-T Easy vector containing the overlap fragment with AatII and EcoRI was followed by dephosphorylation of the pYL156 restriction product to reduce the possibility of pYL156 re-ligating with its own insert. I combined the two restrictions in a ligation reaction, transformed the ligation into *E.*

coli dH5α, and used Sanger Sequencing to confirm clones that had a successful deletion. I named the plasmid at this step "pYL_DEL."

Next, gRNA2 was amplified from the gRNA2-containing binary vector I had previously generated. The forward primer contained an EcoRI tail on the 5' end, and I made use of an XbaI site already present in the gRNA2 vector as the 3' restriction site. I extracted the resulting fragment from the gel and cloned it into a pGEM-T Easy vector. I confirmed positive clones with Sanger Sequencing. Finally, I inserted the gRNA into the MCS using restriction with EcoRI and XbaI followed by ligation. Positive clones were confirmed with Sanger sequencing.

The full map and sequence of pYDg2 can be found in Appendix II.

2.8 GUS histochemical and fluorometric assays

Protocols for GUS assays were based on the method published by Jefferson *et al.* (1987) and Schornack *et al.* (2005).

2.8.1 GUS histochemical staining

GUS Stain:

Ingredient Stock	Amount added for 50 mL	Final Concentration
0.1 M Na_3PO_4	25 mL	50 mM
10% (v/v) Triton-X	1000 µL	0.2% (v/v)
0.1 M Potassium Ferrocyanide	1000 µL	2 mM
0.1 M Potassium Ferricyanide	1000 µL	2 mM
ddH_2O	22 mL	
0.1 M X-GLUC in DMF	1000 µL	2 mM

Incubate at 37°C overnight. Clear tissue in ethanol.

2.8.2 GUS fluorometric assay

Equal leaf spots were punched using the mouth of a 2mL microcentrifuge tube avoiding veins and wound sites, frozen in liquid nitrogen, and stored at -80°C. Frozen tissue punches were ground in a tissue lyser using 3-4 3mm glass beads for 2 minutes. 150µL Extraction Buffer were added to each tube and mixed thoroughly by vortex. Samples were centrifuged for 10 minutes at 13,000 RPM at 4°C and 100µL of supernatant transferred to a new tube. 10µL of the sample was mixed with 90µL Assay Buffer and incubated at 37°C for an hour. 900µL

0.2 M Na_2CO_3 (pH 9.5) was added to each sample to stop the reaction. 300µL of each sample was loaded into a 96 well plate along with dilutions of 4-methyl-umbelliferone as standards and read in a plate reader. Plate readers used were: BMGlabtech CLARIOstar with pre-set 4-MUG settings 360nm (excitation)/450nm (emission); or BMGlabtech FLUOstar Omega with settings 355nm (excitation)/460nm (emission).

Extraction Buffer:

Ingredient Stock	**Amount added for 20 mL**	**Final Concentration**
1M Na_3PO_4	1 mL	50 mM
β-mercaptoethanol	14 µL	10 mM
10% (v/v) Triton X-100	200 µL	0.1% (v/v)
10% (w/v) SDS	200 µL	0.1% (w/v)
0.5M EDTA (pH 8)	400 µL	10 mM
DI water	18.2 mL	

Assay Buffer:

Ingredient Stock	**Amount added for 5 mL**	**Final Concentration**
4-MUG	0.0088 g	5 mM
Extraction Buffer	5 mL	

0.2 M Na_2CO_3:

Ingredient Stock	**Amount added for 50 mL**	**Final Concentration**
Na_2CO_3	1.06 g	0.2 M
DI water	50 mL	

→ pH to 9.5

4-methyl-umbelliferone (MU) stock solution:

Ingredient Stock	**Amount added for 10 mL**	**Final Concentration**
MU salt	0.002 g	1 mM
0.2M Na_2CO_3 (pH 9.5)	10 mL	

1M Na_3PO_4, 10% (v/v) Triton X-100, 10% (w/v) SDS, and 0.5M EDTA (pH 8) were prepared by James Barlow.

Chapter 3: Generation of a CRISPRa system in plants

Introduction

3.1 CRISPRa in plants

CRISPR activation (CRISPRa) functions when deactivated Cas9 (dCas9) fused to a transcriptional activator forms a complex with gRNA and sits on a target DNA promoter region, inducing increased expression of the downstream gene (Gilbert *et al.* 2013, Maeder *et al.* 2013, Perez-Pinera *et al.* 2013). Unlike a CRISPR gene knock out, activation is not dependent on the mutation being out of frame, it is not dependent on degradation of the gene's product protein before sampling, and an effect can be easily observed and quantified even if there is activity on only a single allele. This last quality allows CRISPRa to overcome the challenge of functional redundancy faced by knock out assays. I therefore decided to use CRISPRa as a tool for testing non-transgenic methods of gRNA delivery. At the time of designing this project, there were only two demonstrations of CRISPRa in plants (Piatek *et al.* 2015, Lowder *et al.* 2015).

Piatek *et al.* (2015) generated three plant expression constructs to form their CRISPRa system (Figure 3.1A). The first contained a 2X35S CaMV promoter driving human codon optimised dCas9 fused to either the EDLL domain from the ERF/EREBP family of plant transcription factors or a TAL activation domain (TAD). The second construct contained a GFP:GUS fusion reporter driven by a Bs3 minimal promoter from *Capsicum annuum*, originally published by Römer *et al.* (2009), uncited by Piatek *et al.* (2015). For the gRNA, Piatek *et al.* (2015) cloned each gRNA driven by the *Arabidopsis* U6 promoter into the multiple cloning site of a Tobacco Rattle Virus RNA2 cDNA construct. When they agroinfiltrated all three constructs together using gRNA targeting the Bs3 minimal promoter, they were able to show activation of the GUS reporter gene with either the dCas9-EDLL or the dCas9-TAD.

Lowder *et al*. (2015) set out to create a toolbox for transcriptional regulation in plants. For CRISPRa, they designed a system in which plant codon optimised dCas9 (pco-dCas9) fused to transcriptional activator VP64 combines with gRNA to target a synthetic minimal promoter driving an intron-containing GUS reporter gene. The synthetic minimal promoter contains multiple gRNA binding sites and was chosen in order to avoid the regulatory factors that interact with promoters of endogenous genes (Figure 3.1B).

They first tested their synthetic system using transient expression in *N. benthamiana* by co-infiltrating two expression constructs: one containing the GUS reporter driven by the minimal promoter and one encoding dCas9-VP64 and three gRNAs. They observed activation

of the GUS upon co-expression compared to the reporter construct alone (Figure 3.1C). They then demonstrated endogenous gene activation in Arabidopsis stably transformed with dCas9-VP64 and three gRNAs from a single T-DNA construct. One of the endogenous targets tested was a methylated promoter region, and the dCas9-VP64 was able to activate the downstream gene. This result showed the possibility for using CRISPRa in plants for epigenetic analyses.

The goal of the work presented in this chapter was to develop a system suitable for testing non-transgenic gRNA delivery into plants stably expressing an dCas9-activator and a reporter gene. My design differs from the previous methods (Lowder *et al.* 2015, Piatek *et al.* 2015) because it is based on only the gRNA being kept separate, while the activator and reporter are together in one construct to allow a single transformation step to generate transgenic lines.

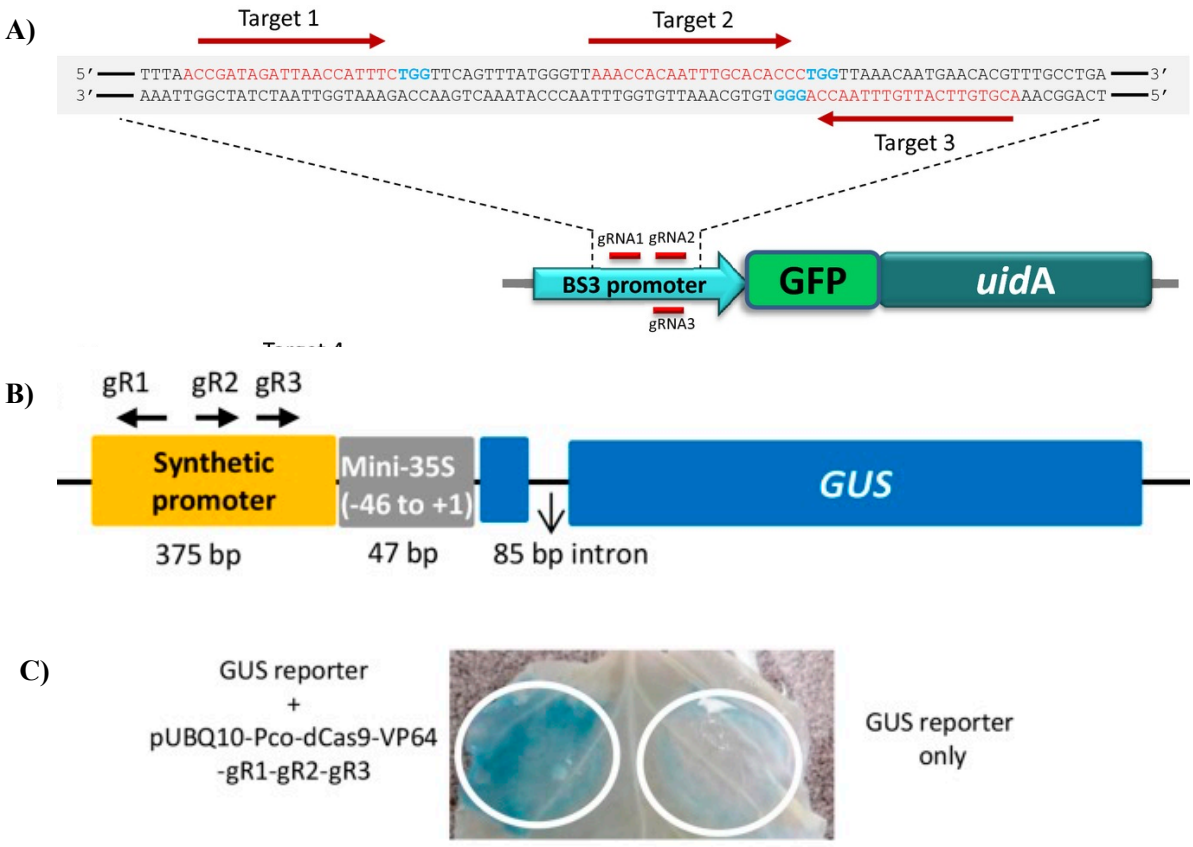


Figure 3.1 Previously demonstrated systems of CRISPRa in plants. A) Schematic of the system used by Piatek *et al.* (2015) in which gRNAs were designed to target a minimal Bs3 promoter to activate GFP:GUS expression when combined with a dCas9-activator. **B)** Schematic of the system used by Lowder *et al.* (2015) in which 3 gRNAs were designed to combine with dCas9-VP64 to target a synthetic minimal promoter driving an intron containing GUS reporter gene **C)** Transient expression assay by Lowder *et al.* (2015) demonstrating that co-expression of dCas9-VP64 and 3 gRNAs targeted to their synthetic minimal promoter can increase GUS expression.
Figures adapted from Piatek *et al.* (2015) and Lowder *et al.* (2015)

Results

3.2 Construct design

3.2.1 dCas9-activator-reporter construct (K-AR)

The design of the dCas9-activator-reporter vector was based on a combination of the systems described in Lowder *et al.* (2015) and Piatek *et al.* (2015), with important modifications. Instead of the human codon optimised dCas9 used by Piatek *et al*. (2015), I chose the plant codon optimised dCas9 from Lowder *et al*. (2015). I decided to use the VP64 transcriptional activator because it been demonstrated in plants by Lowder *et al.* (2015) and there was continued development on the use of dCas9-VP64 in animal systems (Zalatan *et al.* 2015, Konermann *et al.* 2015) which might allow for easy improvement of my system in plants in the future.

For the gRNA, I used an expression method specifically designed for gRNA instead of cloning the AtU6 promoter driving gRNA into the MCS of a viral cDNA expression plasmid as in Piatek *et al*. (2015). This was an illogical design decision for their constructs because the system was not used for viral delivery.

For transient expression assays in *N. benthamiana*, Lowder *et al.* (2015) used a single construct containing the dCas9-VP64 fusion and three gRNAs co-infiltrated with a construct expressing intron-containing GUS under a synthetic minimal promoter. In contrast, Piatek *et al.* (2015) kept all the components in separate constructs, one for the reporter, one for the dCas9-activator, one for gRNA. Unlike Lowder *et al.* (2015), a design feature of my system was keeping the gRNA separate from the dCas9-VP64 in order to be able to test its introduction in trans. My design is distinct from that of Piatek *et al*. (2015), however, because I made a single construct containing the dCas9-VP64 and the reporter gene in order to require only a single tissue culture step to stably transform both components.

Following a similar strategy to the Bs3 minimal promoter used by Piatek *et al.* (2015), I chose to use the Bs4 minimal promoter from tomato (Schornack *et al.* 2005), which had been previously used in testing activation of a reporter gene by TAL effectors (Boch *et al.* 2009). Schornack *et al.* (2005) demonstrated that the Bs4 promoter in tomato can be truncated which causes minimal expression of the Bs4 gene. They showed that the 74bp truncation was not long enough to allow the Bs4 protein to function properly, but it was still viable to drive GUS and showed the lowest level of GUS expression. Therefore, I wanted to use the 74bp sequence. Unfortunately, this strategy was not feasible because no PAM sites exist within the 74bp

region. Therefore, I chose to use the next shortest truncation, 302bp in length, to drive the minimal expression of the reporter.

For the reporter, I chose to use a GFP:GUS fusion as was previously used for CRISPRa (Piatek *et al*. 2015) and for the original demonstration of the Bs4 minimal promoter (Schornack *et al.* 2005). The dCas9-VP64 needed to be under a constitutive promoter, and I chose the commonly used 2x35S promoter from Cauliflower Mosaic Virus.

3.2.2 gRNA selection and construct design

The gRNA constructs were designed to each contain one gRNA targeting the Bs4 minimal promoter in a binary vector. Due to the short length of the 302bp truncated Bs4 promoter, there were only a few options for gRNA target sites, which require the NGG PAM sequence (Jinek *et al.* 2012). I chose three positive sense sites at various locations in the promoter, shown in Figure 3.2A.

To drive the gRNA, I used the *Arabidopsis thaliana* AtU6-26 promoter, based on the system developed by Fauser *et al.* (2014). *Arabidopsis* U6 RNA is transcribed by RNA Polymerase III (Pol III) (Waibel and Filipowicz 1990). A Pol III promoter is commonly used for gRNA expression because the promoter elements are upstream of the transcribed region so they do not interfere with the RNA sequence, their specific transcription start site allows for a clean 5' end of the gRNA, and they have a known termination sequence of a polyT stretch (Gao *et al.* 2018).

While new evidence in mammalian cells suggests the Pol III transcription start site (TSS) might be more complicated than originally thought (Ma *et al.* 2014), it is generally accepted that gRNAs driven by animal and plant U6 promoters should be designed on the basis that Pol III prefers to recognise a G at the +1 position (Ran *et al.* 2013, Parry *et al.* 2016). None of the gRNA sites in the Bs4 302bp promoter began with a G. Therefore, I added a G onto the 5' end of each of my gRNAs because it can increase efficiency of the gRNA without affecting specificity (Ran *et al.* 2013, Parry *et al*. 2016) (Figure 3.2B).

I only included one gRNA per binary vector because the methods of introducing the gRNA in trans in the later parts of my project were limited to the ability to use a single gRNA at a time. Therefore, I made three separate gRNA binary vectors to be able to test the proof of principle that the system can work with a single gRNA.

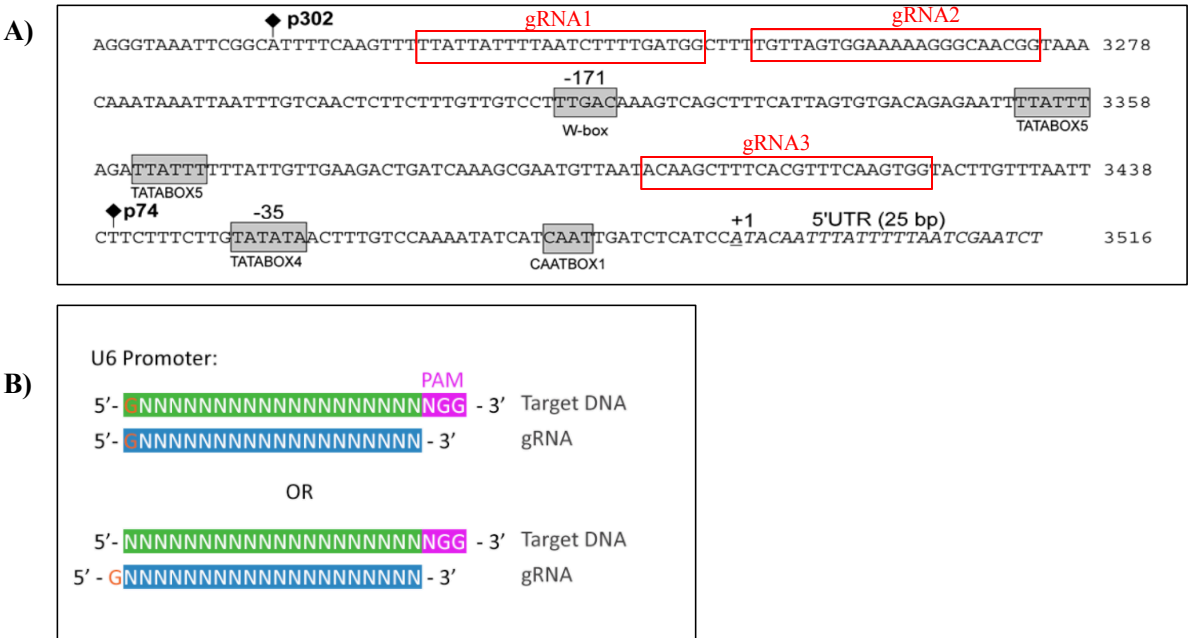


Figure 3.2. gRNA selection and design. A) Shows the sequence of the Bs4 302bp minimal promoter. Red boxes indicate the location of gRNAs selected and their adjacent PAM sequence. Figure modified from Schornack *et al*. (2005). B) Illustration of adding a G to the 5' end of gRNA in order to prepare it for transcription by Pol III, which prefers a G in the +1 location. Figure from ABM Inc.

3.3 Construct generation

3.3.1 dCas9 activator reporter vector (K-AR)

The activator-reporter construct was designed to contain a dCas9-VP64 fusion driven by 2X35S promoter in the same construct as a GFP:GUS fusion reporter driven by the Bs4 minimal promoter. I generated this construct using a variety of molecular cloning techniques, culminating in a multisite Gateway reaction to combine three separate constructs. Gateway cloning matches recombination sequences called "attL/attR" sites (Hartley *et al.* 2000) and I needed to ensure correct assembly in the final reaction using correct sites in the three progenitor constructs (Figure 3.3).

I began with the binary vector and Gateway destination vector pKGWFS7 (Karimi *et al.* 2002), which contains attR1 and attR2 sites followed by a GFP:GUS fusion reporter and confers kanamycin resistance in plants. In between the attR1 and attR2 sites of pKGWFS7, I inserted the sequence encoding the dCas9-VP64 fusion and the Bs4 minimal promoter to drive expression of the GFP:GUS reporter that was already downstream of the Gateway cassette. Insertion of a 2X35S promoter upstream of the Gateway cassette allowed expression of the dCas9-VP64. Since the backbone of this construct is pKGWFS7 and it contains the activator and reporter parts of my system, I named this construct "pK-AR." The complete cloning strategy is illustrated in Figure 3.3 and described in Chapter 2. Primers are in Appendix I and the map and sequence of pK-AR can be found in Appendix II.

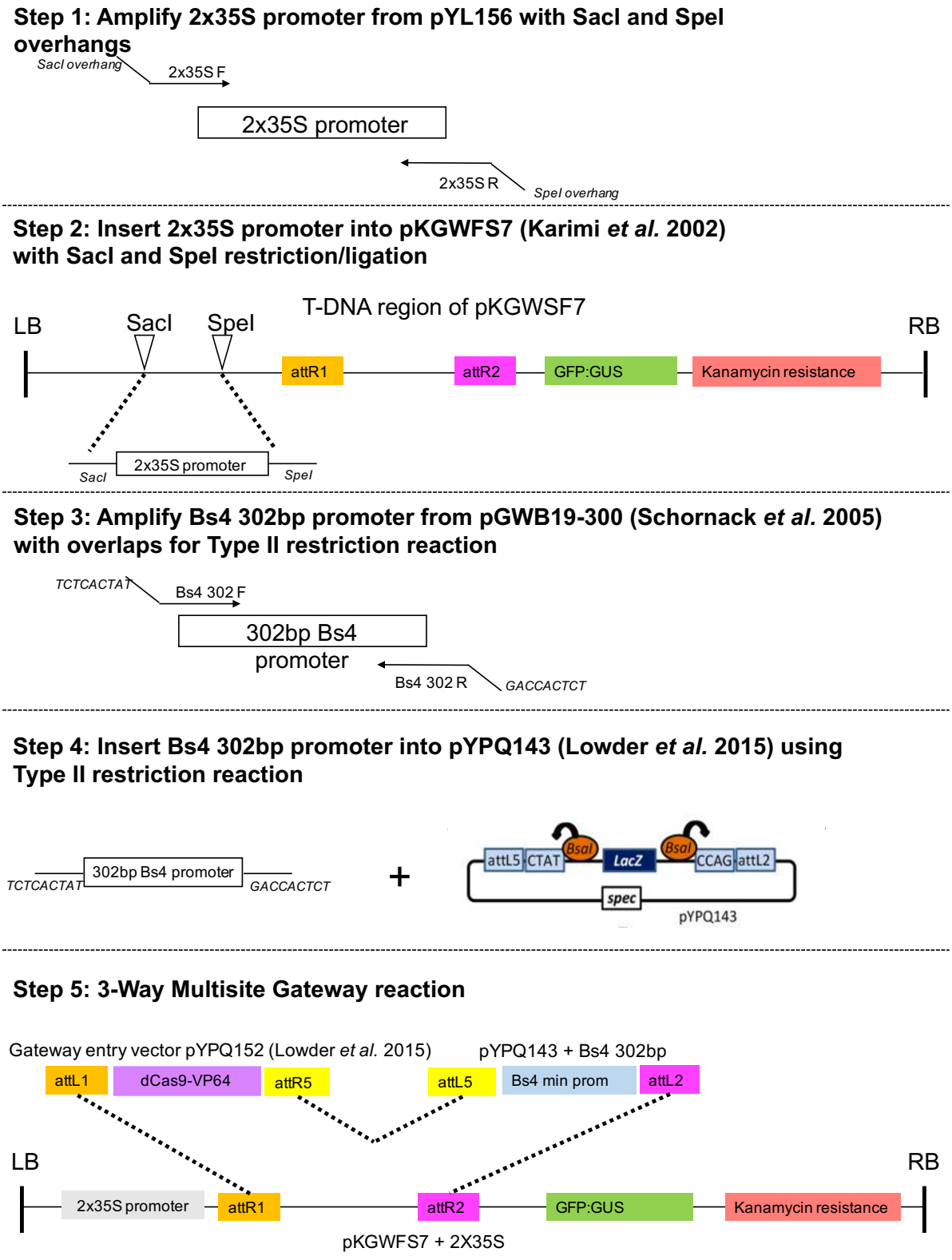


Figure 3.3. K-AR cloning strategy. First, I amplified 2X35S promoter from pYL156 and inserted it into pKGWFS7 using restriction cloning (Steps 1-2). Next, I amplified Bs4 302bp promoter from pGWB19-300 and performed Type II Restriction with BsaI to insert it into pYPQ143 (Steps 3-4). The cloning culminated in a multisite Gateway reaction with pYPQ152, pYPQ143+Bs4 302, and pKGWFS7 + 2X35S (Step 5). Image in Step 4 modified from Lowder *et al.* (2015).

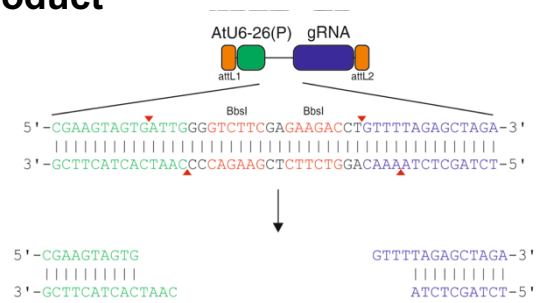
3.3.2 gRNA vector

The gRNA construct needed to be a binary vector that would allow the gRNA to be transiently expressed or stably transformed into *N. benthamiana* separately from K-AR. pEN-Chimera (Fauser *et al.* 2014) is a Gateway entry vector designed for gRNA expression. It includes the AtU6-26 promoter upstream of a BbsI Type II restriction site where the gRNA is inserted and the gRNA scaffold sequence downstream of the insertion site (see Figure 3.4 Step 1).

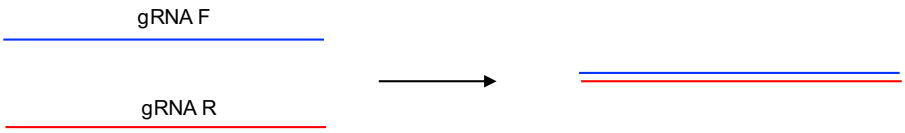
Following the protocol in Schiml *et al.* (2016), I generated three separate pEN-Chimera gRNA constructs. I then used Gateway cloning to insert the gRNA construct from pEN-Chimera into pGWB401 (Nakagawa *et al.* 2007), an empty binary Gateway destination vector with Kanamycin resistance in plants. The final binary vector construct would allow transcription *in planta* of the gRNA driven by the AtU6-26 promoter. The full gRNA cloning strategy is illustrated in Figure 3.4 and is described in Chapter 2. gRNA oligos are listed in Appendix I.

A summary of the constructs in my CRISPRa system can be found in Figure 3.5.

