# ABSTRACT

MILWARD, JONATHAN HUW. Geminivirus: Rep as a Target for Conferring Resistance, the Host's Strigolactone Hormonal Response to Infection, and Host Resistance (Under the direction of Dr. Jose Trinidad Ascencio-Ibáñez).

The Geminiviridae family consists of small single-stranded DNA-based viruses that infect a wide variety of plants. In order to replicate in the plant cell, the virus must take control of the plant's replicative cellular machinery. The Geminiviruses all encode the Replication Associated Protein (Rep) that is the sole viral protein required for replication. Rep is a highly multifunctional protein with many possible binding partners, both viral and host, and it has several different enzymatic activities.

The multifunctional and conserved nature of the Rep protein means that it is a prime target for engineering resistance to Geminiviruses. In-vitro studies have previously screened small peptide aptamers constrained within the active site of thioredoxin, and these studies have determined that they bind to the Rep proteins from the three major *Geminivirdae* genera. They are also able to reduce visible symptoms but do not reduce levels of the virus in plants. The experiments we performed established, using a VIGS-based system of the Cabbage Leaf Curl Virus (CaLCuV) to introduce the peptide sequences, that the A22, A40, and A177 aptamers all appear to reduce visible symptoms in Col-0 Arabidopsis, a susceptible accession.

The recent identification of strigolactones as plant hormones has opened up a new avenue of research into the role they play during geminivirus infection. As there are no currently published studies into the response of strigolactones during geminivirus infection, here we carried out an exploratory study, tagging a strigolactone-induced gene with a GUS reporter in Arabidopsis. These transgenic plants were then infected with a VIGS vector derived from CaLCuV, and at 14 days post-infection, tissue samples were taken from leaf, meristem, and

floral stems and then stained. The experiments showed an up-regulation of the reporter during geminivirus infection in all tissues, indicating a putative increase in strigolactone levels during infection. Supporting this is that during geminivirus infection, the plant hormone auxin is increased; auxin is an up-regulator of strigolactone-synthesizing enzymes. More work will need to be done into what are the downstream effects of strigolactone up-regulation during infection. There are many genes that are induced by strigolactones but repressed by auxin, and determination of the expression levels of these genes will be of particular interest.

The use of VIGS in Arabidopsis testing is a powerful tool for determining the function of genes that are required for proper development of the plant. Arabidopsis lines from different parts of the world show differing effects on silencing, and as such the creation of a library of Arabidopsis responses to VIGS would be a useful tool for scientists. This can be done by screening different lines with VIGS vectors. Work on this has been started, and we contributed to this effort by screening a number of different lines and recording the response. Our work has identified the CS28306 as a possible line for VIGS studies, and additionally the CS22570 line showed a lack of silencing and no apparent symptoms. Further testing on the CS22570 line will have to be undertaken in order to determine the exact nature of the apparent resistance of CS22570 to silencing and symptoms, but it may be of use in subsequent studies on geminiviruses.

# Geminivirus: Rep as a Target for Conferring Resistance, the Host's Strigolactone Hormonal Response to Infection, and Host Resistance.

by
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# DEDICATION

For my Kate

# BIOGRAPHY

Jonathan Milward was born February 12th 1989 in Britain. He graduated from the University of Manchester with a Bachelor's of Science in Biochemistry in 2011, after which he worked in the field of pharmaceuticals before marring his wife, Katherine, and moving to North Carolina. He decided to continue his study of Biochemistry as a Masters Student where he joined the lab of Dr. Jose Trinidad Ascencio-Ibáñez and began work on his Master's thesis.

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# Geminiviruses, an introduction

## Background

The *Geminiviridae* family, so named because of the structure of their twin-icosahedral capsid, are small single stranded DNA (ssDNA) plant viruses. The geminivirus family is composed of nine genera: Mastrevirus, Eragovirus, Becurtovirus, Capulavirus, Curtovirus, Topocurtovirus, Turncurtovirus,Grablovirus, and Begomovirus. Three of these genera have been well characterized: the Mastrevirus, Curtovirus, and Begomovirus, with Begomoviruses comprising the largest of these genera. These viruses are transmitted by insect vectors that feed on plants, and in doing so transmit the virus; different genera of the family have different insect vectors. Geminiviruses have been an increasing threat to crops worldwide, especially in Africa and South America, and increasingly in countries like India (Saeed & Samad, 2017).

Geminiviruses are a highly diverse family in regards to host selection, insect vector, region, and genome. Begomoviruses are whitefly transmitted and normally have a limited host range, and are found in both the Old and New World. This differs from Curtoviruses, which are leafhopper transmitted, and have a large variety of potential hosts but only dicots in New World. *Begomovirus* genomes can be either monopartite or bipartite, with some monopartite begomoviruses having associated satellite DNA (Hanley-Bowdoin, Bejarano, Robertson, & Mansoor, 2013). Curtovirus genomes are solely monopartite.