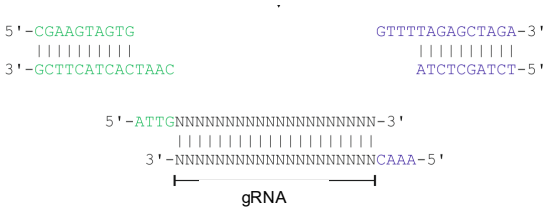
Step 1: Digest pEN-Chimera (Fauser *et al.* 2014) with BbsI and purify the product



Step 2: Anneal gRNA oligos



Step 3: Ligate restricted pEN-Chimera with annealed gRNA oligos, transform into *E.coli* and select positive clones of pEN-Chimera



Step 4: Single site Gateway reaction between pEN-Chimera+gRNA and pGWB401 (Nakagawa *et al.* 2007)

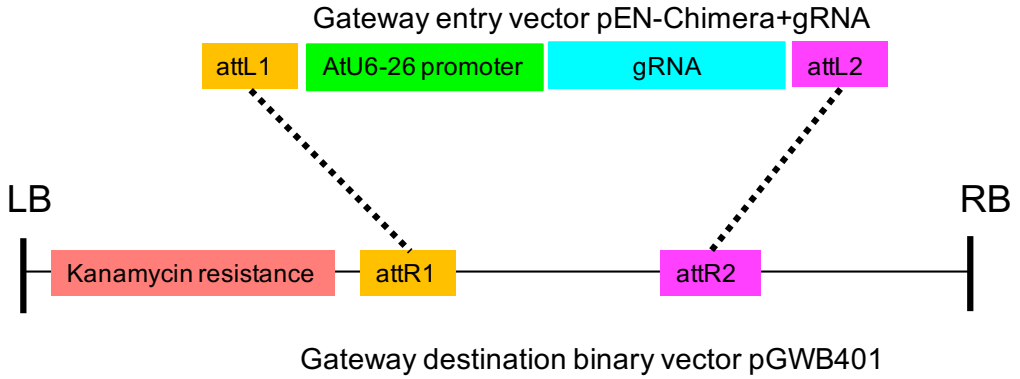


Figure 3.4. gRNA cloning strategy. First, pEN-Chimera (Fauser *et al.* 2014) was digested with BbsI (Step 1). Next, gRNA oligos were annealed and ligated into the restricted pEN-Chimera. Positive clones were selected (Steps 2-3). The final step was a Gateway reaction between pEN-Chimera+gRNA and pGWB401 (Nakagawa *et al.* 2007) (Step 4). Images in Steps 1 and 3 modified from Fauser *et al.* (2014).

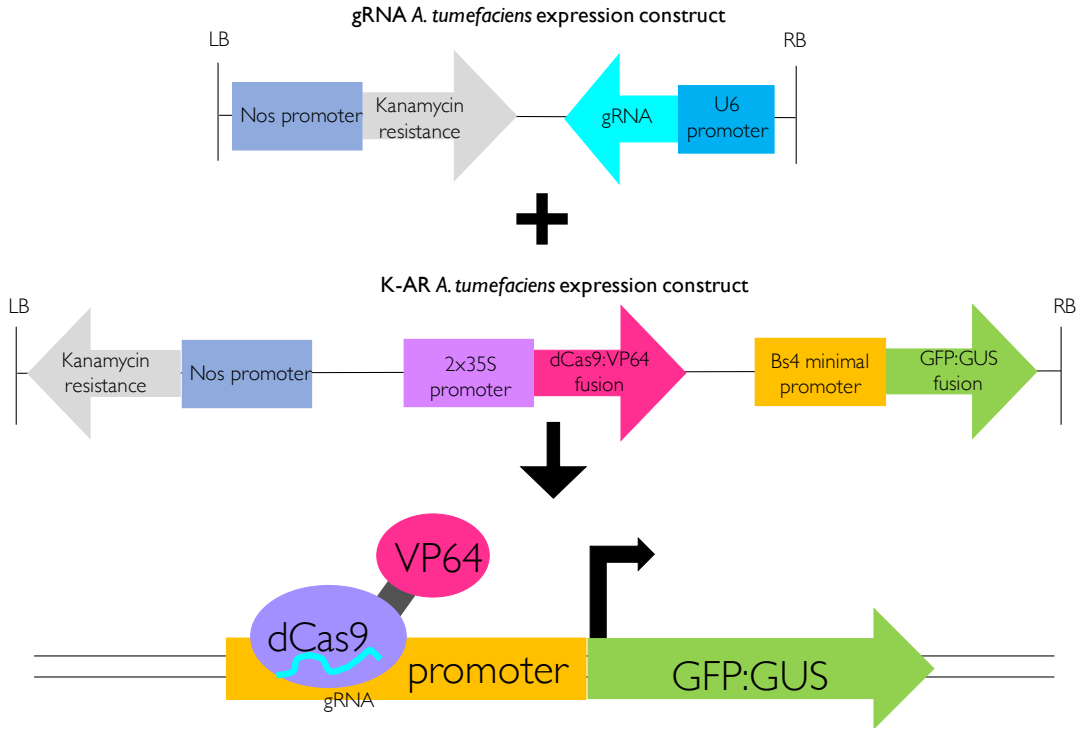


Figure 3.5. Schematic of activator-reporter system. When gRNA is co-expressed with K-AR, the dCas9-VP64 forms a complex with the gRNA and sits on the Bs4 302bp minimal promoter, inducing the increased expression of the downstream reporter.

3.4 Transient expression assays demonstrate functionality of constructs

After assembling the constructs, I transformed them into *A. tumefaciens* GV3101 pSoup and tested their functionality using transient assays in wild type *N. benthamiana*. Constructs transformed into *A. tumefaciens* are depicted in Figure 3.6A and full methods of transient infiltration can be found in Chapter 2. I infiltrated four spots per leaf (Figure 3.6B): 1) 35S GUS positive control + empty *A. tumefaciens* 2) K-AR + gRNA 3) K-AR + empty *A. tumefaciens* 4) gRNA+ empty *A. tumefaciens*. I expected K-AR + gRNA to show activation compared to the basal level of GUS expression present in the spot containing the K-AR + empty *A. tumefaciens*. In the spots where only one experimental construct was used, it was mixed in a 1:1 ratio with empty *A. tumefaciens* in order to maintain equal concentration of individual constructs compared to the K-AR and gRNA mixture. Three days post infiltration (dpi), I sampled the tissue for analysis of GUS expression.

I used ImageJ to quantify the pixel intensity of scanned images of the GUS histochemical assay (Figure 3.6C) to test for GUS activation using gRNA1. There was a statistically significant activation of GUS level in the K-AR + gRNA1 spot compared to the K-AR + empty *A. tumefaciens* (Figure 3.6D). While this was a promising result, it appeared the levels of activation would be quite low. I therefore used the more quantitative and sensitive GUS fluorometric assay in the rest of the activation experiments.

The GUS fluorometric assay at 3dpi (for method see Chapter 2) showed significant increases in GUS expression when a mixture of K-AR + gRNA was co-infiltrated compared to K-AR + empty *A. tumefaciens* for all three gRNAs (Figure 3.7). gRNA2 and gRNA3 showed considerably higher levels of activation than gRNA1.

In order to test the background reading of a non-infiltrated wild type leaf compared to the gRNA + empty *A. tumefaciens* control I had been using as a negative control, I performed a separate experiment to test the readouts of these two conditions. This assay showed no difference in GUS measurement between non-infiltrated wild type leaf and wild type leaf infiltrated with gRNA3+empty *A. tumefaciens* (Figure 3.8).

These transient assays in wild type *N. benthamiana* served as proof of principle of my system, and therefore I was able to move forward with generating stably transformed lines.

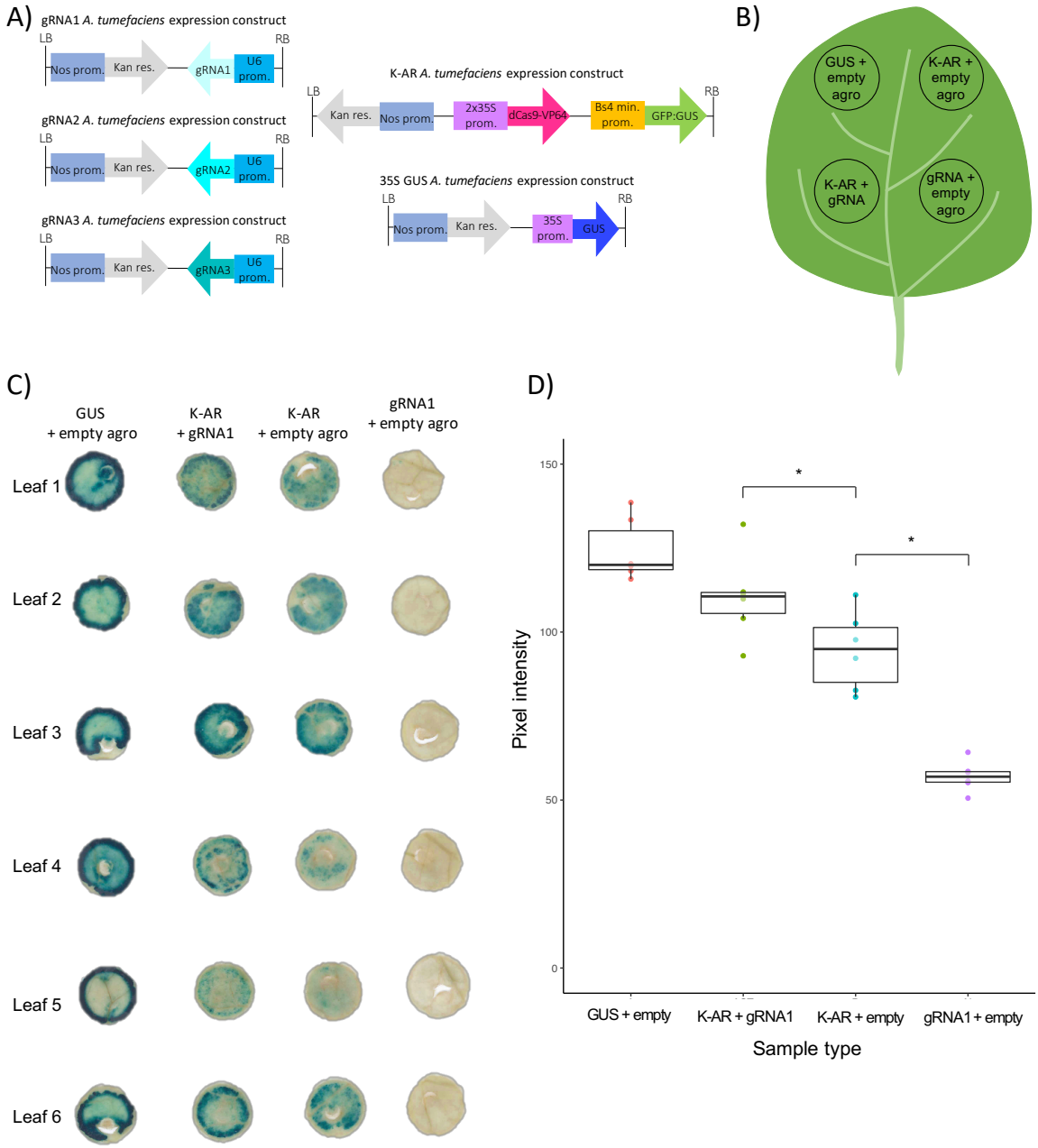


Figure 3.6. Transient expression assays in wild type *N. benthamiana* leaves show activation of GUS reporter by gRNA1. A) All constructs transformed into *A. tumefaciens* for expression assays. B) Diagram of constructs co-infiltrated into leaf spots for transient expression assays. For infiltrations where only one experimental construct was used, it was mixed in a 1:1 ratio with empty *A. tumefaciens* so that there were equal concentrations of individual constructs in each leaf spot. C) Scans of GUS stained leaf discs from a transient expression assay using gRNA1. D) Quantification of the GUS stain in C) by pixel intensity using ImageJ. There were significant differences between K-AR + gRNA1 and K-AR + empty as well as between K-AR + empty and gRNA1 + empty ($p=0.049$, $p=2.6*10^{-5}$, respectively). p-values measured by pairwise t-test.

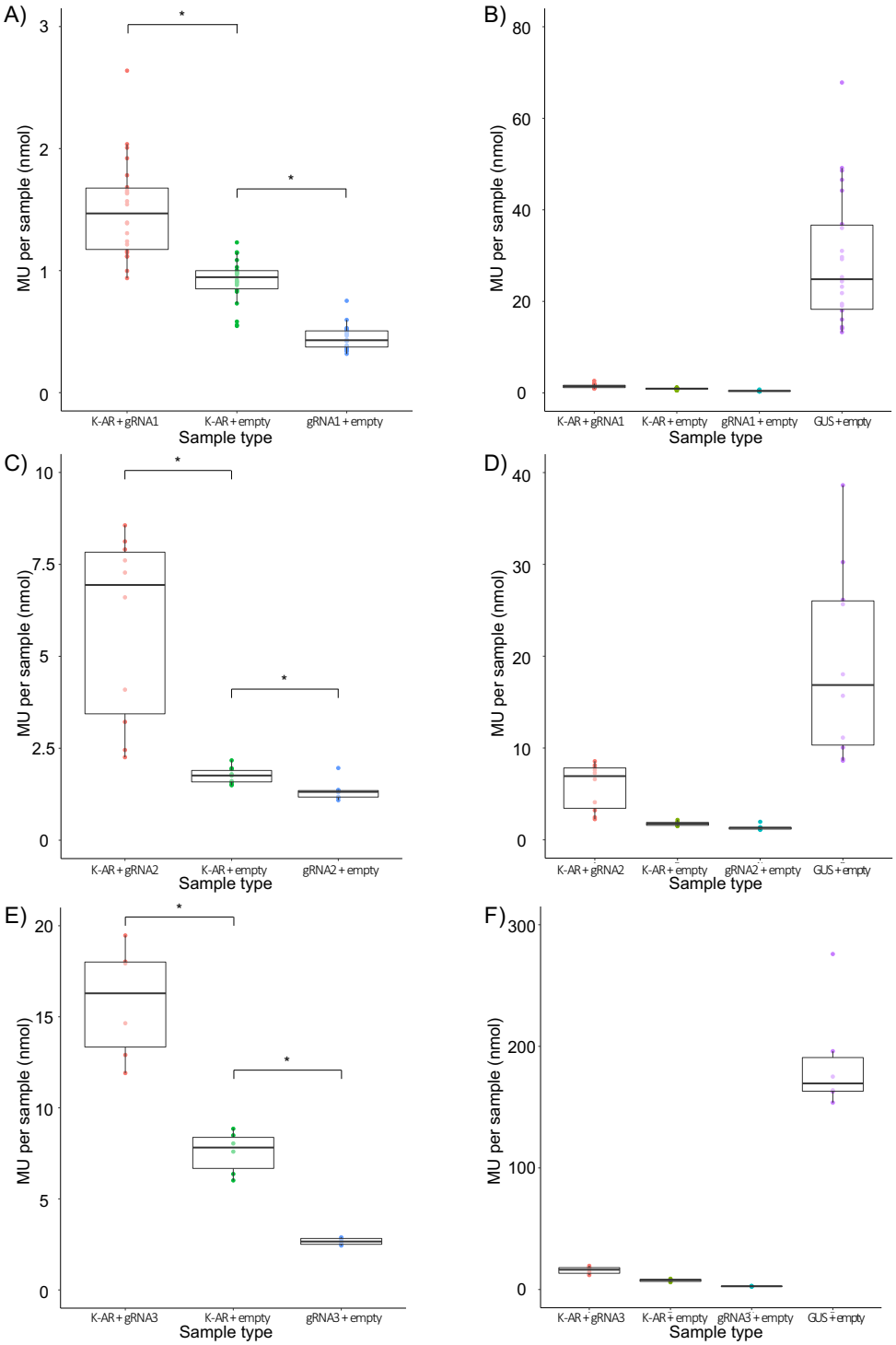


Figure 3.7. GUS fluorometric quantification of transient expression assays in wild type *N. benthamiana* show activation by all three gRNAs. Wild type *N. benthamiana* leaves were infiltrated with 4 spots per leaf as shown in Figure 3.6B. Fluorometric assays revealed GUS levels resulting from co-expression of K-AR and gRNA were significantly higher than K-AR and empty *A. tumefaciens* for each of the 3 gRNAs. **A), C), E)** show fine scale to visualise activation. **B), D), F)** include GUS positive control. **A)/B)** Activation by gRNA1: n= 22 leaves; $p = 2.2*10^{-7}$. **C)/D)** Activation by gRNA2: n= 10 leaves; $p= 7.8*10^{-5}$ **E)/F)** Activation by gRNA3: n= 6 leaves; $p=1.1*10^{-4}$. Reported p-values represent the significance between "K-AR + gRNA" and "K-AR + empty" and were calculated by pairwise t-test. The difference between "K-AR + empty" and "gRNA1 + empty" was also significant in all cases but not relevant for demonstrating functional activation.

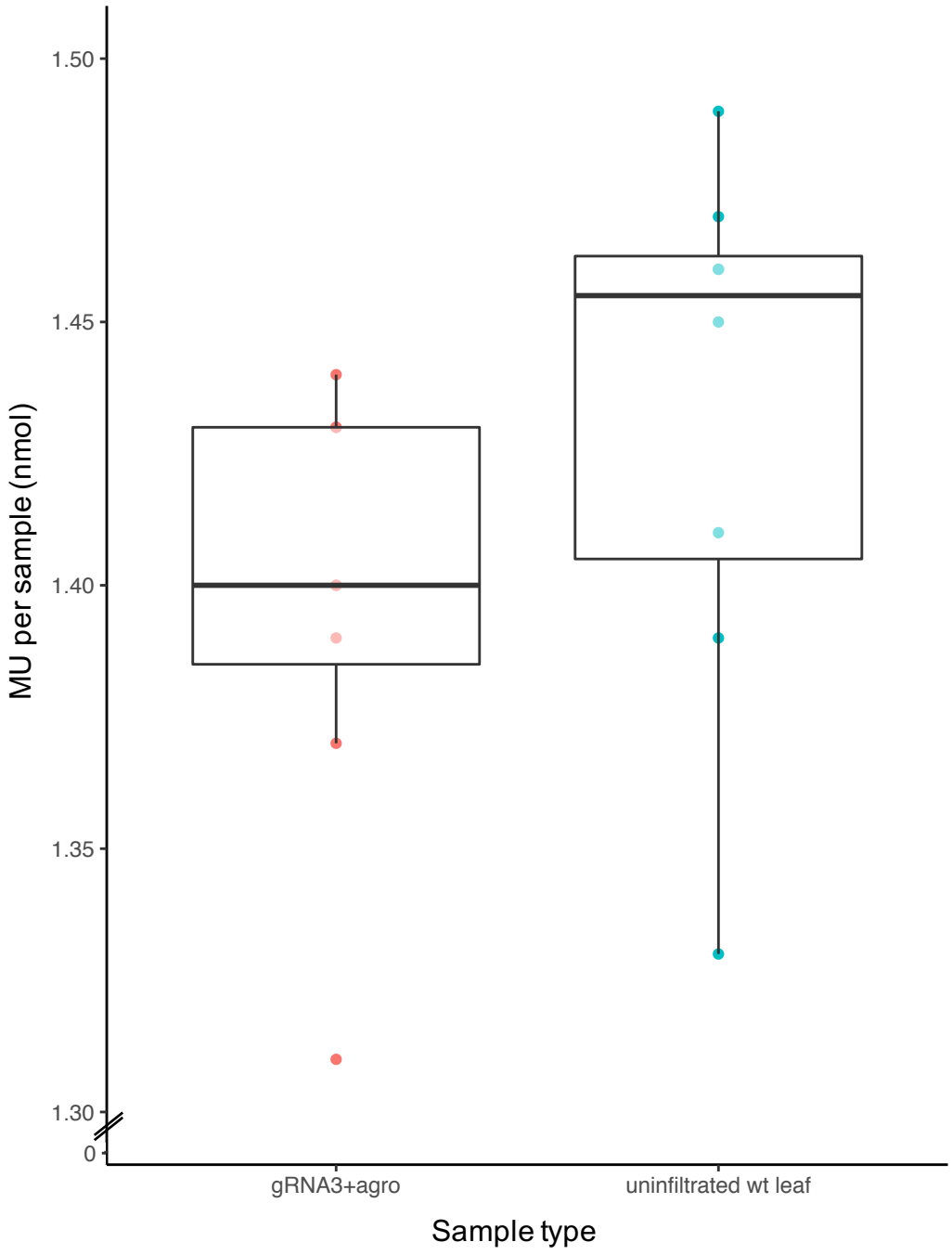


Figure 3.8. GUS fluorometric assay to determine background level of GUS fluorometric assay in wild type leaves. Wild type *N. benthamiana* leaves were infiltrated in one spot with gRNA3 + empty *A. tumefaciens* and the rest of the leaf was non-infiltrated. There was no significant difference between the two conditions. n=8 leaves, p=0.15, pairwise t-test.

3.5 Generation and characterisation of stably transformed lines

The overall aim of this project was to test various methods of delivering gRNA in trans, so I needed to make stable transgenic lines for four separate constructs (one K-AR, one for each of three gRNAs) (see Chapter 2 for transformation protocol).

The gRNA transformations produced 6 gRNA1 T0s, 11 gRNA2 T0s, and 8 gRNA3 T0s. I genotyped 6 T0s from each group using primers located in the backbone of the binary vector, spanning the gRNA insertion site (M13_F and M13_R). All the gRNA T0s tested were positive. These six T0s from each gRNA construct were self-fertilised and seed was harvested.

Transformation yielded 6 K-AR T0s. To determine whether the transgene was successfully integrated, I performed PCRs using primers that spanned the Bs4 minimal promoter and part of the GFP:GUS reporter gene (Bs4p_F and pK_R). 3 out of the 6 K-AR T0s tested positive for the transgene. It is known that transformations are likely to have "escapes," false positive shoots that do not contain the transgene (Estopà *et al.* 2001, Rakosy-Tican *et al.* 2007, Zale *et al.* 2009), so this result was not unexpected. I next checked for transgene expression in these three K-AR T0 lines using RT-PCR with primers in the sequence encoding the dCas9 (KRT_FWD and KRT_REV). Two out of the three were positive, likely due to transgene silencing in one of the lines. The positive line K-AR T0 #6 was self-fertilised. Its seed was collected and sown on soil as the T1 generation.

Primer sequences are in Appendix I.

3.6 Transient assays on K-AR T1 generation show activation ability

To confirm that the K-AR construct could be activated in the T1 transgenic lines, I used transient expression assays on the T1 K-AR leaves with: 1) gRNA2; 2) gRNA3; 3) non-infiltrated leaf; 4) empty *A. tumefaciens*. The GUS fluorometric assay at 3dpi showed activation by gRNA2 and gRNA3 in 4 out of 6 T1 plants (Figure 3.9A). Activation level could be categorised into three main groups: high activation, lower activation, and no activation (circled in Figure 3.9A). The leaf spot infiltrated with empty *A. tumefaciens* also exhibited mild activation compared to the non-infiltrated leaf, but the activation demonstrated by the addition of gRNA was far higher. Genotyping (primers: Bs4p_F and pK_R) revealed that the two plants (#44 and #52) that exhibited background values tested negative for the transgene (Figure 3.9B).

The activation levels and segregation pattern suggest that the K-AR transgene was hemizygous in K-AR T0 #6 and the T1s were either homozygous, hemizygous, or null. T1 #21

exhibited the highest activation level of the T1s in this assay and was likely homozygous, so it was self-fertilised and seed was collected in order to use the T2 generation in later experiments.

Agroinfiltration with gRNA2 showed consistent activation ability in the stable lines, and I therefore chose it as the gRNA used for the rest of the project. RT-PCRs using a primer pair specific to gRNA2 (gRNA2_RT_F and gRNA_RT_R) confirmed that the gRNA2 T0s that had genotyped positive also expressed the transgene. Seed from the positive gRNA2 #24 T0 was sown on soil to produce the T1 generation. T1s were screened for expression with RT-PCR and all tested individuals were positive. Plant gRNA2#24/#1 was self-fertilised and seed was harvested to be sown in the future as the T2 generation.

3.7 Characterisation of K-AR T2 generation

Genotyping and RT-PCR confirmed presence and expression of the transgene in all individual plants of the K-AR#6/21 T2 generation. This pattern further confirms that K-AR#6/21 T1 was homozygous for the transgene, and therefore the T2 plants would also be homozygous.

To confirm function of the activation system in the K-AR T2 generation, I performed transient expression with: 1) gRNA2; 2) 35S GUS; 3) non-infiltrated leaf; 4) empty *A. tumefaciens*. The GUS fluorometric assay showed activation by gRNA2, significantly higher than the slight activation exhibited by empty *A. tumefaciens* infiltration alone (Figure 3.10). I used two independent *A. tumefaciens* cultures for each construct and infiltrated two leaves per plant, one with each group of bacterial cultures, to ensure biological replication. This transient expression experiment demonstrated that the K-AR T2s were suitable for use in further experiments on gRNA delivery in trans.

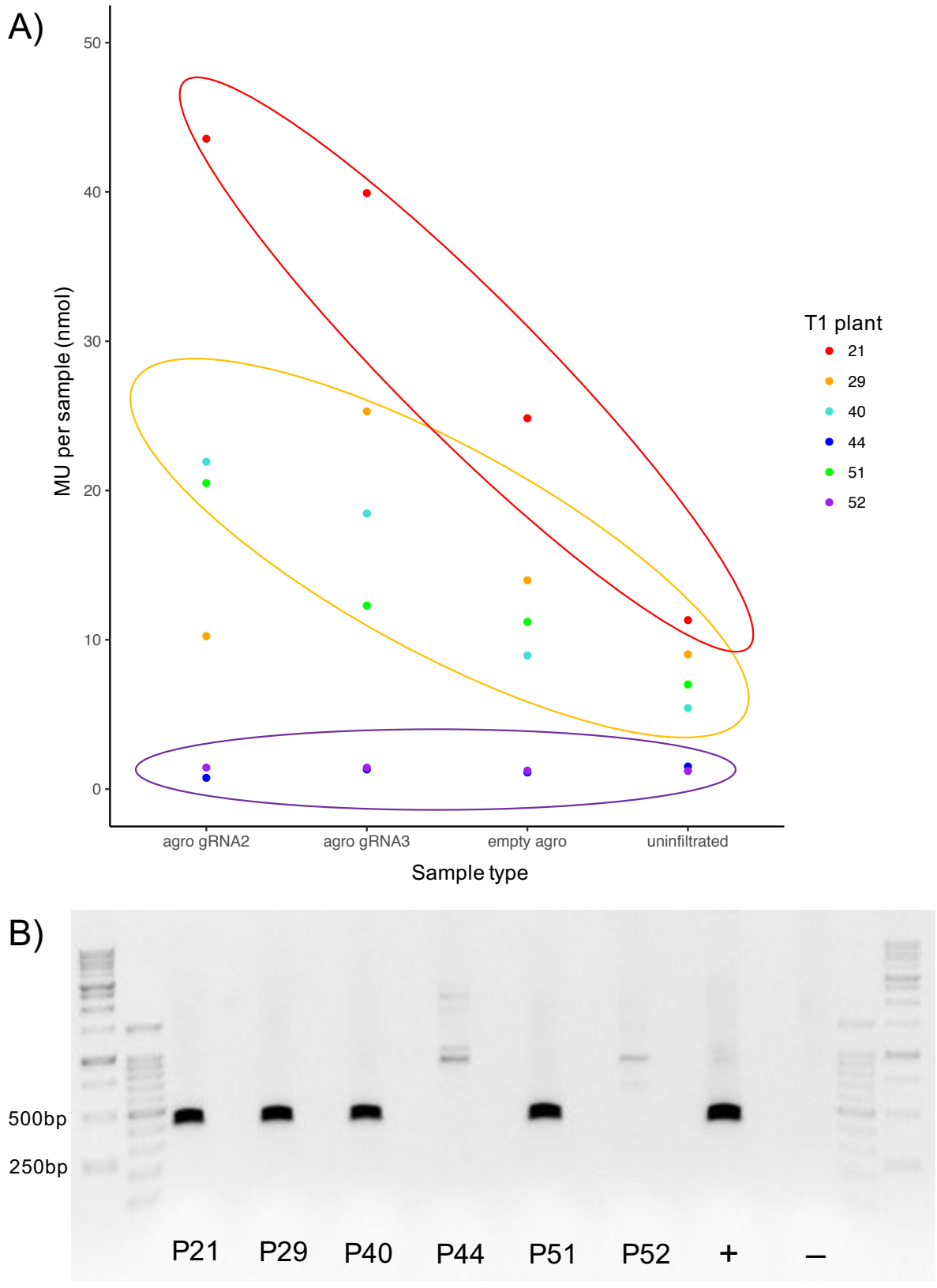


Figure 3.9. GUS fluorometric assay and genotyping of K-AR T1 lines. A) GUS fluorometric assay 3dpi after agroinfiltrating spots of gRNA2, gRNA3, and empty *A. tumefaciens* show the transgene is able to be activated in 4 out of 6 lines. Two of the lines were unable to be activated. Circles are drawn around three groups of activation – high activation, lower activation, no activation. B) Genotyping revealed segregation of the K-AR transgene. The plants lacking the transgene are the plants that did not show GUS activation by the gRNA in the transient expression assay (A).

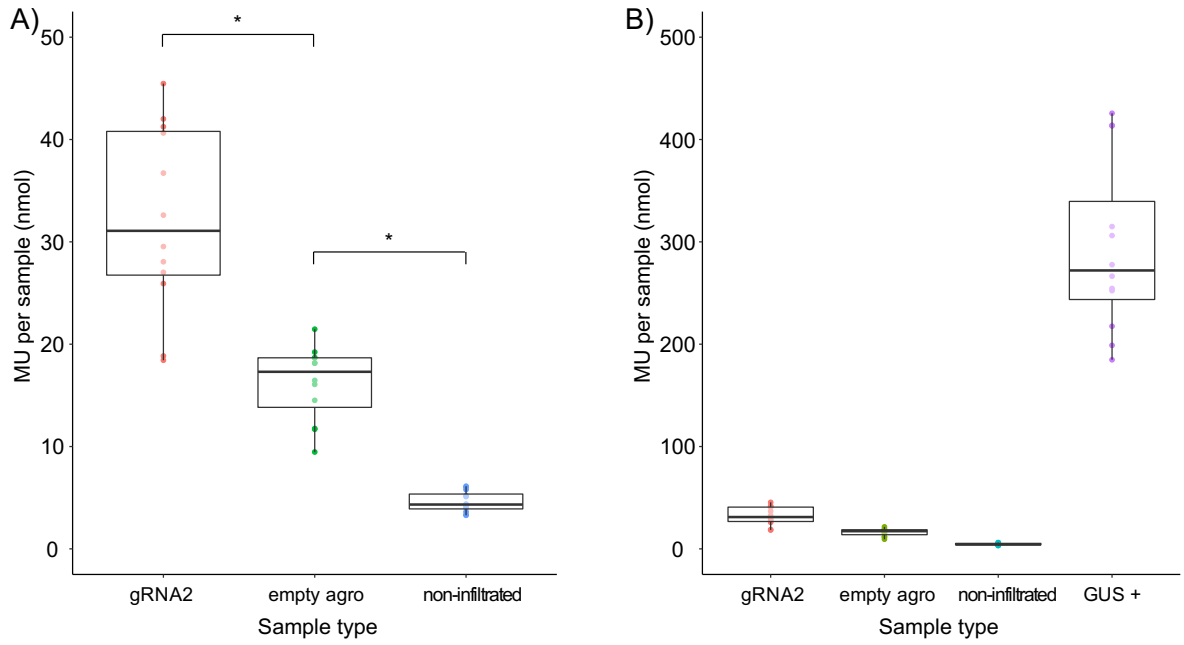


Figure 3.10. Transient expression assays in K-AR T2 lines demonstrated functionality of the system. Leaf spots of K-AR T2 lines agroinfiltrated with gRNA2 showed significant GUS activation compared to spots infiltrated with empty *A. tumefaciens* ($p=1*10^{-5}$). There was also significant activation due to empty *A. tumefaciens* alone compared to non-infiltrated leaf ($p=3.9*10^{-10}$). **A)** shows fine scale to visualise activation while **B)** includes the GUS positive control which confirmed effective infiltration technique.
Two independent *A. tumefaciens* cultures were used per construct and divided into infiltration groups to ensure biological replication. n=12 leaves (6 leaves per bacterial culture group). p-values were calculated by pairwise t-test.

Discussion

3.8 Construct design and transient assays in wt *N. benthamiana*

First, I successfully used molecular cloning to generate constructs for CRISPRa in plants. One construct contains a constitutively expressed dCas9-VP64 activator and a GFP:GUS reporter driven by the Bs4 minimal promoter. I chose to include both the activator and reporter in the same construct so that both components could be stably transformed into *N. benthamiana* in a single tissue culture process. The second construct contains the gRNA to target the Bs4 minimal promoter. When the two constructs are co-expressed, the gRNA-bound dCas9-VP64 targets the Bs4 minimal promoter and increases levels of GUS expression.

Importantly, I needed to demonstrate that a single gRNA is able to activate the system, instead of requiring a multiplexed approach. While multiplexing has been shown to cause higher levels of activation (Maeder *et al.* 2013, Perez-Pinera *et al.* 2013, Piatek *et al.* 2015), the limitations of the future aims of my project (delivering a gRNA via a virus or grafting) required that only one gRNA be used at a time.

The ability of each gRNA alone to activate the system was demonstrated in transient assays in wild type *N. benthamiana* (Figure 3.7). gRNA2 and gRNA3 showed much higher levels of activation than gRNA1. Piatek *et al.* (2015) also noticed variability in the effects of their gRNAs for CRISPRa. They observed that the gRNAs they designed to bind the sense-strand of target DNA were more effective than those binding in the anti-sense direction. All three of my gRNAs are designed to target the sense-strand so this is not the cause of difference.

One theory is that the sequence of the gRNA might affect its activation ability. It has been shown that Cas9 cutting efficiency is influenced by gRNA nucleotide composition (Doench *et al.* 2014), however the same sequence rules did not have a significant effect on CRISPRa (Doench *et al.* 2016). In one of the initial tests of CRISPRa using dCas9-VP64, Maeder *et al.* (2013) reported no correlation between gRNA nucleotide composition and activation level. More research needs to be done to better understand how to predict nucleotide sequences for effective CRISPRa gRNA design.

It is most likely that target site location of my gRNAs affected their activation ability. Piatek *et al.* (2015) suggest that the distance from the TSS might have explained the behaviour of their gRNAs, but do not provide any support from the literature for this claim. This hypothesis has been more carefully considered in mammalian systems and large-scale studies have shown that the ideal target site locations of gRNAs for various CRISPR applications are different (Gilbert *et al.* 2014, Mohr *et al.* 2016). Konermann *et al.* (2015) found that the best

prediction of how effective a gRNA would be for CRISPRa was distance from the transcription start site (TSS), with the highest levels of activation occurring when the target site was within the -200bp to +1bp region of the promoter. Gilbert *et al.* (2014) observed a slightly different optimal window for CRISPRa activity within -400 to -50 bp upstream from the TSS.

All three of my gRNAs are within the ranges suggested by Gilbert *et al.* (2014) and Konermann *et al.* (2015). Perhaps the location of the gRNA relative to various promoter elements might cause the difference in activation ability. For example, distance from a TATA box could be an indicator of activation potential. gRNA2 and gRNA3 are both closer to a TATA box than gRNA1 (Figure 3.2A). As CRISPRa becomes a more widely used technique, it might be useful to use the large data sets to zoom in on the high activation window of -400bp to +1bp to get better information on gRNA placement and promoter elements. In addition, continuing to improve gene annotations and precise locations of promoter elements and the TSS will facilitate designing the best gRNAs for CRISPRa (Mohr *et al.* 2016). It will be important in the future to build a data set for CRISPRa in plants instead of extrapolating observations from studies in mammalian cells.

The activation levels observed in these assays were consistent with other studies using VP64 as an activator (Konermann *et al.* 2015, Li *et al.* 2017). I tested the background of the system in wild type plants comparing leaf spots agroinfiltrated with a mixture of gRNA3 and empty *A. tumefaciens* to non-infiltrated wt leaf spots. These two groups showed no difference in the GUS fluorometric assay confirming that the gRNA alone was not having any effect on the GUS level in wt plants (Figure 3.8).

While the reporter used in this system is a GFP:GUS fusion, I chose only to assay the GUS activity. It has been shown that GFP in a protein fusion is less easily detected from low transcript levels (Martin *et al.* 2009). Since I was anticipating relatively small changes in the amount of reporter transcript, I thought that an enzymatic assay would provide a more reliable readout. In addition, Schornack *et al*. (2005) and Piatek *et al*. (2015) whose work formed a guide for my assays used a GFP:GUS fusion and also only assayed for GUS.

The assays in this chapter used an equal-sized leaf punch as an approximation for an equal amount of protein per sample. There could be slightly different amounts of protein per sample due to conditions like leaf thickness, leaf age, and punch location, but the effects on the results would be random, not causing a clear pattern. Therefore, while there may be mild variability in the data that is not represented in my results, I am confident that the patterns of activation displayed using the leaf punch as an approximation are valid. Future experiments

could measure total protein per sample using the Bradford Assay to provide a more precise quantification.

All the quantification of reporter levels in this chapter should be further confirmed by using qPCR to measure RNA expression. This method is complementary to the enzymatic assay because it measures mRNA instead of protein and would be a clear readout because there is no background level of expression of the GUS transgene in wild type plants.

3.9 Stable transgenic lines showed functional CRISPRa

After demonstrating proof of principle of the system, I generated and characterised stably transformed lines of *N. benthamiana*, transgenic with either the K-AR construct or one of three gRNAs. Piatek *et al.* (2015) did not make any stably transformed lines of their system, limiting them to only testing transient effects. Lowder *et al.* (2015) made stable transgenic lines using a single expression construct including the dCas9-VP64 and three gRNAs designed to target an endogenous gene of choice. Their system successfully activated the target gene, but was not designed for testing methods of gRNA delivery. Therefore, the goal of my transformation lines was to establish the first system in which gRNA could be delivered in trans to a stably transgenic activator-reporter system.

When I agroinfiltrated the T1 generation of a K-AR transgenic line with gRNA targeting the Bs4 minimal promoter, there was significant activation of the GUS reporter compared to the non-infiltrated leaf or leaf infiltrated with empty *A. tumefaciens* (Figure 3.9). This result confirmed that the activator-reporter transgene was functional and able to be activated in my transgenic lines. The T1 plant exhibiting the highest activation and likely to be homozygous was self-fertilised and its progeny were used as the K-AR T2 generation.

Transient expression assays in the T2 generation also displayed significant activation in leaf spots where gRNA had been infiltrated compared with leaf spots of non-infiltrated tissue or tissue infiltrated with empty *A. tumefaciens* (Figure 3.10). Establishing functional transactivation in these K-AR T2s was essential to being able to move forward with experiments exploring various methods of gRNA delivery.

Interestingly, in both the T1 and T2 transient assays, I observed that the leaf spot infiltrated with empty *A. tumefaciens* showed increased GUS expression compared to the non-infiltrated leaf spot. The earlier wild type *N. benthamiana* transient assays showed no difference between these two conditions (Figure 3.8). Additionally, the segregated plants in the T1 generation that were lacking the K-AR transgene did not show activation by *A. tumefaciens*

(Figure 3.9). Therefore, I concluded that the transgenic minimal Bs4 promoter may be slightly activated by the infiltration of *A. tumefaciens*, while significantly less than in the presence of gRNA.

There is evidence to support that non-oncogenic *A. tumefaciens* used in biotechnology can trigger host plant immune responses in the infiltrated region (Pruss *et al.* 2008) and the Bs4 gene is a known part of the disease resistance pathway in tomato (Schornack *et al.* 2004), specifically recognising the AvrBs4 avirulence protein from the bacterium *Xanthomonas campestris* pv. *vesicatoria*. No experiments have been done to show whether the Bs4 promoter or its truncated versions are activated upon infiltration of *A. tumefaciens*, so while the Bs4 protein could be specific to targeting the AvrBs4 protein, the promoter activity could be increased upon signs of bacterial infection.

This hypothesis is supported by the predicted W-box at the -171 position in the Bs4 promoter (Schornack *et al.* 2005). The W-box is a conserved DNA sequence that acts as a binding site for WRKY transcription factors, which behave as regulators for plant pathogen defence (Pandey and Somssich 2009). The pathogen-associated molecular pattern (PAMP) of bacteria such as *A. tumefaciens* induces expression of WRKYs (Pandey and Somssich 2009). The PAMPs from *A. tumefaciens* are part of the bacteria's virulence process (Djamei *et al.* 2007), not restricted to the tumour-inducing genes that have been removed in the 'disarmed' strains used in biotechnology (Hellens *et al.* 2000). Van Verk *et al*. (2008) discovered a WRKY transcription factor (NtWRKY12) in *N. tabacum* that binds in the pathogenesis-related gene *PR-1a* promoter. They observed that when they infiltrated *N. tabacum* leaves with *A. tumefaciens*, expression of both *PR-1a* and *NtWRKY12* increased. Pruss *et al.* (2008) did not mention WRKYs but also reported that *A. tumefaciens* infiltration induced expression of *PR-1* in *N. tabacum*.

Therefore, it would be reasonable that the infiltration of *A. tumefaciens* into my K-AR T2 lines would induce the WRKY transcription factors, which would recognise the W-box in the Bs4 minimal promoter, and increase expression of the GUS reporter. If I were to redesign the construct, I would perhaps consider selecting a minimal promoter that is not involved in plant immune response, or check for the presence of a W-box. However, for my project, the important result was that the gRNA infiltrated spot showed significantly higher GUS expression than the activation exhibited in the leaf spot infiltrated with empty *A. tumefaciens*.

3.10 Conclusion

The results in this chapter serve as the proof of principle of the activator-reporter system and demonstrate the functionality of the K-AR T2 stable transgenic lines. Therefore, I was confident moving forward with using these materials to test various non-transgenic gRNA delivery methods by CRISPRa.

Chapter 4: Transactivation using a viral vector

Introduction

4.1 Tobacco Rattle Virus in biotechnology

Tobacco Rattle Virus (TRV) is frequently used in plant biotechnology as a delivery vector for engineered genetic sequences. It has a rod-shaped structure and the protein subunits are arranged in a helix (Hull 2002) (Figure 4.1A). TRV is an RNA virus with a genome comprised of two strands of positive sense RNA (Figure 4.1B). TRV RNA1 encodes for replicase proteins, a movement protein, and a 16K suppressor of silencing (Macfarlane 2010). This suppressor of silencing is one of the reasons TRV is so commonly used in biotechnology, because it is thought to allow the virus to invade plant meristems (Martin-Hernandez and Baulcombe 2008).

TRV RNA2 encodes the viral coat protein and two non-structural proteins (2b and 2c) that are not essential for viral infection (Macfarlane 2010). The 2b non-structural protein is recorded either as 29.4K (Hernandez *et al.* 1995) or 40K (Visser *et al.* 1999). Sequencing differences established by Visser *et al*. (1999) that suggested the protein actually consists of additional amino acids caused the reclassification.

Once in the plant cell, the RNA-dependent RNA polymerase (RdRP) encoded on TRV RNA1 is translated and replicates the viral genome (Newburn and White 2015). First it copies the genomic RNAs 3’ to 5’, generating negative sense genomic RNA molecules, which are then copied 3’ to 5’ to produce positive sense genomic RNA.

Viral genomes include genes for several proteins. To ensure translation of internal open reading frames (ORFs) that do not begin at the 5’ end of the genomic RNA, TRV produces subgenomic RNAs (sgRNA). These sgRNAs can be thought of as 5’ deletion derivatives of the genomic RNA because they have the same 3’ end as genomic RNA, but different 5’ start sites (Miller and Koev 2000). There are two proposed mechanisms for the generation of sgRNA (Miller and Koev 2000, Sztuba-Solinska et al. 2011). “Internal initiation” involves the negative sense RNA acting as the template for both sgRNA transcription and genome replication. In this model, RdRp promoter sites exist both at the ends of the genomic RNA for replication and internally in the genomic RNA for sgRNA production (Miller *et al.* 1985).

In an alternative “premature termination” model, the RdRP stops at a certain location on the genomic RNA as it transcribes the negative strand (Sit *et al.* 1998). This process results in a subgenomic length negative sense RNA strand which then serves as a template for positive sense sgRNA synthesis. These two models are summarised in Figure 4.2.

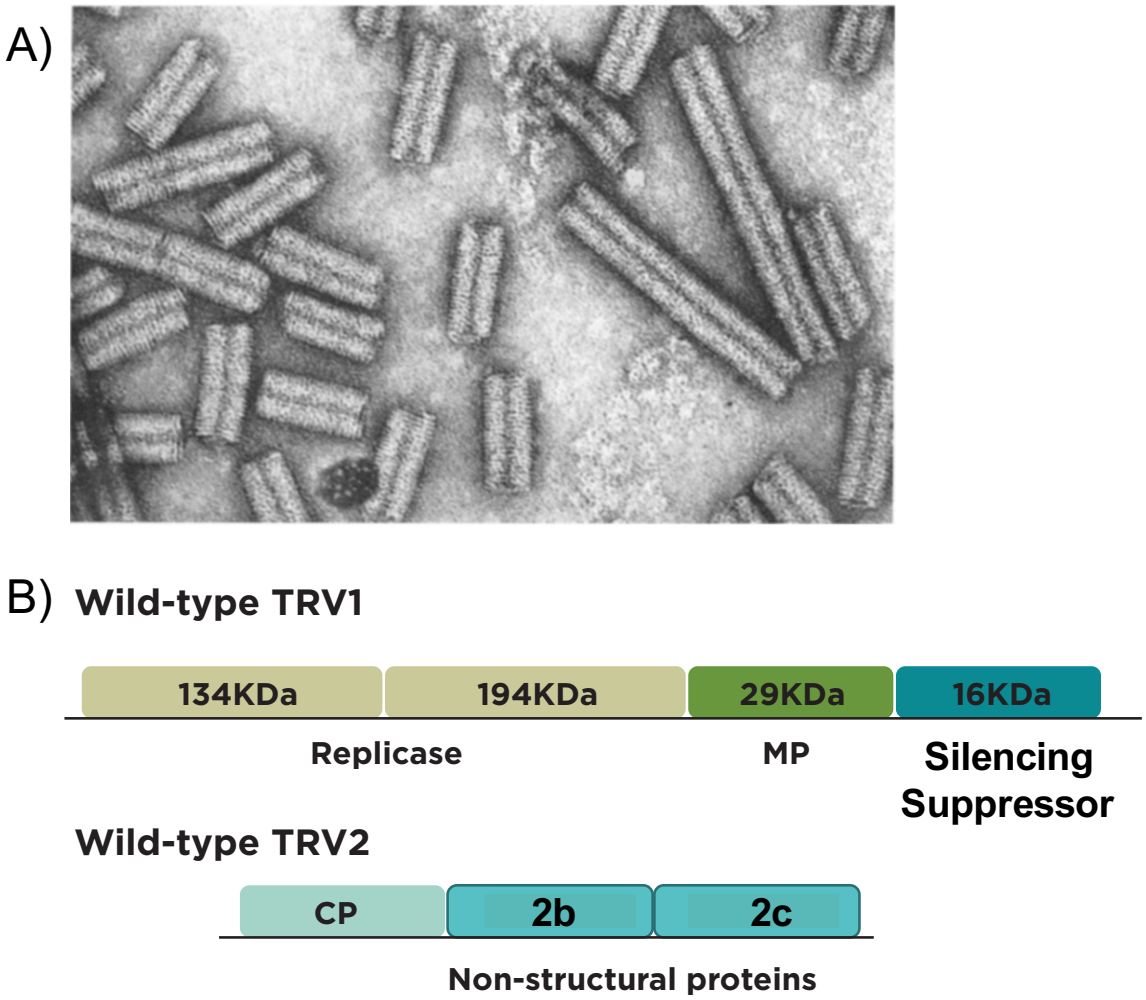


Figure 4.1. Physical and genomic structure of TRV. A) Electron microscopy image of TRV showing its helical, rod-shaped structure. Image from Offord (1966) B) Schematic of TRV genomic RNA structure. RNA1 contains replicases, a movement protein (MP), and a suppressor of silencing. RNA2 includes the coat protein (CP) and two non-structural proteins, "2b" and "2c." Diagram modified from Senthil-Kumar and Mysore (2014).

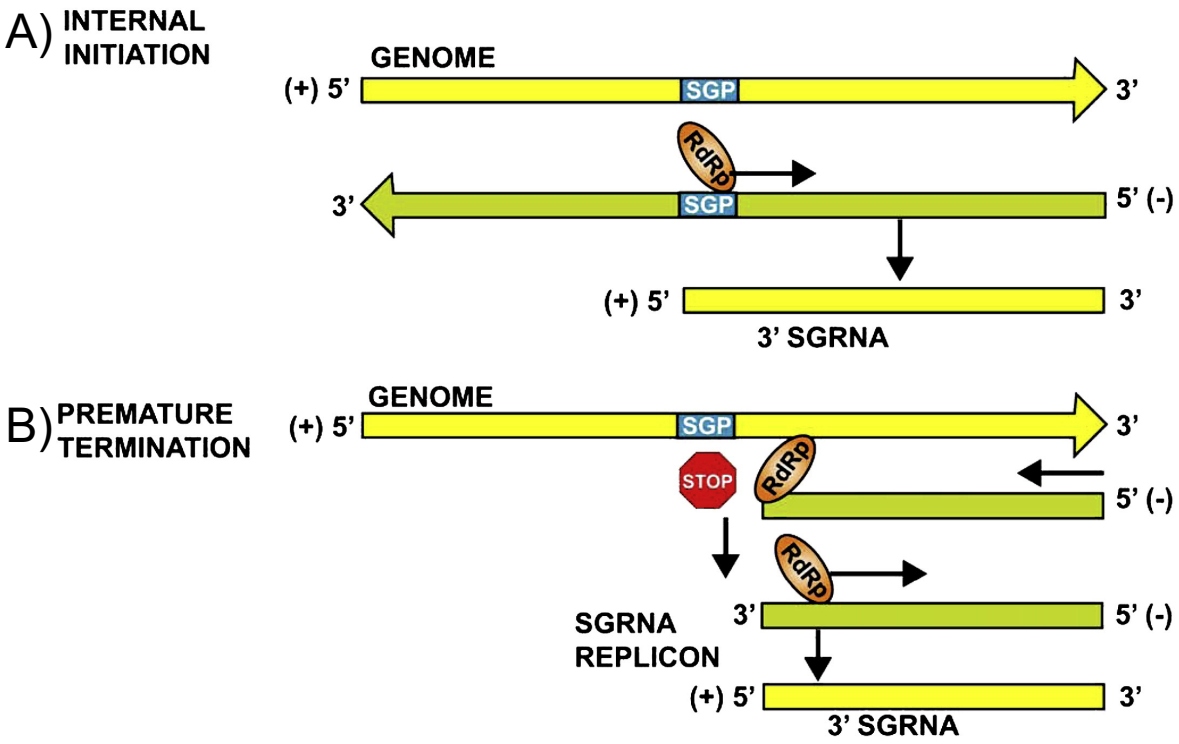


Figure 4.2. Diagram of sgRNA production models. A) Shows "internal initiation" model in which a subgenomic promoter (SGP) causes the RNA-dependent RNA Polymerase (RdRP) to begin transcribing at a point in the middle of the viral genome instead of at the 3' end, generating a positive sense sgRNA strand. B) Shows "premature termination" model in which the SGP acts as a signal for the RdRP to stop reading, generating a negative sense sgRNA strand which is then read 3' to 5' to generate the final positive sense sgRNA. Figure from Sztuba-Solinska *et al.* (2011).

In plant RNA virus vectors, the inserted sequence is usually part of a sgRNA. In TRV RNA2, an engineered insert can replace the non-essential 2b and 2c sequences downstream of the RdRP promoter (MacFarlane 2010). The size limit for an insert sequence in TRV is around 1.5kB (Burch-Smith *et al.* 2004). This size limitation means that TRV as a vector for CRISPR in plants can only accommodate the gRNA, while the 4kB Cas9 sequence has to be expressed separately.

At the start of my PhD, there had been three attempts to perform Virus Induced Gene Editing (VIGE) in plants. Ali *et al*. (2015) showed that it is possible to use TRV as a vector to deliver gRNA and cause mutation in transgenic Cas9-expressing *N. benthamiana*. They reported the mutations were detected in two progeny lines. Yin *et al*. (2015) used cabbage leaf curl virus, a geminivirus with a DNA genome, to deliver gRNA into transgenic Cas9-expressing *N. benthamiana*. While their system had a high efficiency of systemic genome editing in the infected plant, they were unable to show inheritance of the induced mutation, which the authors anticipated due to the limitations of the geminivirus used. Yin *et al.* (2015) also questioned the inheritance of Ali *et al.* (2015)'s system. Baltes *et al.* (2014) used bean yellow dwarf virus (BeYDV) lacking the coat and movement proteins as a vector to deliver the sequences for both the Cas9 and gRNA to *N. tabacum*. Gene editing mutations were successfully induced but limited only to the directly infected tissue because the virus could not spread cell-to-cell or systemically.

Clearly, there is scope for further optimisation of VIGE as a reliable method for gRNA delivery for heritable CRISPR gene editing. My approach, described in this chapter, uses a modified TRV vector for delivery of a gRNA to my CRISPRa system (Chapter 3) because TRV has a high chance of invading the meristem and causing heritable mutations. The advantage of using a transactivation system for optimising the VIGE method is that even low activity can be detected and phenotypes can be observed without waiting for residual protein product to degrade.

Results

4.2 Virus Design and Construct Generation

4.2.1 Vector selection

In order to infect *N. benthamiana* with TRV, binary Ti plasmid vectors for expression in plants containing the cDNA for TRV RNA1 and TRV RNA2 were generated first by Ratcliff *et al.* (2001) and later by Liu *et al.* (2002) based on TRV isolate PPK20. Direct infiltration of these vectors using *A. tumefaciens* causes the viral genomic RNA to be transcribed in the host plant so that the virus can then replicate and spread systemically. Both TRV RNA2 vectors include a multiple cloning site (MCS), designed for easy insertion of engineered sequences. In addition, the vector pair from Liu *et al.* (2002) includes a self-cleaving ribozyme at the 3' end of the viral genomes to make sure there is a precise end for viral replication (Figure 4.3). For my project, I chose to use the vector pair from Liu *et al.* (2002) due to their demonstrated higher virulence (Liu *et al.* 2002). These vectors are "pYL192" encoding TRV RNA1 and "pYL156" encoding TRV RNA2. Since only TRV RNA2 needs to be engineered with synthetic sequence, I left pYL192 unchanged. I modified pYL156 in order to efficiently express my gRNA.

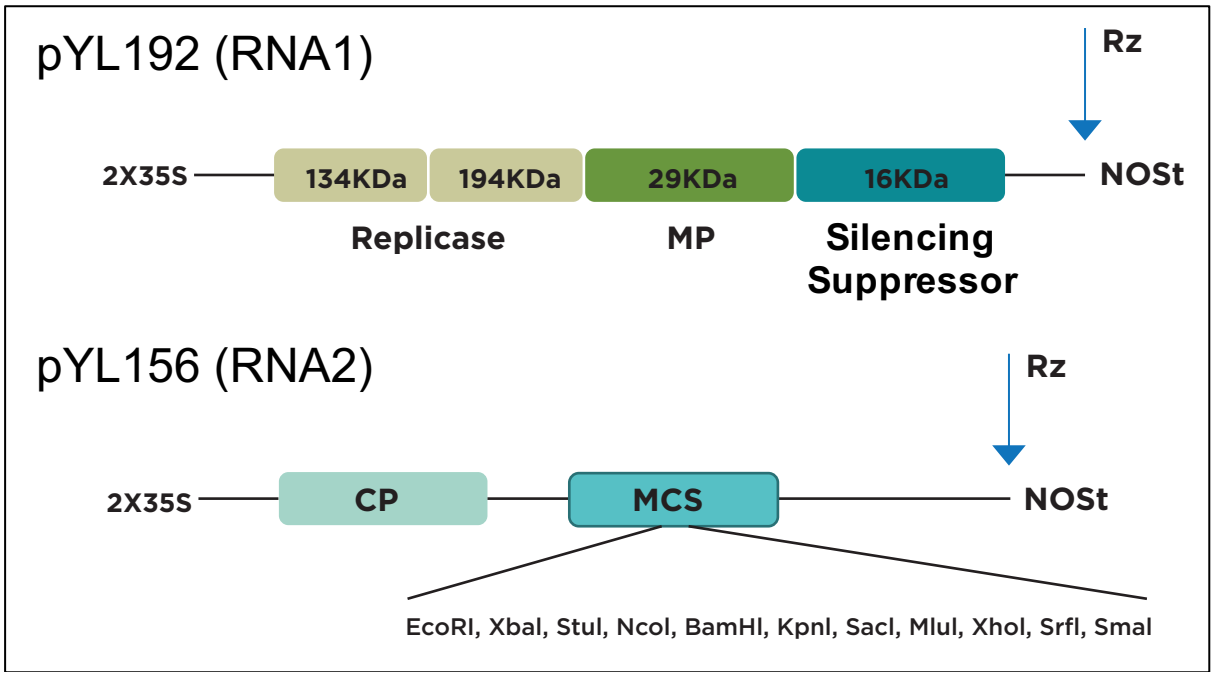


Figure 4.3. TRV cDNA regions of pYL192 and pYL156. pYL192 and pYL156, generated by Liu *et al.* (2002), include a 2X35S promoter driving the viral cDNA and a self-cleaving ribozyme sequence at the 3' end of the viral genomic cDNA. pYL156 was engineered with a multiple cloning site for insertion of desired sequence fragments. Diagram adapted from Senthil-Kumar and Mysore (2014).

4.2.2 TRV RNA2 modifications

I had two main considerations when designing my TRV RNA2 construct to deliver gRNA. First, I wanted to have as few non-essential bases as possible since it is known that smaller inserts are more stable in plant virus vectors (Avesani *et al.* 2007, Bruun-Rasmussen *et al.* 2007, Burch-Smith *et al.* 2004). The second main consideration was to ensure that the gRNA sequence started as close to the viral transcription start site (TSS) as possible in order for the gRNA to have an accurate 5' end. The 5' sequence of the gRNA aligns with the complementary target DNA and additional sequence could interfere with the targeting mechanism.

The TRV subgenomic RdRP promoter is not precisely defined (Miller and Koev 2000), but is generally thought of as the regions between ORFs on a viral genomic RNA. pYL156 includes the region in between the coat protein and 2b non-structural protein as the subgenomic RdRP promoter before the engineered MCS. Based on the findings of Hernández *et al*. (1995) and the reporting of Goulden *et al.* (1990), I located the transcription start site of TRV RNA2 sgRNA in strain ppk20 (Figure 4.4). I then analysed the sequence of pYL156 to find any non-essential bases between the TSS of the sgRNA and the MCS where my gRNA would be inserted.

There was a region of 303 bp in between the TSS and the MCS that corresponded to residual bases from the 5' end of the 2b non-structural protein gene, which could interfere with the processing of the gRNA. It has been shown that the 2b protein can increase the ability of TRV to invade roots and meristems (Valentine *et al*. 2004) so I thought that could be a possible reason for its sequence being present in the vector. However, Valentine *et al*. (2004) make it clear that the construct from Liu *et al.* (2002) lacks the 2b gene. Analysis of the plasmid confirmed that the residual bases corresponded to a severely truncated fragment of the 2b gene that would not have been functional. Removing them would therefore reduce the amount of additional sequence at the 5' end of my gRNA.

In order to remove the non-essential bases in between the TRV promoter and the MCS, I used an overlap PCR deletion approach (Figure 4.5). The resulting plasmid was called "pYL_DEL." I then inserted the gRNA into the multiple cloning site of pYL_DEL via restriction and ligation. I chose to use gRNA2 because of its consistent ability to activate the system in transient assays (see Chapter 3). The final TRV2 construct with the proper deletion of bases and insertion of gRNA2 was called "pYDg2."

The complete cloning method is outlined in Figure 4.5 and described in Chapter 2. Primers are in Appendix I and the map and sequence of pYDg2 can be found in Appendix II.

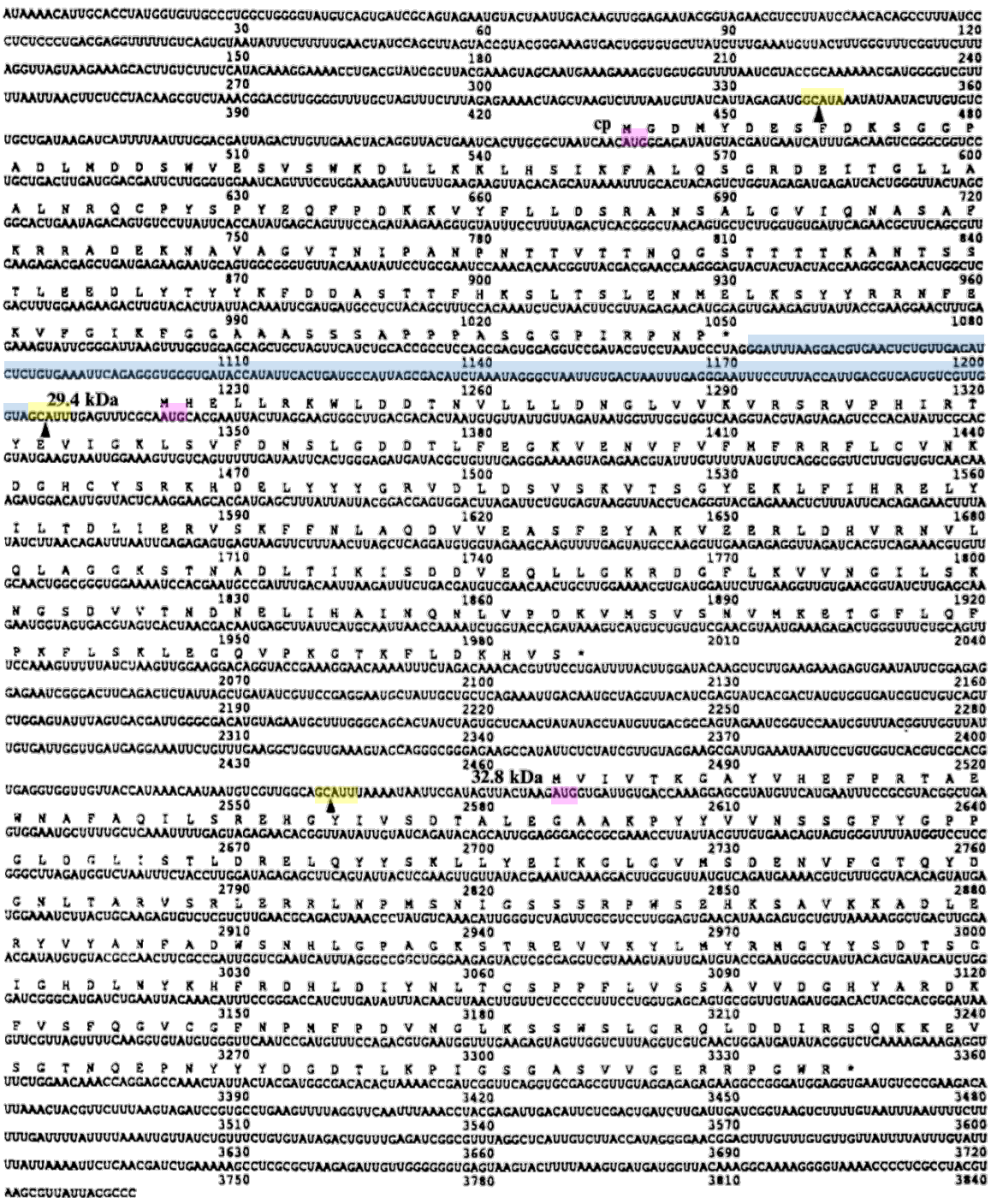


Figure 4.4. Annotated sequence of TRV ppk20 RNA2. Yellow boxes mark the sequence indicative of transcription start site, and black arrows deliniate 5' termini of sgRNAs. Pink boxes indicate translation start sites of ORFs. Blue represents the region thought of as an RdRP subgenomic promoter, the sequence in between the end of the coat protein and the next ORF. Sequence from Hernandez *et al*. (1995). Annotations by me, partially based on information in Goulden *et al.* (1990).

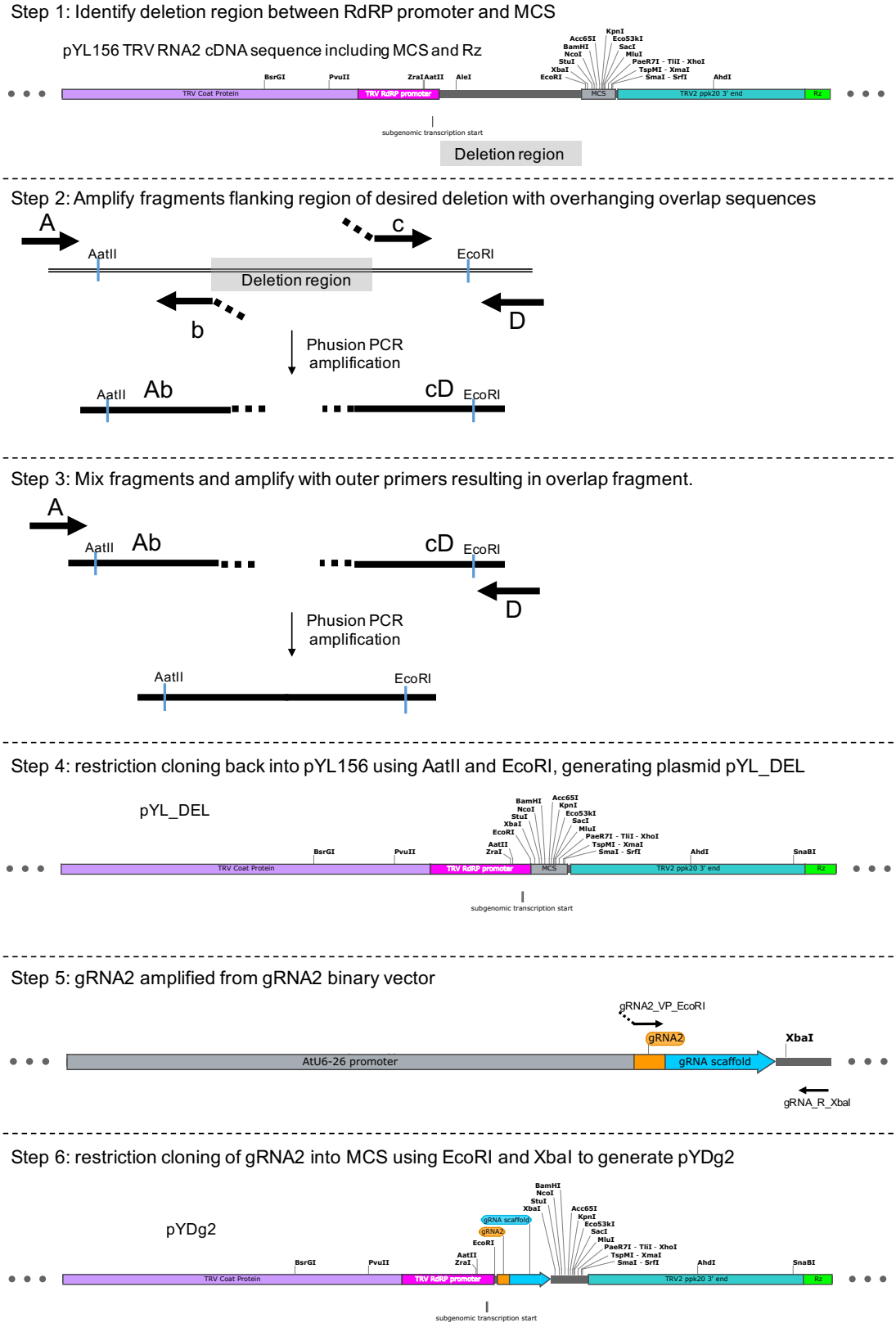


Figure 4.5. Cloning schematic to generate pYDg2, a gRNA2 TRV RNA2 vector. First, I used overlap PCR to delete a 303 bp non-essential fragment. Then I used restriction cloning to insert gRNA2. Schematics generated using Snapgene.

4.3 gRNA TRV activates GUS expression in directly infiltrated tissue

With the pYDg2 construct complete, I could test transactivation of my reporter system using gRNA delivered via TRV. Direct infiltration of viral constructs in *A. tumefaciens* into *N. benthamiana* is a commonly used method in plant biotechnology for virus inoculation (Macfarlane 2010). I transformed the viral constructs pYL192, pYL156, and pYDg2 into *A. tumefaciens* strain GV3101 and cultured them appropriately to prepare for direct infiltration into *N. benthamiana* K-AR T2s (Chapter 3). I used a 1:1 mixture of pYL192 and pYDg2 as "gRNA2 virus" and a 1:1 mixture of pYL192 and pYL156 as "wt TRV virus." I used two independent bacterial cultures of each construct to ensure biological replication. I infiltrated two leaves per plant of 4-week old *N. benthamiana* K-AR T2s with either gRNA2 virus (8 plants) or wt virus (8 plants). Experimental procedure is outlined in Figure 4.6 and infiltration methods can be found in Chapter 2.

At 3 days post infiltration (dpi) into the K-AR T2s, I harvested one infiltrated leaf for analysis. I used a 2mL microcentrifuge tube to punch an equal disc out of each leaf to be used in the GUS fluorometric assay and saved the rest of the leaf for RNA extraction. The GUS fluorometric assay shows significant activation of GUS in the gRNA2 virus infiltrated leaves compared with the wt virus infiltrated leaves (Figure 4.7).

RT-PCRs on RNA from the infiltrated leaves using primers located in the TRV RNA2 viral genome spanning the insertion site showed that each experimental group contained the correct virus and the single strong band indicates that at 3dpi in the infiltrated leaf, the insert is stably expressed in the gRNA2 virus (Figure 4.8). Primers for RT-PCR can be found in Appendix I.

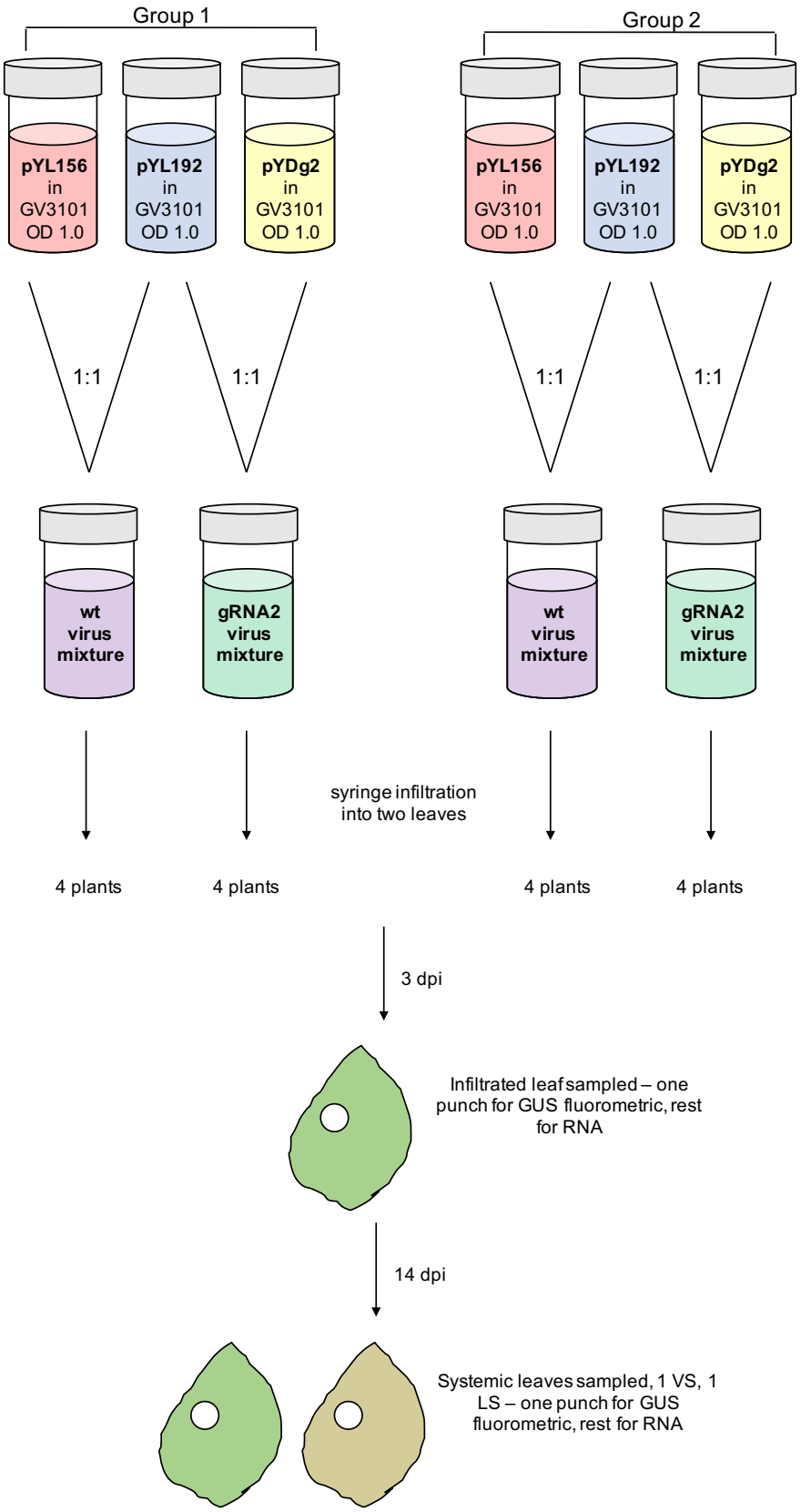


Figure 4.6. Diagram of virus infiltration and sampling method. First, I cultured viral vectors in *A. tumefaciens* GV3101 to OD 1.0. I used 1:1 ratio combinations to make a gRNA2 virus and a wt virus for infiltration into 4 week old *N. benthamiana* K-AR T2s. I sampled infiltrated leaves at 3dpi and systemic leaves at 14dpi to use for GUS fluorometric and RNA assays.

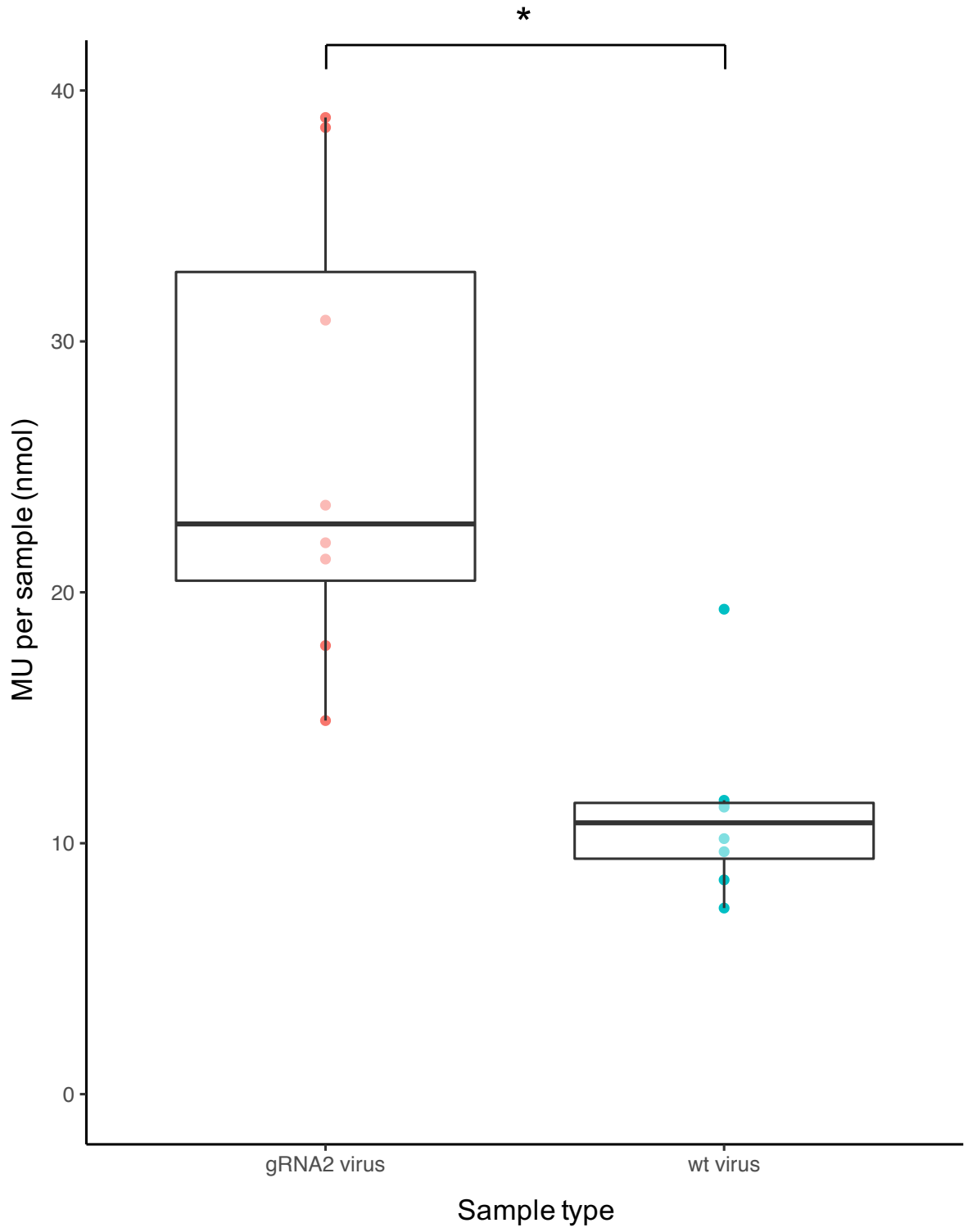


Figure 4.7. GUS fluorometric assay of directly infiltrated leaves 3dpi. The leaves agroinfiltrated with gRNA2 virus constructs showed significantly increased levels of GUS expression compared to leaves agroinfiltrated with wt virus constructs ($p=8*10^{-4}$, pairwise t-test). gRNA2 virus n=8 leaves, wt virus n=8 leaves.

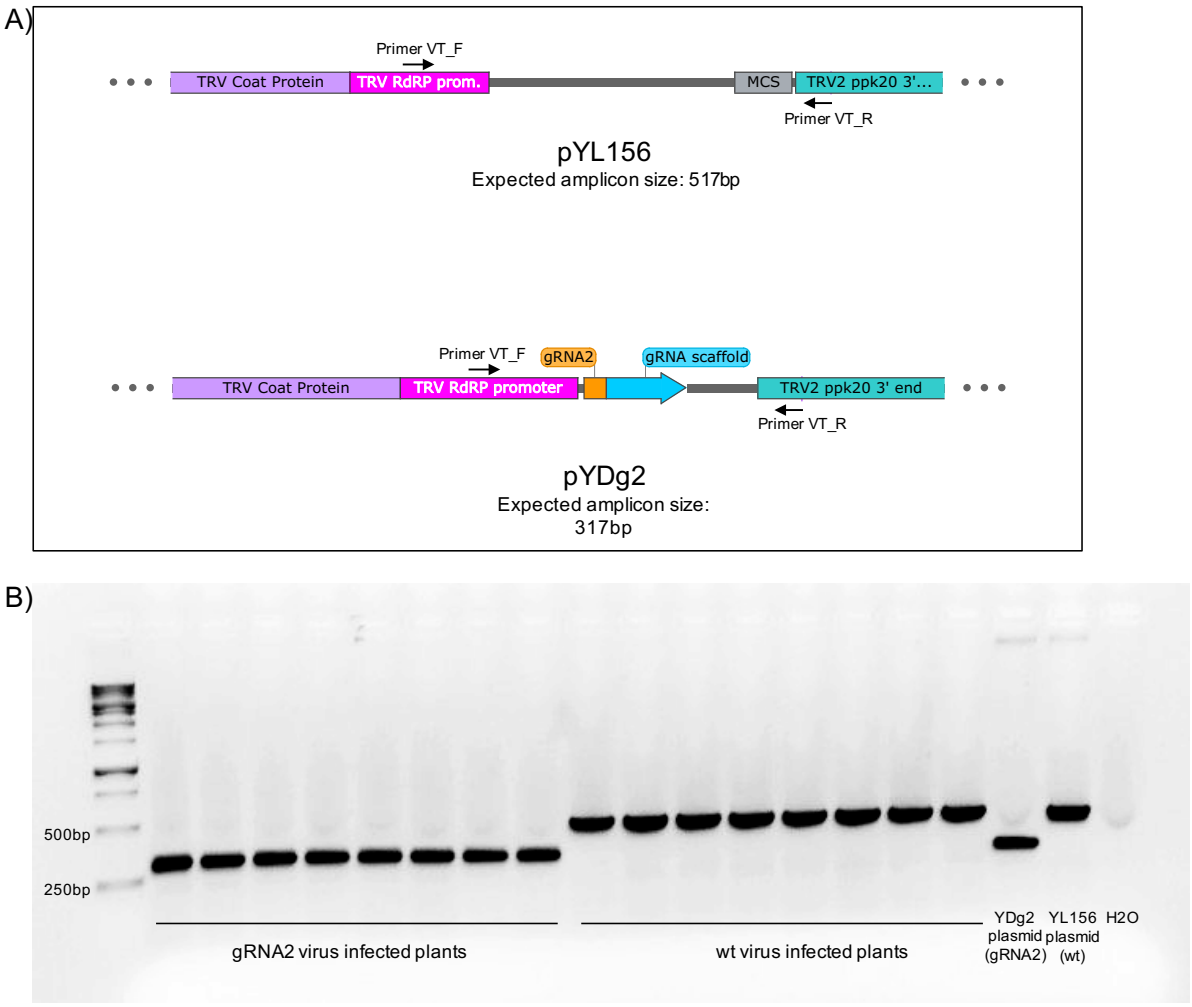


Figure 4.8. RT-PCR of virus infiltrated leaves 3dpi. A) Primers were used that span the insertion site on RNA2 to be able to distinguish between gRNA2 virus and wt virus. The expected band for the wt virus was 517bp and for the gRNA2 virus 317bp. The reason the gRNA2 virus is a smaller size, despite it having an insert is because the wt virus is the undeleted version of pYL156. 517bp - 303bp deletion = 214bp. 214bp + 103bp gRNA2 = 317bp. Schematics generated using Snapgene. B) RT-PCR shows the bands are of the correct size and show no insert loss at this time point.

4.4. gRNA TRV activates GUS expression in mildly symptomatic systemic leaves but not in very symptomatic systemic leaves

The next question was whether the gRNA2 virus could activate in systemic (not directly infiltrated) leaves. At 14 dpi, I collected tissue from “less symptomatic” and “very symptomatic” systemic leaves. The “less symptomatic” leaves were always the newest leaves on the plant, while the “very symptomatic leaves” were closer to the position of the leaves that had been directly infiltrated. As in the previous assay, I used a 2mL microcentrifuge tube to punch an equal disc out of each leaf to be used in the GUS fluorometric assay and saved the rest of the leaf for RNA extraction.

The GUS fluorometric assay (Figure 4.9) showed that in the “less symptomatic” leaves, there is still a pattern that the plants infected with the gRNA2 virus exhibit higher GUS expression than the plants infected with the wt TRV. In contrast, in the “very symptomatic” leaves, there is no difference between the two conditions but background GUS expression levels are much higher than previously observed activation.

RT-PCRs on the RNA from the systemic leaves (Figure 4.10) revealed instability of the insert in the virus vector. In the “less symptomatic” leaves there was a band of the correct size for the gRNA2 virus, but at a lower abundance than the signal from the wt virus. The “very symptomatic leaves” showed degradation of the gRNA insert, with weak bands of many different sizes in the gRNA2 virus samples, while the wt virus samples still resulted in a single, strong band of the correct size.

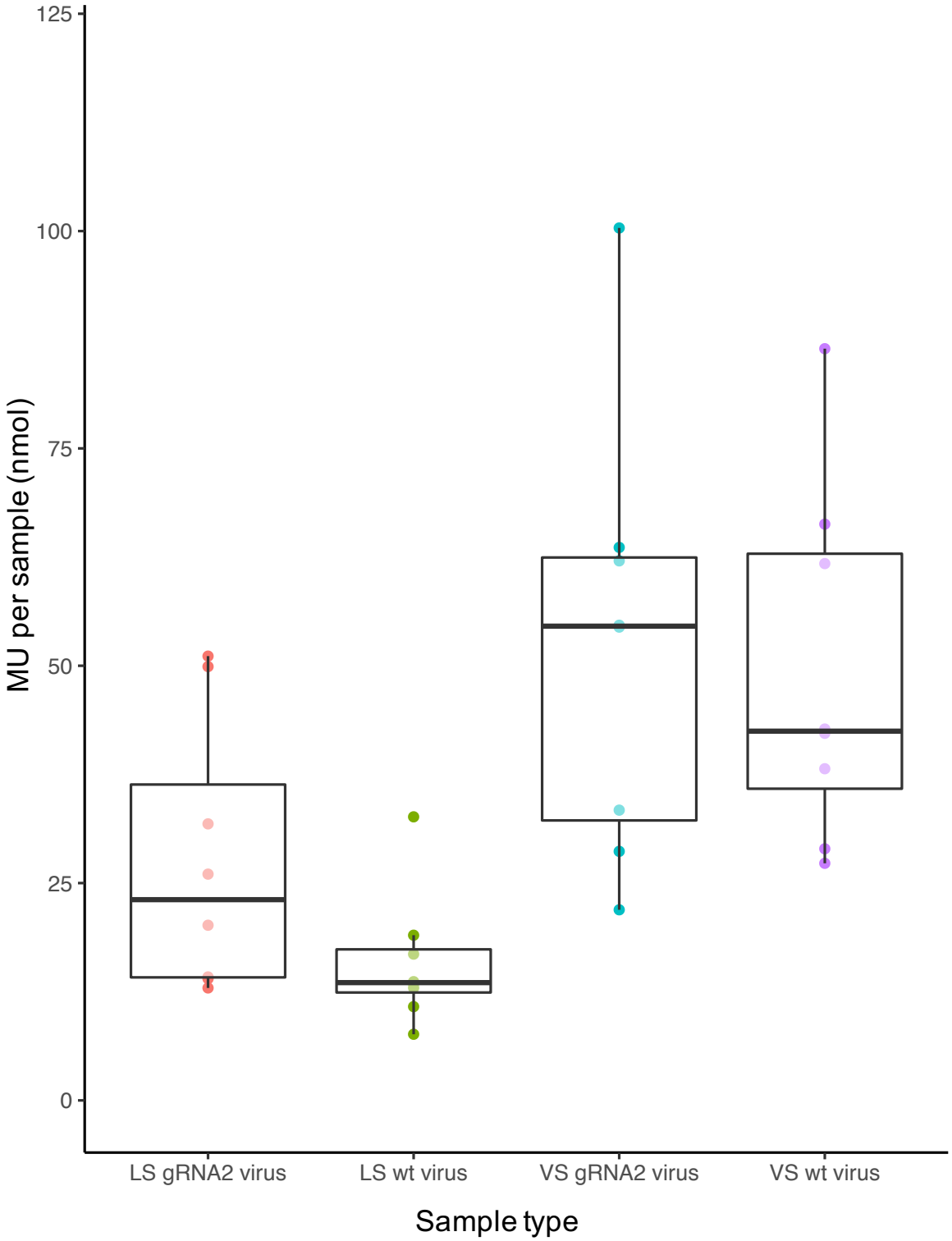


Figure 4.9. GUS fluorometric assay of systemic leaves 14dpi. LS = Less Symptomatic, VS = Very Symptomatic. In the LS condition, the systemic leaves infected with gRNA2 virus seem to show increased levels of GUS expression compared to systemic leaves infected with wt virus, but the difference is not statistically significant (p=0.078, pairwise t-test). Any pattern of activation is lost in VS leaves (p=0.79) and the level of GUS is much higher than anticipated. LS gRNA2 virus n=8 leaves, VS gRNA2 virus n=8 leaves, LS wt virus n=8 leaves, VS wt virus n=8 leaves.

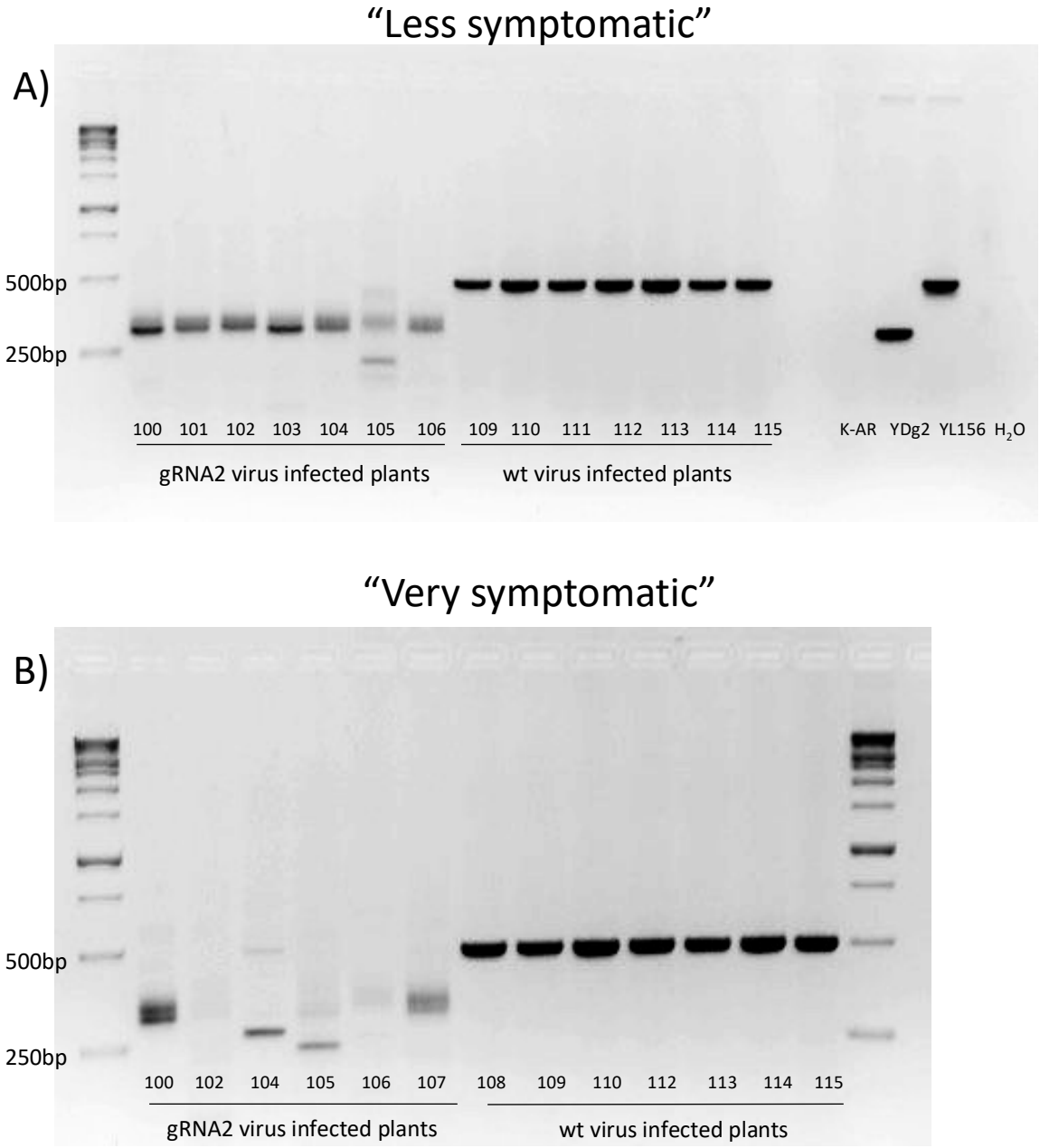


Figure 4.10. RT-PCR of virus infected systemic leaves 14dpi. Primers were used that span the insertion site on RNA2 to be able to distinguish between gRNA2 virus and wt virus, as in Figure 4.8. A) Less symptomatic systemic leaves. B) Very symptomatic leaves. Controls shown are a non-infected K-AR transgenic plant, the two TRV RNA2 plasmids to show correct size, and water.

4.5 Discussion

I have described the design, construction, and testing of a TRV vector for CRISPR gRNA delivery. In this section, I will discuss the preliminary evidence showing that these gRNAs are effective for transactivation of the reporter in my CRISPRa system (Chapter 3) and suggest future directions for validation and optimisation.

4.5.1 TRV assays in *N. benthamiana*

My preliminary evidence indicates that the GUS reporter gene in my CRISPRa system was able to be transactivated by gRNA delivered in a TRV vector. The directly infiltrated leaves of *N. benthamiana* K-AR T2s showed clear activation of the GUS reporter when infected with my gRNA2 TRV compared to leaves infected with wt TRV (Figure 4.7).

In systemic "less symptomatic" leaves, there was also activation of the GUS reporter (Figure 4.9). In contrast, "very symptomatic" systemic leaves showed unexpectedly high levels of GUS with both the wt and gRNA constructs. This observation suggests that the GUS gene was somehow activated by a mechanism other than the gRNA guiding dCas9-VP64 to the minimal promoter.

The RT-PCRs from these samples show intact gRNA2 virus and wt virus in the respective infiltrated leaves (Figure 4.8). However, in more symptomatic leaves, there was instability of the gRNA2 virus (Figure 4.10). Even wild type TRV is known to recombine to modify the ORFs on RNA2 (Hernandez *et al*. 1996) so I propose that my engineered TRV recombined to negatively select against the gRNA2 insert. The instability of the gRNA2 insert explains why the less symptomatic leaves showed less GUS activation than infiltrated leaves.

Recombination, however, does not explain why the GUS transgene driven by the minimal promoter was overexpressed in the "very symptomatic" leaves. One potential reason is that the GUS reporter transgene was silenced in the K-AR T2 plants. It is known that transgenes are frequently subjected to epigenetic silencing in plants (Rajeevkumar *et al*. 2015) and there is direct evidence of GUS transgene silencing in *N. tabacum* (Day *et al.* 2000). In this scenario, in the absence of the virus, the transgene expression was low. Upon infection with either wt or gRNA TRV, the RNA1-encoded suppressor of silencing released this transgene silencing and caused high background levels of GUS. The TRV 16K suppressor of silencing has been shown to interfere with silencing of endogenous genes during viral infection in addition to its role of reducing the immune response to the virus (Ghazala *et al.* 2008, Martínez-Priego *et al*. 2008) so this is a likely explanation.

The “less symptomatic” leaves were farther away from the initial infiltration, while the “very symptomatic” leaves were close to the infiltrated leaves on the plant. Therefore, in the “very symptomatic” samples, the virus probably had a longer amount of time to replicate, recombine to select for the preferred version without intact gRNA, and cause symptoms of viral infection including suppression of transgene silencing.

4.5.1.1 Preliminary activation results require further confirmation

These GUS activation and insert stability data are preliminary and further experiments are needed to confirm the results. Repeat assays should include the infection of wt *N. benthamiana* with both wt and gRNA2 virus constructs alongside the K-AR T2 *N. benthamiana*. This additional control would investigate the hypothesis that the viral release of transgene silencing caused the increased level of GUS in very symptomatic systemic leaves. If this theory is correct, it would be expected that very symptomatic wild type leaves would show no difference compared to less symptomatic non-infected wild type leaves in the GUS fluorometric assay, with both conditions exhibiting background levels. In contrast, the K-AR T2 very symptomatic leaves would show the same pattern as the results in this chapter, with very high GUS levels in both wt virus and gRNA2 virus infected tissue.

GUS RNA expression data determined by qPCR is necessary to serve as a confirmation of the activation observed in the fluorometric assays. Using qPCR, the system could also be tested using a gRNA targeting an endogenous gene. Demonstrating activation of a non-silenced endogenous gene would avoid the issue of the viral infection’s potential effect on transgene silencing.

4.5.1.2 Preliminary viral gRNA2 insert stability results require further confirmation

The RT-PCRs presented in this chapter were originally intended as a diagnostic test to confirm that the leaf samples were infected with the correct virus. Unfortunately, these experiments are lacking a no-RT control. The no-RT control is essential to validate the observations because in the infiltrated leaves, any residual DNA from the pYDg2 plasmid used in the agroinfiltrations would amplify during PCR and give a false result. The samples were treated with DNase during RNA extraction, but a no-RT reaction would confirm that the bright single bands representing a stable gRNA virus in the infiltrated leaves was indeed from viral RNA and not residual plasmid DNA.

The systemic leaves were in theory never exposed to agroinfiltration, but the no-RT is still necessary to ensure that the result is purely from viral RNA and not from plasmid

contamination. A non-infected plant as a negative control did demonstrate that the signal was coming from the virus and not an endogenous location. (Figure 4.10). The RNA from the non-infected plant was good quality based on quantification, but testing that an endogenous gene (e.g. GAPDH) is able to be amplified from the sample would verify its reliability as a negative control for the virus assay.

Another improvement would be to change the RT-PCR primer pair spanning the gRNA2 insertion site in order to amplify from the negative sense strand of RNA2. The primers used in this chapter were designed to amplify the positive sense strand of RNA. Therefore, in infiltrated leaves, it cannot be concluded whether the amplification signal is a product of viral replication or merely the initial RNA transcript from the pYDg2 plasmid. Negative sense viral RNA is a fundamental part of the TRV replication process (explained in Section 4.1). Therefore, testing for its presence would illustrate whether the virus is actively replicating in infected tissue.

The relative replication competence of the different viral constructs should also be assessed. Using a second primer pair spanning a region of the conserved coat protein sequence would show relative amounts of TRV RNA2 accumulation in tissues infected with wt or gRNA2 TRV. This control could be measured by analysing band strength in RT-PCR or performing qPCR. It would also be interesting to include the intermediate TRV RNA2 construct pYL_DEL in this analysis to determine whether the viral replication ability is affected by the deletion of bases in between the subgenomic promoter and the MCS or only once the gRNA2 insert has been added in pYDg2, if there are any differences at all.

4.5.2 Virus optimisation and future directions

I aimed to generate an improved TRV vector for delivering gRNA to my CRISPRa system. With the knowledge that plant viral vectors are more stable with smaller inserts (Avesani *et al.* 2007, Bruun-Rasmussen *et al.* 2007, Burch-Smith *et al.* 2004), I removed 303 unnecessary bases before the MCS in the pYL156 TRV RNA2 vector. This deletion also meant that my gRNA would start immediately after the viral TSS, ensuring a precise 5’ start to the gRNA. I considered engineering a ribozyme sequence at the 3’ end of the gRNA so that there would be a precise cut-off, but this strategy would interrupt viral replication so it was not possible to be sure of the 3’ end. The gRNA was functional even without 3’ cleavage so it does not seem like an essential modification.

I considerably reduced the size of insert compared with Ali *et al.* (2015), who kept the non-essential bases before the MCS and on top of that inserted a subgenomic RdRP promoter

sequence from Pea Early Browning Virus, which was not needed because the TRV subgenomic promoter was already present in the construct. When they tested for viral presence in the infiltrated and systemic leaves of their plants via RT-PCR, their TRV RNA2 amplicon was located in the coat protein sequence, and not related to the gRNA insertion site. Therefore, they would not have been able to detect insert stability. My results suggest that while the wild type parts of their virus may have been intact, they probably had substantial insert removal before the virus even reached a meristem to invade. Therefore, it is unsurprising that their reported inheritance efficiency was so low, and potentially not even valid (Yin *et al.* 2015).

The preference of the virus to remove gRNA inserts is problematic for the use of viral vectors to deliver gRNA for CRISPR gene editing. When I first saw the results from Ali *et al.* (2015) which suggest that VIGE in plants would be possible using TRV, I assumed that their extremely low efficiency of mutation inheritance was caused by the virus not being able to act efficiently in the meristem, where the germ line cells are formed. Even TRV, which has a suppressor of silencing that allows meristem invasion, is only able to remain in the meristem for a short amount of time, and the plant defences are high to try to eliminate the virus (Martin-Hernandez and Baulcombe 2008).

I therefore initially thought my transactivation system would be useful to study and optimise the spatial aspects of gRNA delivery via a virus, dissecting out the meristem to measure GUS activation level. While my system still would allow this assay, I think the problem with the method lies before invasion of the meristem. If the virus with the gRNA insert is already being selected against in the leaves, only a small fraction of the virus with intact insert would ever reach the meristem.

It still could be possible to use viral gRNA delivery for CRISPR gene editing, but my results indicate that optimisation should probably focus on stability of the insert in the virus, because proper and precise editing will not occur if the virus successfully invades the meristem but is no longer carrying an intact gRNA. One strategy for improving stability would be to examine the structure of the insert instead of only the length. Ding *et al*. (2018) found that modifying the secondary structure of their insert in BMV led to increased insert stability. This outcome makes sense because the RNA structure could be important in the template switching mechanism that is the cause of many types of viral recombination (Bujarski 2013, Sztuba-Solinska *et al.* 2011).

Further optimisation strategies and relevant new improvements to VIGE are discussed in Chapter 6. My positive readout strategy will be a useful tool for future analysis; even at low efficiency, activation is easily assayed and quantified.

Chapter 5: Graft delivery of gRNA

5.1 Introduction

Grafting is frequently used in both crop production and basic research to obtain desired phenotypic traits and study long distance systemic movement of signalling molecules through the plant vasculature. *N. benthamiana* is able to be grafted, even to other species in heterografts (Notaguchi *et al.* 2015, Xia *et al.* 2018). There are previous demonstrations of movement of siRNA, miRNA, and mRNAs across graft junctions in *N. benthamiana* (Voinnet *et al.* 1998, Bai *et al.* 2011, Kasai *et al.* 2010, Xu *et al.* 2013).

Typically, mobile RNA follows the direction of phloem flow from photosynthetic source tissues like mature leaves to sink tissues like roots and flowers (Kehr and Kragler 2018). In grafting experiments, the RNA of interest is expressed in source tissue and movement across the graft junction is demonstrated by sampling the destination sink tissue (Figure 5.1).

Normally the sink tissues in the scion are in meristems and flowers, but a technique called mentor grafting (Figure 5.2) can force additional sinks in the shoot, encouraging movement from the rootstock into the scion (Goldschmidt 2014). This method involves removing all mature leaves of the scion after the graft has healed, forcing the scions to rely completely on the rootstock for nutrients. The mentor grafting procedure switches the role of the scion from source tissue into sink tissue. New developing leaves that grow from the scion are nutrient sinks and are sampled for RNA assays. Mentor grafting has been previously used successfully in *N. benthamiana* (Kasai *et al.* 2011).

The aim of the experiments in this chapter was to explore whether grafting could be a potential method for delivering CRISPR gRNA in trans. I based my work on the questions: 1) Can a gRNA enter the phloem channel and move systemically? 2) If gRNA is mobile, is it still functional when it reaches its destination tissue?

There is no information on whether gRNA can move systemically in a plant which therefore makes it an exciting concept to investigate for both basic science and future applications.

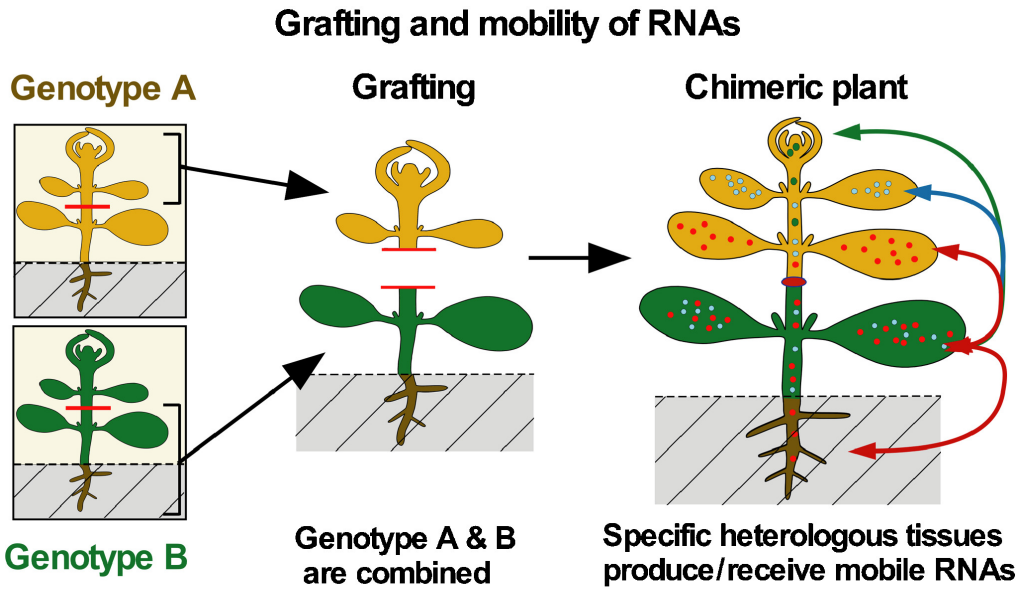


Figure 5.1 Grafting to test for RNA mobility. Plants of two types, here represented by genotypes, are grafted together in order to observe movement of molecules like RNA from source tissue to sink tissue. Figure from Kehr and Kragler (2018).

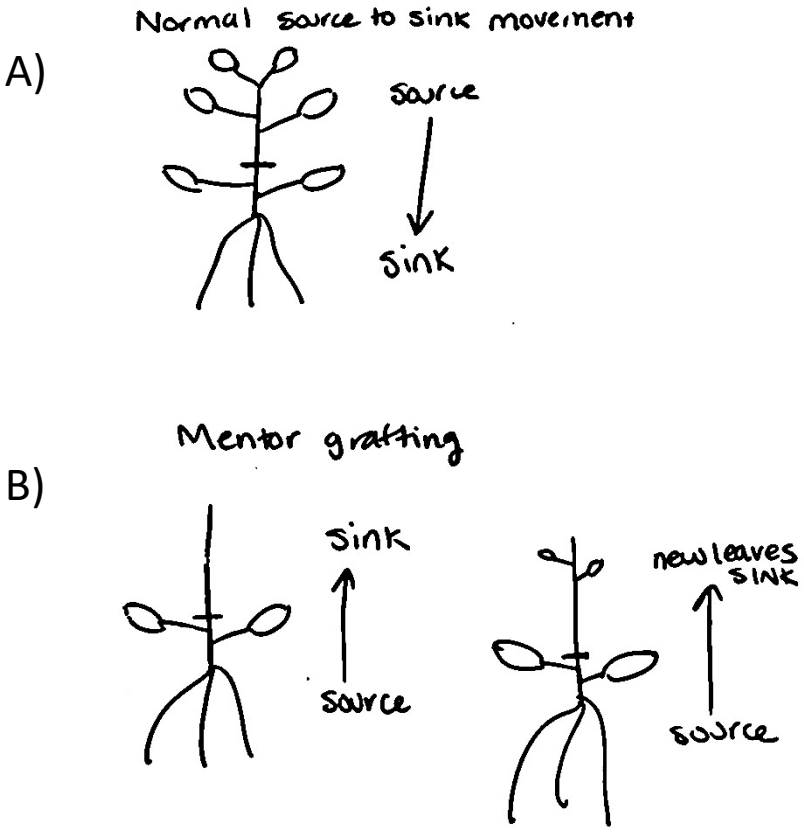


Figure 5.2 Mentor grafting schematic. A) The direction of phloem flow normally moves from photosynthetic source tissues in the shoot to sink tissues in the root. B) The technique of mentor grafting involves cutting off the leaves of the shoot in order to induce new growth, forcing a sink in the scion.

5.2 Results

Grafting experiments were carried out to test whether gRNA could move systemically in plants. I also performed crosses intended to be a control for function of gRNA produced from a transgene.

5.2.1 Grafting design

Reciprocal experimental and control grafts were designed in order to test for gRNA movement (Figure 5.3). Experimental grafts consisted of T2 transgenic gRNA2 *N. benthamiana* grafted to T2 transgenic K-AR *N. benthamiana*. Detail about the generation of these T2 transgenic lines is in Chapters 2 and 3. Before grafting, I confirmed transgene presence in each individual plant via PCR. Control grafts followed the same set up, but with wild type *N. benthamiana* grafted to T2 transgenic K-AR plants.

It is known that RNA generally moves systemically from source to sink (Kehr and Kragler 2018). Therefore, for grafts containing gRNA2 plants as the scion and K-AR as the stock, it would be expected that gRNA2 would move from shoot to root, following the source to sink gradient (Figure 5.3). In the controls, no gRNA2 should be detected in the sink tissue because there would be no gRNA2 being produced in the wild type scion. The strategy of reciprocal grafting was used in order to make it possible to force a sink in the scion via mentor grafting, changing the direction of gRNA2 movement from root to shoot. The control grafts would not show any gRNA2 in the shoot sink tissue because it would not be present in the root source.

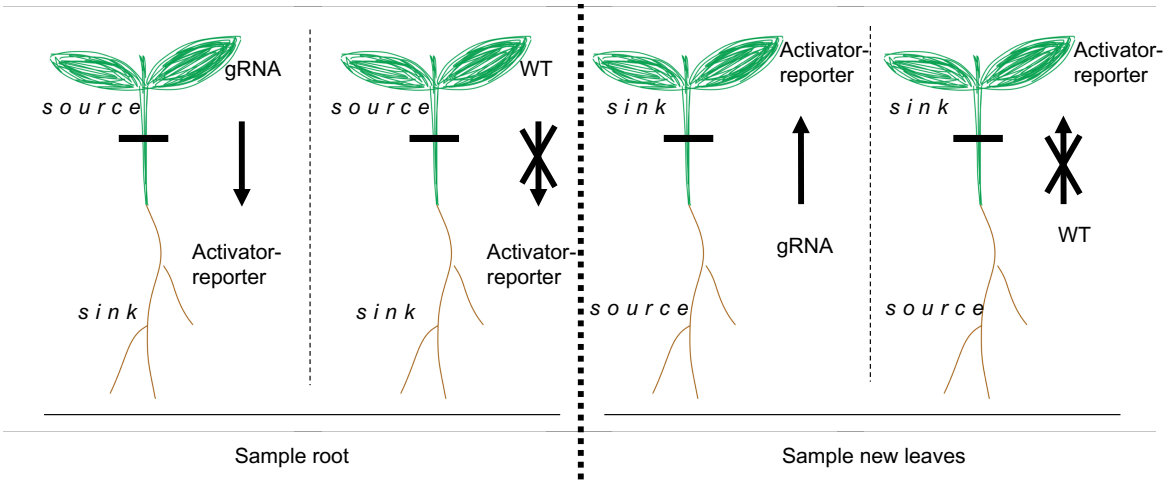


Figure 5.3. Grafting design. Reciprocal grafts between K-AR and gRNA2 T2s, and K-AR T2s and wild type plants were designed to allow assaying gRNA movement in both directions across the graft junction.

5.2.2 RT-PCR results from graft roots inconclusive about gRNA mobility

I expected that mobile gRNA2 would be in low abundance in destination sink tissues. Therefore, I chose to test for the presence of gRNA2 by RT-PCR because the amplification is highly sensitive. In order to detect a mobile version of gRNA2, I designed RT-PCR primers within its sequence (Figure 5.4A). Primer sequences can be found in Appendix I.

I started by testing the roots of grafts in which K-AR was the stock because it would be more likely to detect movement from the shoot to the naturally strong sink tissue of the root than via a mentor grafting approach. The expected result from the RT-PCR was to find a 95 bp fragment representing gRNA2 in the roots of experimental grafts that had gRNA2 scions, but not in the roots of control grafts that had wild type scions. Eleven wt/K-AR grafts and eight gRNA2/K-AR grafts were sampled and root RNA was extracted. Positive control samples (gRNA2 transgenic roots) showed a clear band for gRNA2 (Figure 5.4B), demonstrating that gRNA2 can be detected in roots via this method when it is in high enough abundance. There was no indication of mobile gRNA2 in any of the grafted root samples (Figure 5.4B).

5.2.3 Crossing of T1 lines intended as a positive control for activation

gRNA would be produced transgenically in the T2 plants used for grafting. As a control for this type of expression, I crossed a stable K-AR T1 line with a stable gRNA2 T1 line (Figure 5.5) in order to validate the functionality of my CRISPRa system when the gRNA is expressed via a transgene. Plants expressing both K-AR and gRNA2 were expected to exhibit GUS activation compared to uncrossed K-AR T2 lines. My full *N. benthamiana* crossing method (found in Chapter 2) required development to establish correct timing for the flower to be mature enough to develop properly, but not already have been self-pollinated. I was able to collect seeds from 14 of the cross attempts.

I sowed F1 seeds from 7 of the 14 crosses alongside K-AR T2s to be able to compare plants of the same age and used OneStep RT-PCR to confirm expression of both transgenes in 6 of the 7 progeny sets (Figure 5.6). Three of the progeny sets were generated with K-AR as male and three as female. I selected 4 plants from each of the 6 successful crosses that were positive for both transgenes to be sampled for GUS expression. I punched equal leaf discs with a 2mL microcentrifuge tube to be used in the GUS fluorometric assay. At the same time, I harvested leaf punches from the 8 K-AR T2s of the same age. The fluorometric assay did not show any difference in GUS expression between the crosses and the K-AR T2s (Figure 5.7).

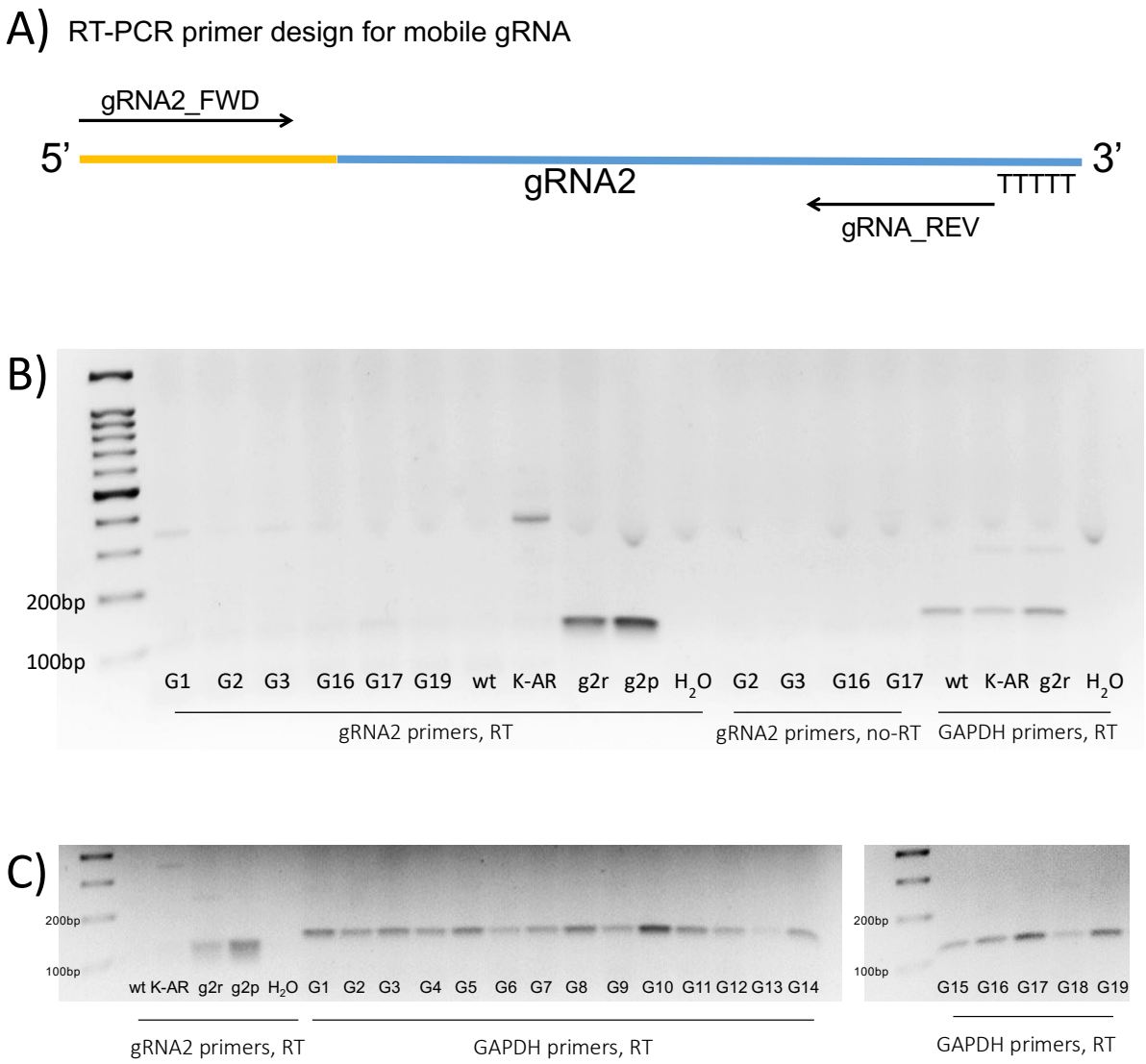


Figure 5.4. gRNA2 primer design and graft root RT-PCR results. A) RT-PCR primers were designed to only amplify inside the gRNA in order to be able to detect it in its potential mobile form. B) RT-PCRs were carried out on graft root RNA to test for gRNA2 movement from shoot to root. This gel shows samples which are representative of all the grafts tested. G1, G2, G3 = wt shoot/K-AR root grafts. G16, G17, G19 = gRNA2 shoot/K-AR root grafts. wt = non-grafted wt root. K-AR= non-grafted K-AR T2 root. g2r = gRNA2 T2 root. g2p = gRNA2 plasmid. Using the gRNA2 primers (shown in A), RT-PCR did not show a gRNA2 band in roots of either control grafts or experimental grafts. wt and K-AR samples were negative controls, and did not have a gRNA2 band as expected. g2r was a positive control and the clear band demonstrates gRNA2 is detectable via RT-PCR in gRNA2 transgenic roots. g2p has the same clear band which is a positive control for primer function and band size. No-RT controls for the grafts also did not show a band. GAPDH testing confirmed that the lack of gRNA2 bands was a true result and not due to insufficient RNA quality. GAPDH results for wt, K-AR, and g2r samples is included in B). The GAPDH results for all the grafts are on the gels shown in C) along with repeated results for control samples (wt, K-AR, g2r, g2p, H2O) with gRNA2 primers.

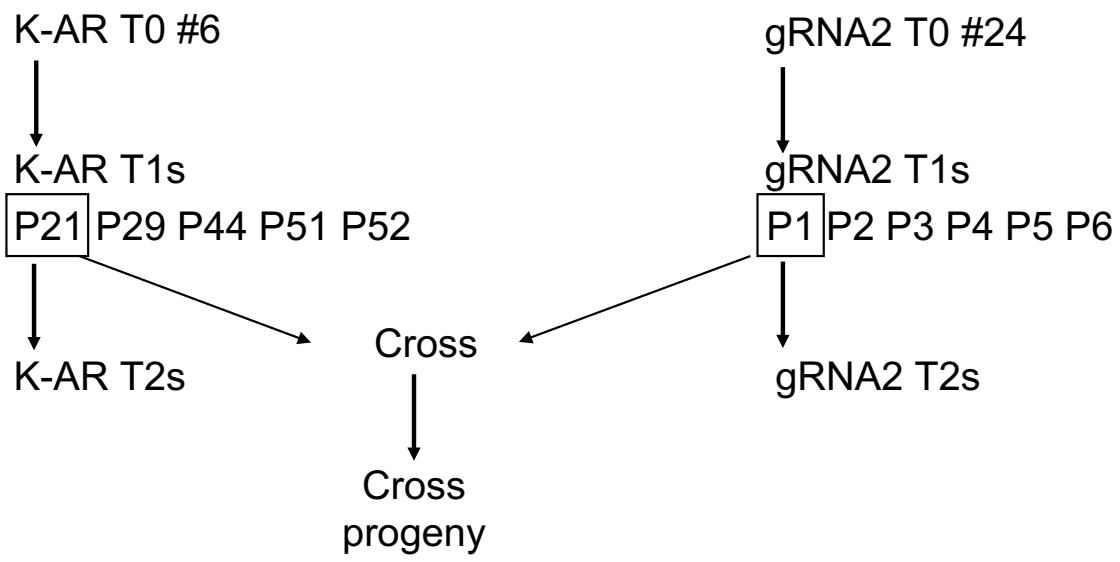


Figure 5.5. Crossing scheme. K-AR T1 P21 and gRNA2 T1 P1 plants were crossed multiple times, in both directions (as the male and as the female). The progeny were sown and compared to K-AR T2s for GUS level.

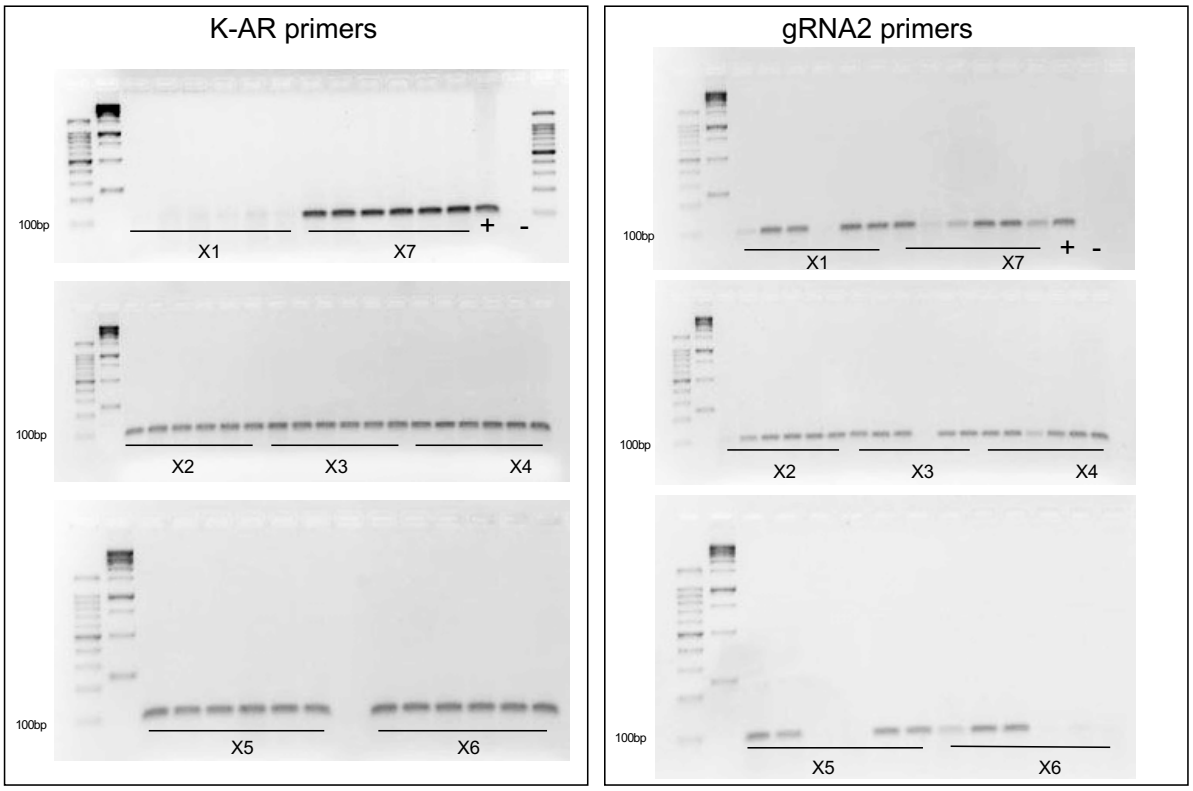


Figure 5.6. RT-PCRs show successful crossing of F1 transgenic lines. Progeny sets from six individual crosses were tested for expression of both K-AR and gRNA2 transgenes. Crosses 1-4 (X1-X4) were performed using K-AR#6/21 as the male and gRNA2#24/1 as the female. X5-X7 were performed in the opposite direction. K-AR primers: + = K-AR plasmid, – = H_2O. gRNA2 primers: + = gRNA2 plasmid, – = H_2O. For the fluorometric assay (see Figure 5.7), the following were the cross progeny plants used: X2 #3,4,5,6/X3 #1,2,3,5/X4 #2,4,5,6/X5 #1,2,5,6/X6 #1,2,3,4/X7 #1,4,5,6.

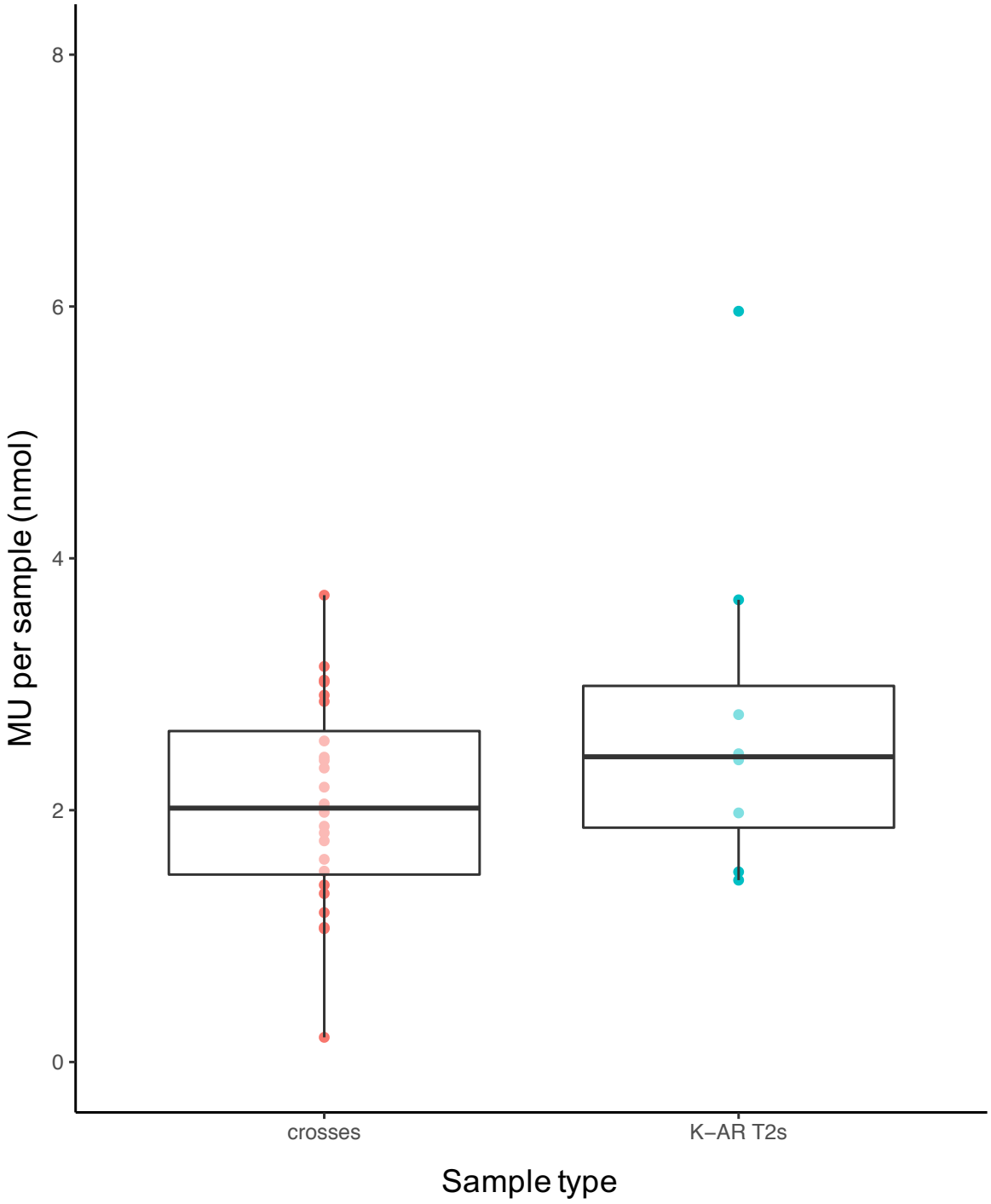


Figure 5.7 GUS fluorometric assays of K-AR T2 lines compared to crosses. There is no significant difference (p=0.095) between the GUS level in the crosses compared to the K-AR T2s. K-AR T2s n=8. Crosses n=24. 6 cross progeny sets are represented, 4 plants per set.

5.3 Discussion

The aim of the work presented in this chapter was to explore the use of grafting as a method of gRNA delivery by investigating the questions 1) Is gRNA mobile across a graft junction? and 2) If gRNA is systemically mobile, is it still functional upon reaching its destination tissue?

RT-PCR results from grafting experiments were unable to demonstrate movement of gRNA2 across a graft junction (Figure 5.4). However, the possibility of gRNA mobility should not yet be eliminated. Crosses which were meant to act as a positive control for gRNA2 activation from a transgene did not produce the desired effect (Figure 5.7). It is likely that there was not enough gRNA2 produced in the transgenic gRNA2 lines to activate the system (discussed below), and therefore it probably also would not have been in high enough abundance to be detected in its mobile form.

In retrospect, I should have focused first on functionality of transgenic and mobile gRNA2 instead of testing for movement. Ensuring functionality of the gRNA2 transgenic lines before using them for grafting would have increased the potential to detect gRNA2 systemic movement. Additionally, GUS assays could have been used to determine whether mobile gRNA2 is able to activate the system. Even if gRNA is mobile, lack of function post-movement would discard this strategy for gene editing applications.

5.3.1 Crosses were unable to function as a control

In order to focus on functionality, it would be important to confirm that gRNA produced from a transgene is able to activate the system. The crosses presented in this chapter were meant to act as that control, but they were ineffective.

The crosses performed between K-AR and gRNA2 T2 lines were successful in the sense of both transgenes being expressed in F1 progeny (Figure 5.6). However, the GUS fluorometric assay showed no difference in GUS expression between un-crossed K-AR T2 plants and the crossed F1s (Figure 5.7). The anticipated result was clear activation due to both transgenes being constitutively expressed in each cell, so this observation was surprising.

It is unlikely that the K-AR transgenes were no longer able to be activated after crossing because their functionality had been tested by transient assay (Chapter 3). A more likely explanation is that the transgene in the gRNA2 T2 line was not functional perhaps because the expression level was too low. I characterised the gRNA2 lines for expression of the gRNA with RT-PCR, but did not test them for function. Therefore, the selected gRNA T2 line could be

non-functional in the system. This could be tested by doing transient expression assays in the parental gRNA line, following the same strategy used to test function in the K-AR lines (Chapter 3). First, a modified K-AR construct containing only the minimal promoter driven reporter would need to be generated by removing the dCas9-VP64. When infiltrated separately into wt *N. benthamiana*, spots infiltrated with the original K-AR construct and the modified K-AR construct (K-R) should show the same level of GUS expression because there would be no gRNA present. In contrast, on gRNA transgenic lines, the K-AR infiltration should show increased levels of GUS compared to the K-R infiltration. A confirmed functional gRNA line could then be used for crossing with K-AR.

Expression level of the transgenes could also have played a role in the result of the crosses. Previous demonstration of the functional CRISPRa system in stable K-AR transgenic lines (Chapter 3) was via agroinfiltration assays, in which very high copy number of the gRNA was introduced to each cell of the infiltrated spot. The integrated transgene is likely not transcribed as abundantly, and may even be partially silenced. To test this hypothesis, I would use qPCR to measure the gRNA expression level in the gRNA transgenic lines compared to gRNA expression level in wt or K-AR leaves agroinfiltrated with the gRNA construct. If the transgenic lines show lower expression levels, the role of silencing could be explored by transiently expressing a silencing suppressor such as P19 (Voinnet *et al.* 1999, Silhavy *et al*. 2002, Voinnet *et al.* 2003: retracted) in gRNA2 transgenic lines and comparing gRNA transcript abundance in the infiltrated spots and non-infiltrated leaf.

In addition to lower expression level of the gRNA transgene in stably transformed lines compared to agroinfiltration, the crosses also cause the dosage of each transgene to be reduced. Dosage effect describes how copy number of a gene can proportionally affect the quantity of the gene's product (Birchler and Veitia 2012). Therefore, after crossing the F1 transgenic lines there would be fewer copies of the gene producing gRNA2 and fewer copies of the K-AR gene to be activated. In order to cause the observed result, the amount of activation caused by the gRNA would have to make up for the different dosage amount.

This phenomenon could be tested by sampling the cross F1s that express K-AR but are lacking the gRNA transgene and comparing them to the F1s from the same cross that do express both transgenes. In my experiment, there were too few of these plants to have been able to draw any conclusions. In the future, I would choose one cross and sow out many F1s to have a large enough sample size to test for dosage effect.

5.3.2 Testing the function of mobile gRNA using CRISPRa

After validating that gRNA can activate the GUS reporter in a crossing situation, my CRISPRa system would also allow mobile gRNA functionality to be easily assayed. Even a low abundance of gRNA moving across a graft junction could have a measurable activation effect. To test for this, I would do qPCR for GUS, comparing the expression levels in K-AR sink tissues with mobile gRNA and the K-AR sink tissues that had been grafted to wt plants. If the GUS level is increased, it would show that the gRNA is able to transactivate the system, and therefore is functional after movement.

5.3.3 Improved grafting method for higher throughput

I reciprocally grafted six-week old transgenic K-AR *N. benthamiana* lines with either transgenic gRNA2 lines or wt plants in order to determine if gRNA can systemically move through the phloem. In the future, I would design these experiments to be done via micrografting and hydroponic growth. This method has been successfully used in *N. benthamiana* (Xu *et al.* 2013) and would allow for a much higher throughput. In addition, the suspension of roots in water would provide an easier sampling system than soil-grown mature plants.

5.3.4 Conclusion

Functional gRNA T2 lines and successfully activated crosses should be established before continuing grafting experiments. Further suggestions for how to improve the effectiveness of crosses and grafts are discussed in Chapter 6. In the future, my positive readout assay will still be useful in investigating grafting as a gRNA delivery method because of the ability to identify activity caused by low amounts of systemically mobile gRNA.

Chapter 6: General Discussion

Developing gene editing technology in plants is useful for both crop improvement and basic research; knock-out lines for most genes exist for Arabidopsis, but are not available for other model species like *N. benthamiana* and important crop plants (Bally *et al.* 2018). The work presented in this thesis aimed to address the challenges of efficient delivery mechanisms for CRISPR gene editing in plants. In this chapter, I will recapitulate the project goals, discuss my findings, suggest future directions in context with recent advancement, and mention the policy implications for new CRISPR technology.

6.1 Summary of Thesis Aims

The first goal of the work in this thesis was to set up a system to facilitate optimisation of CRISPR component delivery to plants, especially as a research tool. The protein components in this system would be expressed transgenically and the gRNA introduced into the plant in trans. I then aimed to test the system by delivering gRNA via a virus or by grafting. Since gRNA is the only variable part of the CRISPR system, this strategy would reduce the amount of tissue culture required to produce plants with the desired traits.

My approach was based on the recognition that new delivery systems might only operate at a low efficiency and that loss of function of a target gene function might be difficult to observe phenotypically. For that reason, I developed a system based on gene activation in which low activity might be easier to detect, especially if histochemical methods could be performed as an initial screen. Using the activation system to optimise a delivery method would then allow future application of efficient external gRNA delivery for gene editing.

6.2 Discussion of findings

6.2.1 Developing a CRISPRa system for testing gRNA delivery methods

First, I generated a CRISPRa system designed for introducing gRNA in trans, separately from the activator-reporter construct. I validated the proof of principle by using fluorometric assays to demonstrate gRNA-induced transactivation of the GUS reporter gene in transient expression experiments (Figure 3.7). I generated stable transgenic lines of my constructs and confirmed that the CRISPRa system was functional in the T1 and T2 generation stable activator-reporter lines by transiently expressing gRNA (Figures 3.9, 3.10).

6.2.2 Demonstration of TRV gRNA delivery for CRISPRa

My CRISPRa system was designed to be used for optimising various VIGE methods by providing an easy assay to determine functional gRNA delivery. Of the previously attempted methods (Ali *et al.* 2015, Yin *et al.* 2015, Baltes *et al.* 2014), a TRV vector has the highest chance of causing gene edited progeny by seed due to its ability to invade plant meristems (Martìn-Hernàndez and Baulcombe 2008). The first demonstration of VIGE with TRV struggled to show sufficient inheritance (Ali *et al.* 2015, Yin *et al.* 2015), so I focused my work on identifying the system's obstacles in order to improve it for the future.

I tested viral delivery of gRNA for CRISPRa by inoculating *N. benthamiana* K-AR T2 plants with TRV carrying gRNA2 in its genomic RNA2. Quantitative GUS assays showed activation in those plants infected with gRNA2 virus compared to plants infected with unmodified wild type TRV. This effect was significant in directly infiltrated leaves (Figure 4.7). There was a pattern in mildly symptomatic systemic leaves (Figure 4.9), but the activation was reduced. RNA analysis showed instability of the gRNA2 insert in these systemic leaves compared to intact wild type TRV (Figure 4.10A). Very symptomatic systemic leaves located closely to the infiltrated leaves entirely lost the pattern of activation and showed almost complete removal of the correct size gRNA2 insert (Figures 4.9, 4.10B).

This thesis is the first demonstration that viral gRNA delivery can be used for the purpose of CRISPRa. Using the CRISPRa system allowed easy analysis of the efficiency of viral delivery as the gRNA insert was recombined out of the viral genome and preferentially selected against in systemic leaves.

This observation suggests that more work needs to be done on viral insert stability. Even if the virus is indeed reaching the germline cells, there may be low abundance of the virus containing the correct gRNA insert. Not only does this make the intended effect minimal, but there could also be recombination variants that cause unintended editing effects, which damages the precision reliability of CRISPR edits. Therefore, my results question whether VIGE is an accurate and efficient delivery mechanism.

6.2.3 Inconclusive Graft Delivery of gRNA

I also began to explore gRNA delivery via grafting. T2 generation gRNA2 lines were grafted with K-AR T2 lines and sink tissue was sampled to test for long distance gRNA movement through the phloem. The results are currently inconclusive due the negative results from RT-PCRs of graft roots (Figure 5.4) in which mobile gRNA was not found. Starting with a focus on function instead of trying to find movement would have more directly determined if graft delivery of gRNA would be a useful tool for biotechnology.

As a control to understand whether gRNA produced from a transgene was functional for my CRISPRa system, I performed crosses between K-AR and gRNA2 T1s. GUS fluorometric assays revealed no difference in expression level between the cross F1 progeny and K-AR T2 lines (Figure 5.7). I expect that further experiments, described in Chapter 5, would show that the amount of gRNA produced from the transgene was not enough to function in the CRISPRa system. Lowder *et al.* (2015) were able to quantify activation in stably transformed lines using the AtU6 promoter to drive gRNA expression; however, they used multiplexing of three gRNAs simultaneously and therefore the activation was likely amplified compared to the effect from one gRNA.

6.3 Recent developments in CRISPRa and VIGE

6.3.1 Second generation CRISPRa for enhanced activation

The increased expression resulting from my CRISPRa system was effectively quantified by fluorometric assays, but a higher level of activation would be beneficial for even quicker analysis of transactivation by only GUS histochemical staining. Over the course of my PhD, second generation CRISPRa systems have been developed that result in higher levels of activation (Figure 6.1). These systems rely on the recruitment of multiple activation domains by one dCas9/gRNA complex. Tanenbaum *et al.* (2014) modified the dCas9 component of the system to achieve this result. They developed a system in which dCas9 fused to a repeating peptide array called SunTag carries multiple copies of VP64-antibody fusions to the target location when in complex with a gRNA. Papikian *et al.* (2019) applied the SunTag-VP64 activation system to Arabidopsis, successfully activating genes and transposable elements.

Instead of changing the dCas9, it is also possible to alter the gRNA structure for increased activation technology. Zalatan *et al.* (2015) designed scaffold RNA (scRNA), adding RNA aptamers that recruit specific RNA-binding proteins (RBPs). Transcriptional regulators are fused to the RBPs that attach to the scRNA, achieving multiplexed activator or repressor

activity at the target site. The synergistic activation mediator (SAM) method is also based on modified gRNA. A dCas9-VP64 fusion forms a complex with gRNA containing two stem loops associated with the bacteriophage coat protein MS2 (Konermann *et al*. 2015). Additional VP64 fused to the MS2 protein is also expressed from the system and links to the stem loops, causing the dCas9 to be attached to many copies of VP64 instead of one. Lowder *et al.* (2018) demonstrated the use of SAM in plants, showing three to four fold higher transcriptional activation compared to their first study of CRISPRa using first generation dCas9-VP64 (Lowder *et al.* 2015).

Alternate approaches engineered fusions of multiple activator domains in order to achieve increased activation. The dCas9-VPR technique (Chavez *et al.* 2015) augments activation compared to dCas9-VP64 by using dCas9 fused to a tripartite activator composed of VP64, the activator domain of nuclear factor kappa B (p65), and Epstein-Barr virus R transactivator (Rta). An even larger activator was developed by Li *et al.* (2017), who combined six TALE activation domains with VP128 (8 times VP16) to create dCas9-6TAL-VP128, renamed as dCas9-TV. Their activator showed vast improvement over dCas9-VP64 (Figure 6.2), for which their reported activity was similar to what I observed in my own dCas9-VP64 assays.

Strategies for epigenome engineering are also being developed using second generation dCas9-based strategies. For example, instead of recruiting activator domains to the SunTag system, the catalytic domain of demethylase TET1 has been fused to dCas9, causing increased expression of targeted genes. This technique was first used in animals, but was recently applied to plants by Gallego-Bartolomé *et al.* (2018) who showed activated expression of a highly methylated epiallele and methylated regions of a transposon in Arabidopsis when targeted with the SunTag-TET1 system.

Incorporating these stronger activation techniques into my system would provide a more robust result that could be used for rapid assessment of gRNA delivery methods.

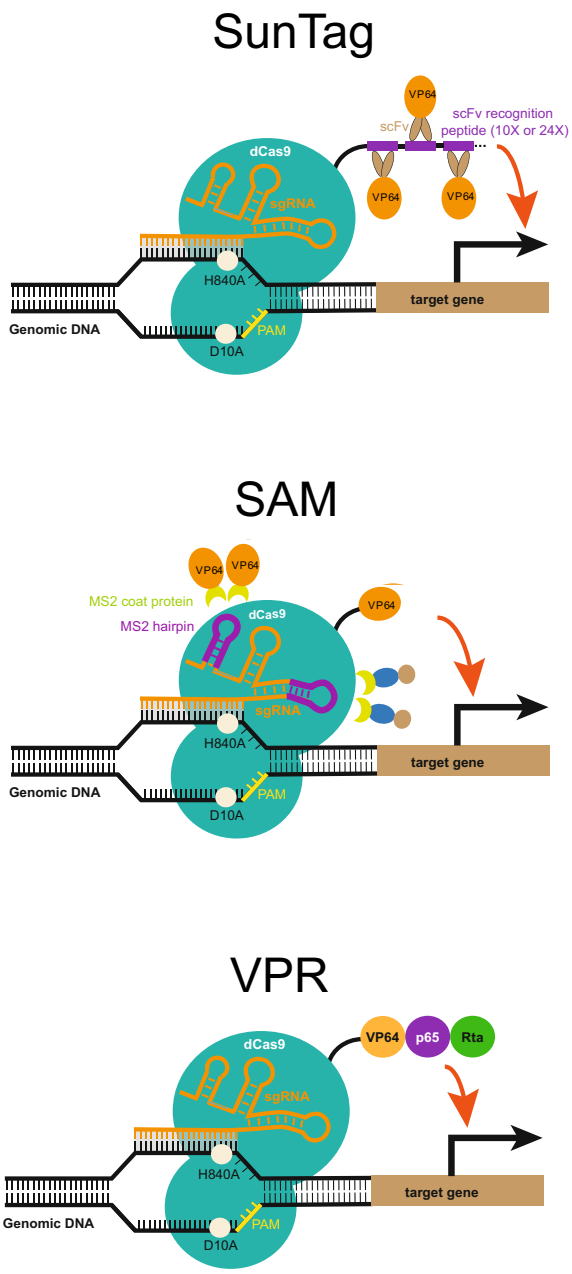


Figure 6.1. Second generation CRISPRa systems for enhanced activation level. The SunTag system uses small peptide repeats fused to dCas9 to recruit multiple copies of antibody single chain variable fragment (ScFV) fused to VP64. SAM uses stem loops on the gRNA to cause binding with bacteriophage coat protein MS2 fused to transcriptional activators. In plants, it was applied to bind to multiple copies of VP64 (Lowder *et al.* 2018). VPR uses a tripartite activator of VP64, p65, and Rta fused to dCas9 to initiate activation. Figure modified from Chen and Qi (2017).

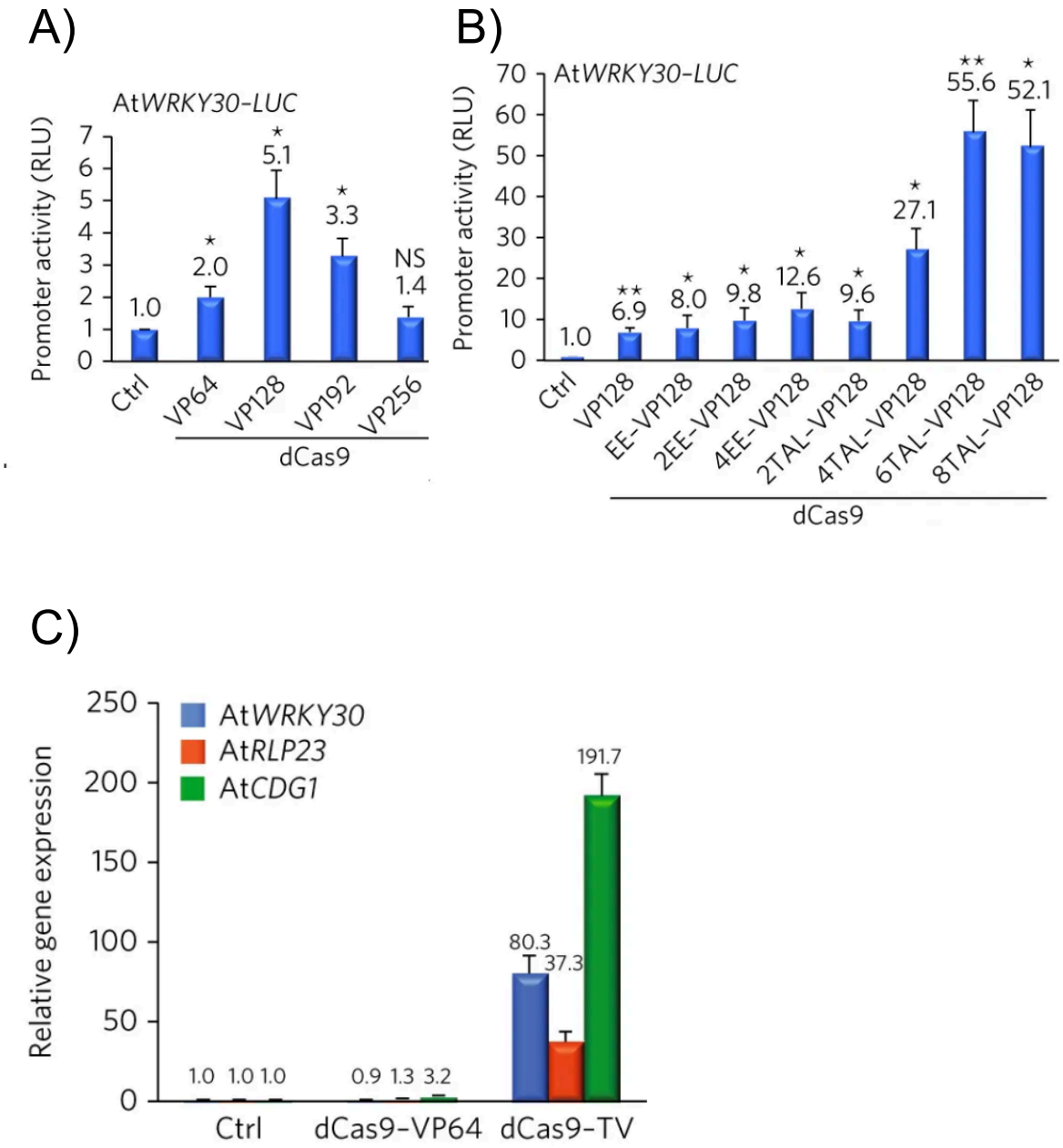


Figure 6.2 dCas9-TV is able to activate target genes at a much higher level than dCas9-VP64. A) and B) are Luciferase measurement assays in Arabidopsis protoplasts in which gRNA is targeted to the AtWRKY30 promoter driving LUC. RLU = relative luciferase unit A) Reveals VP128 to be the VP16 repeat with the highest level of activation, 5.1 times higher than the control. In contrast VP64 only showed a 2 fold increase. B) dCas9-6TAL-VP128 (dCas9-TV) shows a 55.6 fold activation. C) When targeted to activate endogenous genes, qPCR quantification shows dCas9-TV exhibits high activation levels compared to dCas9-VP64. Figures modified from Li *et al.* (2017)

6.3.2 Advancement in vectors available for VIGE

During the course of my PhD, other groups continued to experiment with VIGE. Ali *et al.* (2018) expanded upon their original work (Ali *et al.* 2015) with TRV in *N. benthamiana* by applying their vector to Arabidposis and showing successful CRISPR mutations in infected plants. They also demonstrated the use of PEBV-mediated gRNA delivery in *N. benthamiana* and observed that it had higher rates of mutation compared to their first generation TRV vector. Unlike their original TRV VIGE publication, they did not test for inheritance in their follow up work, instead only mentioning the theoretical possibility of edited progeny due to the fact that PEBV and TRV are known to be able to infect meristematic tissues (Wang *et al.* 1997). They suggest that modified tissue from the infected plant could be placed in tissue culture and regenerated plants could be screened for the mutation.

Other groups focused on local gRNA delivery using viral vectors instead of attempting to achieve systemic spread into meristematic tissues. For example, Cody *et al.* (2017) used tobacco mosaic virus (TMV) RNA-based overexpression (TRBO) to develop a transient screening tool. TRBO is a mutant form of TMV lacking the coat protein (Lindbo 2007). It is therefore unable to move systemically but can still move cell-to-cell. This vector was chosen for gRNA delivery because of its previously demonstrated ability to produce high amounts of desired protein and stay localised to the infected regions (Lindbo 2007, Cody *et al.* 2017). The VIGE method using TRBO aimed to saturate the infiltrated cells with gRNA to increase editing efficiency. The experiments showed high editing rates (60-70%). Since the method was developed only for local expression, inheritance was not a priority and was not tested.

Gil-Humanes *et al.* (2017) built on the work of Baltes *et al.* (2014), using geminivirus-based replicons to deliver both gRNA and Cas9 using wheat dwarf virus (WDV) to induce targeted mutations in cereals. They were also able to cause specific insertions by homologous recombination (HR) by including the repair template in the viral replicon with the Cas9 and gRNA. As in the work of Baltes *et al*. (2014), their approach is based on localised infection, using vectors lacking the movement protein and coat protein in order to allow for large insert size. The study was carried out mainly in protoplasts which would be subjected to tissue culture in order to generate the desired edited plant, but the method eliminates the need for any stable integration of transgenes for editing cereals. Geminiviral delivery of CRISPR components has also been demonstrated in tomato (Dahan-Meir *et al.* 2018) and rice (Wang *et al.* 2017), establishing it as a method that can be used for many valuable crop species.

Jiang *et al.* (2019) used beet necrotic yellow vein virus (BNYVV) which has a genome comprised of four or five single-stranded RNAs. Only RNA1 and RNA2 are essential for viral

replication and therefore the other RNAs can be engineered to express genes of interest. This study successfully demonstrated BNYVV delivery of gRNA for causing CRISPR mutations in infected plants Cas9 transgenic *N. benthamiana*.

BNYVV RNA2, RNA3, RNA4, and RNA5 can exhibit internal deletions (Jiang *et al.* 2019), similar to what has been observed in TRV RNA2 (Hernandez *et al*. 1996). My work suggests that insert stability in RNAs that undergo recombination may be a limitation of using viral delivery (Chapter 4). However, Jiang *et al.* (2019) observed intact gRNA via RT-PCR in systemic leaves 5 weeks post infiltration. While it is possible that the two viruses naturally behave differently, there is also a notable difference in the construction of the gRNA expression strategy. My strategy was to place the gRNA downstream of a subgenomic RdRP promoter in order to ensure the correct 5' sequence. In contrast, Jiang *et al.* (2019) designed their construct so the gRNA is directly after the sequence encoding the BNYVV RNA4 protein p31. This approach may be useful in future VIGE design, if indeed attaching the gRNA to a viral protein sequence increases insert stability.

Perhaps the most promising result from Jiang *et al.* (2019) for the future of VIGE is their demonstration of simultaneous expression multiple reporters from one viral vector due to the many RNA strands of the BNYVV genome. The largest size insert they tested was 2650nt, which is larger than most viruses are able to tolerate, but still not enough to carry Cas9 (~4kb). However, as bacteria continue to be screened for orthologs of Cas9, there could be potential for introducing both parts of the CRISPR machinery with BYNVV if a small enough ortholog is found. The BYNVV system would still hold advantages over other plant viral vectors because it could accommodate both components in a single vector, while there can be complications caused by the use of multiple vectors at once or co-infection of the same vector backbone with different inserts (Cody *et al.* 2017, Jiang *et al.* 2019). Inheritence of mutations were not tested in this study, but BYNVV is transmitted by the fungus *Polymyxa betae* (Hull 2002) and not by seed, therefore it is unlikely that components delivered by BYNVV would be able to enter the germline.

Hu *et al.* (2019) demonstrated the use of barley stripe mosaic virus (BSMV) for gene editing in *N. benthamiana*, wheat, and maize. BSMV has a tripartite RNA genome consisting of RNAα, RNAβ, and RNAγ. Two versions of the gRNA delivery vector were tested in *N. benthamiana* – one inserting the gRNA in the place of the coat protein on RNAβ and one inserting the gRNA directly after the sequence of protein γb on the RNAγ strand (Figure 6.3). Successful editing occurred when the viral constructs were agroinfiltrated in conjunction with

a Cas9 expression plasmid. At 14dpi, systemic leaves were agroinfiltrated with the Cas9 construct and displayed targeted mutations when sampled 4 days later.

Interestingly, the results showed higher editing efficiency from the latter vector design in systemic leaves. This is consistent with my observation from the work of Jiang *et al.* (2019) where gRNA inserted immediately following a viral protein sequence may be better maintained than when it is downstream of a subgenomic RdRP promoter. The study did not include RT-PCRs of the gRNA site from systemic leaves, but it would be useful to investigate whether the insert was partially lost when using the RNAβ vector design.

The BSMV-induced mutations in *N. benthamiana* were passed to the next generation when plants were regenerated from edited leaf tissues via tissue culture (Hu *et al.* 2019). BSMV is known to be seed transmitted (Carroll 1972), so there is potential for BSMV VIGE to create heritable mutations when used with a Cas9 expressing transgenic line in the future.

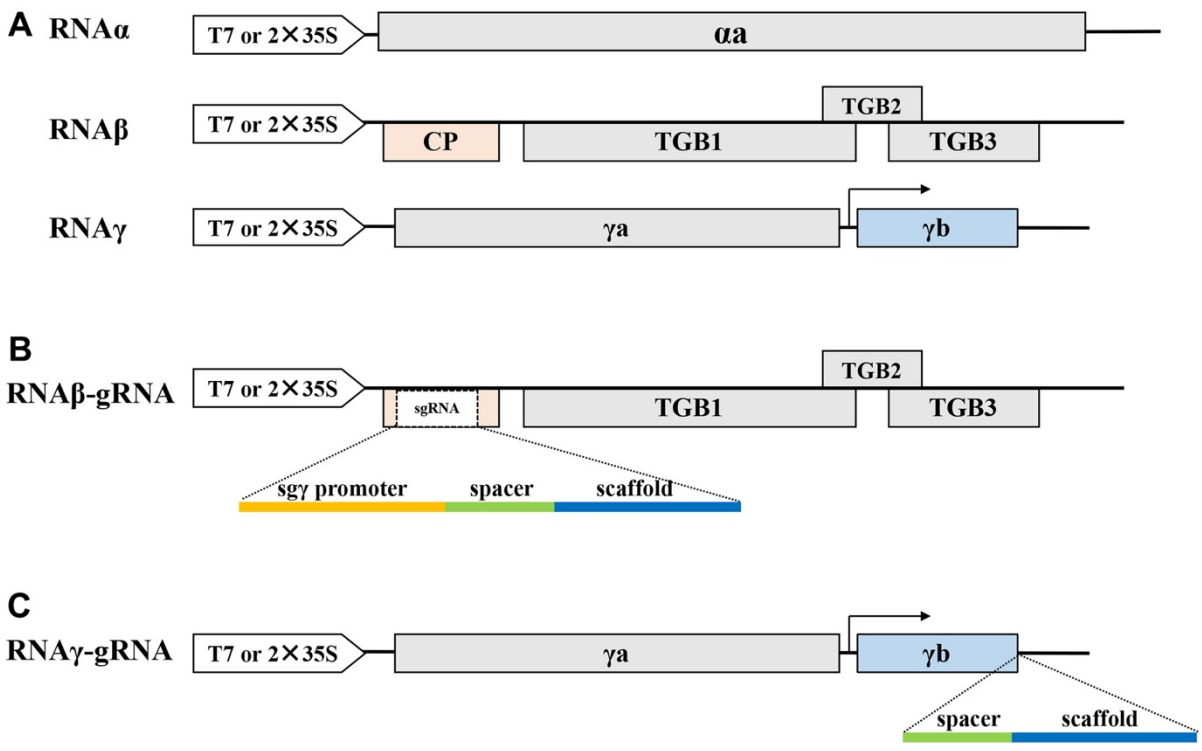


Figure 6.3 Schematic of BSMV vector constructs. A) The vector constructs for the expression of wild type BSMV tripartite genome. B) Vector design in which gRNA is inserted following a subgenomic RdRP promoter (sgγ promoter) in the place of the coat protein on RNAβ C) Vector design in which gRNA is inserted on RNAγ directly downstream of the sequence encoding protein γb. Figure from Hu *et al.* (2019).

6.4 Future work to further advance project aims

6.4.1 Potential modifications to the CRISPRa system for testing gRNA delivery in trans

Reverting to the native RNA organisation of CRIPSR, the crRNA and the tracrRNA could be separated, with the tracrRNA included in the activator-reporter construct. If the tracrRNA sequence is already transgenic in the plant along with the dCas9-VP64, the only component that needs to be added is the 20nt crRNA. The short length of the crRNA could allow it to be more stable in a viral vector. Alternatively, it may be possible to spray 20nt crRNA onto plants as is done with siRNAs in spray-induced gene silencing (SIGS) (Koch *et al.* 2016).

It could be useful in future work to use a gain of function system for heritable gene editing using out of frame reporters. When a gRNA is targeted to the out of frame portion of the reporter, the editing event causes a frame shift allowing the reporter to be expressed (Yin *et al.* 2015). This method relies on mutations, and not all mutations would cause the necessary frame shift. However, it would not require any currently existing protein to degrade, and correctly mutated lines could be quickly screened. The frame shift, and therefore the reporter expression, would be inherited in the next generation allowing for easy determination of inheritance.

6.4.2 Future optimisation of viral gRNA delivery for CRISPRa

The recent advances to the VIGE method are promising, but overcoming meristem exclusion to enter the germline and avoiding extra tissue culture are still challenges. The modified geminiviral vectors have the advantage of being able to deliver all components non-transgenically but are unable to invade the meristem. TRV, PEBV, and BSMV vectors have the potential to enter the germline, but inheritance has not yet been solidly demonstrated. In addition, my work indicated that insert stability may be an issue that should be addressed in order to ensure precise editing events.

All the recent publications have tested VIGE by screening for phenotypes caused by mutation. CRISPRa provides distinct advantages for method optimisation. It delivers a quantifiable gain of function result, allowing detection of even very low activity. In addition, it is not necessary to wait for the degradation of residual gene product in order to observe the phenotype. In this section, I will describe potential VIGE optimisation strategies using CRISPRa.

6.4.2.1 Multiplexing gRNAs for increased activation and multiple edit sites

The system I developed provided preliminary evidence that CRISPRa can be achieved through viral delivery of gRNA. In order to fulfil the potential of being a powerful screening tool, the activation level could be further enhanced. One strategy is to combine viral delivery with second generation CRISPRa methods. Another possibility is to increase activation by gRNA multiplexing within the viral vector.

Multiplexing of gRNAs has been shown to increase activation level (Cheng *et al.* 2013, Maeder *et al.* 2013, Perez-Pinera *et al.* 2013, Piatek *et al.* 2015), but currently the TRV technology has only been used with one gRNA at a time to ensure proper viral replication. Introducing components like self-cleaving ribozymes or tRNA precursors that are cleaved post transcription (Xie *et al.* 2015) would interrupt the RNA genome and impede replication. Hu *et al.* (2019) engineered a BSMV RNAγ to carry two gRNAs without any space between them and demonstrated multiplexed editing of the targets. Cody *et al.* (2017) successfully applied the same approach in their TMV-based TRBO system. This strategy of simply adding gRNAs back to back would theoretically be possible in the TRV system, and could be a way to increase activation of CRISPRa, in addition to achieving multiplexed targeted mutations when applied for gene editing.

6.4.2.2 Using gene silencing knock down lines to promote viral infection

Weakening the plant's immune response would make it easier for the virus to access the meristem and could lead to greater insert stability because of decreased selection pressure. *N. benthamiana* knock down lines deficient in essential RNA silencing machinery such as DCL proteins already exist and have been shown to exhibit increased levels of TRV infection compared to wt plants (Katsarou *et al.* 2019). RDR6 seems like another likely knock down target for decreasing silencing ability, but it has been shown previously that using RDR6 knock down lines for infection does not increase level of TRV accumulation (Schwach *et al.* 2005) and the process of TRV meristem invasion is independent of RDR6 (Martìn-Hernàndez and Baulcombe 2008).

Crossing *N. benthamiana* DCL knock down lines (Katsaurou *et al.* 2019) with my K-AR T2 lines would generate a hyper-susceptible host plant in which the virus would have a better chance of invading the meristem with an intact gRNA insert. Testing reporter activation levels in dissected meristems of plants infected with gRNA TRV compared to wild type TRV would be a simple indicator of the spatial aspects of the delivery method. The method optimised

with CRISPRa could be applied with catalytically active Cas9 to cause mutations in the germline cells for edited progeny.

6.4.2.3 Placement of gRNA in viral vector could affect insert stability

Stability of the gRNA insert could also be addressed by experimenting with placement of the gRNA in the viral genome. The results from BSMV (Hu *et al.* 2019) and BNYVV (Jiang *et al.* 2019) have shown gRNA insert stability when it is placed immediately following the sequence for a viral protein on a non-essential strand of the vector's RNA genome. In contrast, the current work in tobraviruses (this thesis, Ali *et al.* 2015, Ali *et al.* 2018) designed the gRNA to follow a subgenomic RdRP promoter, in the place of where a viral protein would natively be. My results suggested recombination to remove the insert, leading to a mixture of insert fragment sizes in very symptomatic systemic leaves (Figure 4.10). Perhaps engineering my TRV vector to include the gRNA insert directly following the coat protein sequence on RNA2 would enhance stability in the future.

6.4.3 Future work for grafting

Transgenic lines should be screened for the highest gRNA expression, which would reveal differences transgene silencing may play on the amount of gRNA available to the system. It is also possible that using a stronger promoter would produce higher levels of gRNA. Pol III promoters are usually used for gRNA expression because of their specific transcription start site allowing a clean 5' end of the gRNA and known termination sequence (Gao *et al.* 2018). Unfortunately, there is a limited amount of characterised Pol III promoters (Gao and Zhao 2014), restricting the variety of expression levels possible. In contrast, many characterised Pol II promoters exist, but RNAs derived from them are subjected to post-transcriptional processing that is not useful for gRNA which needs a precise 5' end to match the target gene sequence (Gao and Zhao 2014).

Recently, a method allowing gRNA to be expressed transgenically from any promoter was developed (Gao and Zhao 2014). The study demonstrated that gRNA flanked by two self-cleaving ribozymes on either side and driven by a Pol II promoter is able to be transcribed and then processed to the desired length. Therefore, use of a well-characterised strong promoter for expression of gRNA may be possible to ensure there is enough produced by the transgene in order to function in crossing and grafting. Using one of the second generation CRISPRa systems which increase the efficiency of the gRNA combined with expressing the gRNA from a stronger promoter could further boost the system.

6.5 Future applications of gRNA delivered to CRISPRa system in trans

6.5.1 Uses for virus induced CRISPRa

Beyond an optimisation tool for gene editing, virus induced CRISPRa could have its own applications. One use of CRISPRa could be for screening regions of the genome that are amenable to editing via VIGE to aid in better experiment design. Instead of mutation screens, qPCR for endogenous gene activation would be an indicator of whether the editing location is able to be targeted.

Viral CRISPRa could also be used for gene function assays. Similar to the use of VIGS for gene repression, using a viral delivery for CRISPRa using either transcriptional activators (e.g. dCas9-VP64) or epigenetic modifiers (e.g. dCas9-Tet1) would allow for temporal control of gene activation. This method would be particularly useful for experimenting with genes for which improper regulation during development would be lethal. Additionally, viral CRISPRa could be used as a transient way to activate certain genes in the meristem to induce desired traits in the progeny caused by overexpression at a specific developmental time point.

6.5.2 Potential use of graft delivery of gRNA

Grafting is used frequently in crop production, including on the industrial scale (Haroldsen *et al.* 2012). Therefore, it is reasonable to imagine that introducing gRNA for CRISPR via grafting would be a technique that is attractive to the food production industry. It would reduce some of the uncertainty caused by repeated tissue culture because the genome of the end product is from the consistent Cas9/dCas9 line, not the variable gRNA line. However, in terms of time required, graft delivery is not the most efficient because of the constraint of generating stably transformed gRNA lines.

If gRNA introduction by grafting is not functional, testing for gRNA mobility could still have valuable implications for basic research. A sensitive assay such as RNA sequencing would be able to establish RNA movement even if it is at very low abundance and is not functional upon reaching destination tissue. If mobility is established, determining exactly how the gRNA moves could provide information on trafficking processes for many classes of RNAs.

6.6 Tissue culture-free systems for CRISPR gene editing in plants

Due to the importance of efficient plant gene editing technology for both basic research and agricultural applications, many groups are focused on alternate CRISPR delivery methods in plants. In addition to the development of VIGE, other non-transgenic delivery strategies for CRISPR exist such as injecting the assembled Cas9/gRNA RNPs into protoplasts (Woo *et al.* 2015). Similar to geminiviral VIGE, this method eliminates initial tissue culture to introduce transgenes, but still requires plant regeneration after delivery of the CRISPR components. Three recently established techniques stand out as having high potential because they do not require any tissue culture and are possible in a wide variety of plant species.

6.6.1 Induction of *de novo* meristems for CRISPR gene editing

Originally developed to aid genetic transformation in recalcitrant species (Lowe *et al.* 2016), overexpression of morphogenic genes to induce meristem formation has now been applied for gene editing. Existing shoot meristems were removed from transgenic *N. benthamiana* plants expressing Cas9. T-DNA constructs containing combinations of developmental regulation genes and gRNA in *A. tumefaciens* were then perfused into the cut sites (Figure 6.4) (Maher *et al.* under review, communication with Voytas lab). A proportion of the resulting *de novo* meristems produced CRISPR-edited shoots that did not contain transgenes for the developmental regulators or gRNA. Progeny from these shoots exhibited the edited mutations, demonstrating inheritance without the use of tissue culture.

The main benefit of this approach is the reduced time required to produce a gene edited plant. However, while repeated traditional tissue culture using hormone-containing media is not used, cells are still induced into an un-differentiated callus state before new meristems are formed. The de-differentiation process may still cause epigenetic mutations, and therefore this method does not yet fully overcome the tissue culture bottleneck.

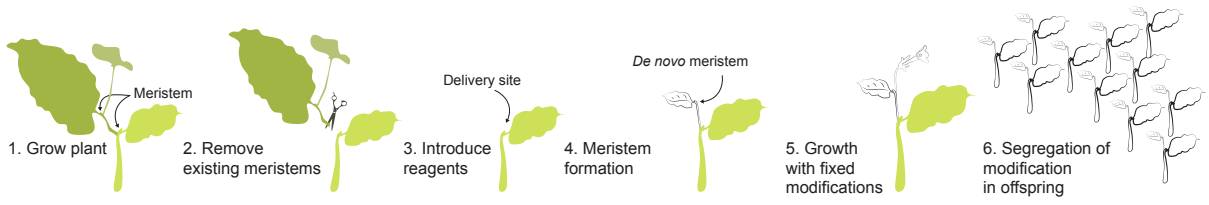


Figure 6.4. Induction of *de novo* meristems for gene editing. Shoot meristems are removed from transgenic plants expressing Cas9. Developmental regulators and gRNA are delivered to the cut site in *A. tumefaciens*. The shoot from the induced meristem has undergone gene editing and the mutation is inherited to the next generation. Figure from Maher *et al.* (under review).

6.6.2 Haploid induction for CRISPR gene editing

The natural process of haploid induction has also been harnessed for gene editing (Kelliher *et al.* 2019). The study demonstrated that when pollen of a plant transgenic for Cas9 and gRNA is crossed to the egg of a haploid inducer line, the resulting haploid progeny contain the desired mutation and are lacking both CRISPR transgenes. This process is possible because the two genomes form a transient zygotic state, during which time the editing occurs. The male genome is then eliminated during the haploid induction step of reproduction. Kelliher *et al.* (2019) first performed this method in maize (Figure 6.5A), then showed it was possible in dicots by experiments in Arabidopsis. Finally, they demonstrated that pollen from maize expressing the CRIPSR components could edit an elite wheat variety (Figure 6.5B), based on the knowledge that when maize pollen fertilises wheat ovules, the maize chromosomes are eliminated (Laurie *et al.* 1988, Mochida *et al.* 2004). The wheat haploid embryos therefore had undergone CRISPR editing, and were free of the Cas9 and gRNA transgenes that were only carried in the maize pollen.

This haploid induction method is a considerable step forward for efficient gene editing in two very valuable crop species and the demonstration in Arabidopsis showed that it can be applied to many more plants in the future. The edited plant line has never been exposed to the tissue culture process, but the necessity to generate a transgenic editor line containing the Cas9 and gRNA for each new genomic target is still a limitation to the throughput of the method.

6.6.3 Nanoparticle DNA delivery for CRISPR gene editing

Another exciting advance has been in the field of nanoparticle delivery. Nanoparticle introduction of biomolecules to plants is a growing area of research due to the method's demonstrated success in animal systems (Cunningham *et al.* 2018). Demirer *et al.* (2019) showed successful delivery of plasmid and linear DNA into mature plants using carbon nanotubes (CNT), which are below the size exclusion limit of the cell wall and therefore able to diffuse into plant cells. They suggest that CNT delivery of CRISPR components would allow transient expression, inducing transgene-free targeted gene edits.

The proposed nanoparticle-mediated CRISPR in plants is tissue culture-free, transgene-free, and gRNAs can be easily interchanged for high throughput editing. The technique has high potential, but a key outcome that is currently unknown is whether the method can successfully target germline cells, generating heritable mutations. Nanopoarticle DNA delivery to plants is still in its infancy, and further optimisation will likely yield substantial developments for biotechnology.

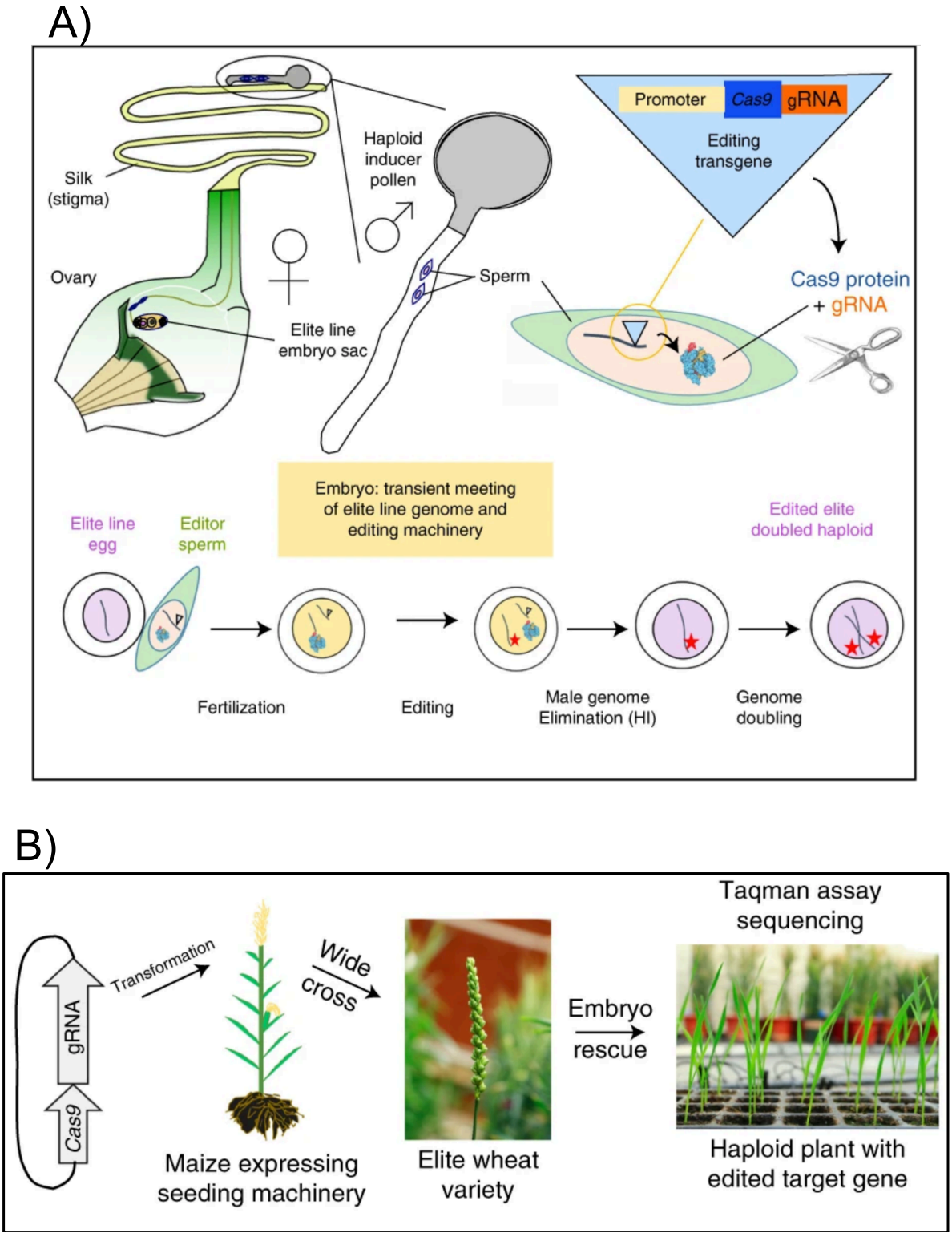


Figure 6.5. Haploid induction gene editing method. A) Haploid-inducing maize pollen transgenic for Cas9 and gRNA fertilises the egg of an elite maize line resulting in CRISPR edited, transgene-free haploid progeny. B) Maize pollen transgenic for Cas9 and gRNA fertilises the egg of an elite wheat variety, yielding haploid, edited, transgene-free wheat progeny. Figure modified from Kelliher *et al.* (2019).

6.7 Policy implications of gene editing in plants

6.7.1 Implications of VIGE for biosafety

The United States Defense Advanced Research Projects Agency (DARPA) released a plan for a programme called “Insect Allies” in which insect vectors such as aphids carrying engineered plant viruses would be released into a field for the purpose of rapid gene editing of crops in order to respond to potential threats to the national food supply (DARPA, 2016). This strategy caused recent uproar in the scientific community regarding containment concerns and worry that the technology could turn into a bioweapon (Reeves *et al.* 2018). The DARPA project is an extreme example of how innovation in viral delivery is full of potential, but each development must proceed with caution when entering the field.

The VIGE technology discussed in this thesis would be performed in laboratory biocontainment. Additionally, even though it can invade the meristem, TRV is not seed transmissible in *N. benthamiana* (Martìn-Hernàndez and Baulcombe 2008) so progeny plants from my system would not carry the virus. However, the viruses that have the highest chance of effectively inducing heritable mutations in the meristem are seed transmissible. Therefore, future strategies could show inherited CRISPR mutations, but the progeny may be infected with the virus. Even other researchers focused on VIGE highlight the consideration required when using functional viruses in biotechnology (Cody *et al.* 2017). It will be important to check the seed transmissibility of the viral vector for each proposed technology before allowing the offspring plants to be grown in the field. It may be possible to treat infected progeny in order to remove viruses with methods like thermotherapy (Wang *et al.* 2018) to ensure safe containment.

6.7.2 Implications for crop biotechnology regulation

I have described how the use of transgene-free CRISPR methods are scientifically useful for increasing efficiency of gene editing. They also carry with them important implications for crop biotechnology policy.

The use of genetically modified crops is highly contested around the world (Huang *et al.* 2016). First generation genetically modified organisms (GMOs) involved stably integrating a transgene into the plant to confer a desired trait and most of the regulation of crop biotechnology regulation is based on this method (Voytas and Gao 2014). The rapid development of CRISPR has sparked the question of how gene edited plants should be regulated.

For example, a recent case brought before the European Court of Justice (Case C-528/16) was on the topic of whether gene edited crops should be regulated in the same strict manner as transgenic GMOs, set out in the GMO Directive (Directive 2001/18). There is an exemption in the GMO Directive for products derived from mutagenesis techniques. To date, this has been applied to varieties developed from methods such as chemical and radiation induced mutagenesis. Proponents for CRISPR crops argued that a gene edited product is indistinguishable from one derived from other types of mutagenesis and therefore should fall under the mutagenesis exemption. The Court ruled in the opposite direction, citing that the mutagenesis exemption does not apply to products that have been generated via a process that includes a transgenic step. This is indeed the ruling consistent with the regulation laid out in the GMO Directive, based on the necessity to stably transform the Cas9 and gRNA to induce the edit before removing the transgenes via crossing to yield the final product.

In this chapter, I have discussed multiple new methods of performing CRISPR gene editing in plants that do not involve a transgenic step, such as geminiviral VIGE, haploid induction, and nanoparticle diffusion. These technologies all have the potential to circumvent this ruling and all other regulation worldwide that is based on transgenic methods. As plant biotechnology advances away from relying on transgenic processes, policy in many countries will need to be redefined.

6.8 Conclusion

Developing sustainable agricultural practices is an urgent global priority as we are confronted with the effects of climate change, pest migration, and population increase (FAO 2016). The majority of the scientific community feels that gene editing in plants is a key technology for defending crops against impending threats and ensuring food security for humans (Huang *et al.* 2016). The research presented in this thesis contributes to the fast-paced work being done around the world on improving CRISPR delivery to a wide variety of plant species by providing a tool for method optimisation and insight into the potential of VIGE. Each step in the evolution of efficient CRISPR in plants brings the technology closer to being able to reliably and rapidly serve the needs of global agricultural systems.

Appendix I: List of primers

Primer sequences are printed 5’ to 3’.

Chapter 3

Primers used to amplify 2x25S from pYL156 with SacI and SpeI overhangs

2x35S F	GATCGAGCTCCTTGCATGCCTGCAGGTCAAC
2x35S R	GATCACTAGTCCTCTCCAAATGAAATGAACTTCC

Primers used to amplify Bs4 302bp promoter with flanking BsaI and overlaps

Bs4 302 F	GGGGTCTCACTATATTTTCAAGTTTTTATTATTTTAATCTTTTGATGGCTTTTG
Bs4 302 R	GGGGTCTCACCAGAGATTCGATTAAAAATAAATTGTATGGATGAGATC

gRNA annealing oligos

gRNA1F	ATTGGTTATTATTTTAATCTTTTGA
gRNA1R	AAACTCAAAAGATTAAAATAATAAC
gRNA2F	ATTGGTGTTAGTGGAAAAAGGGCAA
gRNA2R	AAACTTGCCCTTTTTCCACTAACAC
gRNA3F	ATTGGACAAGCTTTCACGTTTCAAG
gRNA3R	AAACCTTGAAACGTGAAAGCTTGTC

pK-AR Sequencing primers

Seq1	CGCGGCTGAGTGGCTCCTTCA
Seq2	GATGACGCACAATCCCACT
Seq3	GCACGAGAGACACCCAATCTTCGG
Seq4	AGATACGATGAGCACCACCAGG
Seq5	GTTGATAAGGGAGCTTCTGCTCAG
Seq6	CTCTTACCTTCAAGGAGGACATCC
Seq7	GCTTGTTGAGACCAGACAGATC
Seq8	GGAGGATTCGATTCTCCAACCGT
Seq9	CCACCAGTCTATCACCGGACTT
Seq10	GCGTTCCCTCTAGATAACGCA

Genotyping of gRNA T0 transformants

M13_F	GTAAAACGACGGCCAGT
M13_R	CAGGAAACAGCTATGAC

Genotyping of K-AR T0 transformants

Bs4p_F	GCAGGATCCCCAAGTGGTGGCTA
pK_R	GCCGTCCAGCTCGACCAGGAT

RT-PCR of gRNA2 T0 transformants

gRNA2_RT_F	GTGATTGGTGTTAGTGGAAAAAGGGCAAG
gRNA_RT_R	CACCGACTCGGTGCCACT

RT-PCR of K-AR T0 transformants

KRT_FWD	CGGAGGATTCGATTCTCCAACCGTTG
KRT_REV	GCCTGTCCAGCCTTCTTGGTAGC

Chapter 4

Primers used in overlap PCR and gRNA2 restriction cloning

DP_A	GCAGCTGCTAGTTCATCTGCACCG
DP_B	AGAGAATTCTGCGAAACTCAAATGCTA
DP_C	AGTTTCGCAGAATTCTCTAGAAGGCCT
DP_D	CGCCGATCTCAAACAGTCTATACAC
gRNA2_F_EcoRI	GATCGAATTCGTGTTAGTGGAAAAAGGGCAAGTTTTAGAGCTAG
gRNA_R_XbaI	GCCAACTTTGTACAAGAAAGCTGGGTC

Primers used for TRV RNA2 insertion site RT-PCR

VT_F	CACTGATGCCATTAGCGACATCT
VT_R	CAGACACGGATCTACTTAAAGAACCG

Chapter 5

GAPDH_F	AGCTCAAGGGAATTCTCGATG
GAPDH_R	AACCTTAACCATGTCATCTCCC
K_F	ATCCACCAGTCTATCACCGGACTTTACGAG
K_R	GCCTGTCCAGCCTTCTTGGTAGCAG
gRNA2_FWD	GTGTTAGTGGAAAAAGGGCAAG
gRNA_REV	ACCGACTCGGTGCCAC

Appendix II: Construct maps and sequences

pK-AR

Binary vector with 2X35S driven pcodCas9-VP64 and Bs4 minimal promoter driven GFP:GUS

2X35S, pcodCas9, VP64, Bs4 302bp, GFP:GUS

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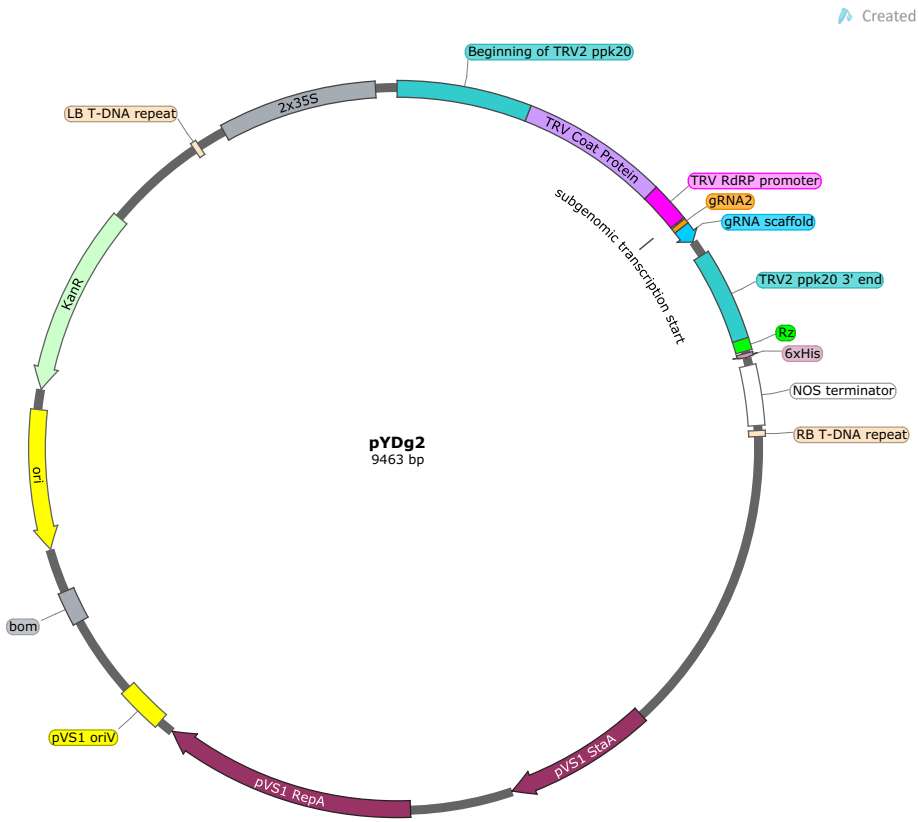
Binary TRV RNA2 vector containing gRNA2



TRV Coat Protein, TRV RdRP promoter, gRNA2, gRNA scaffold

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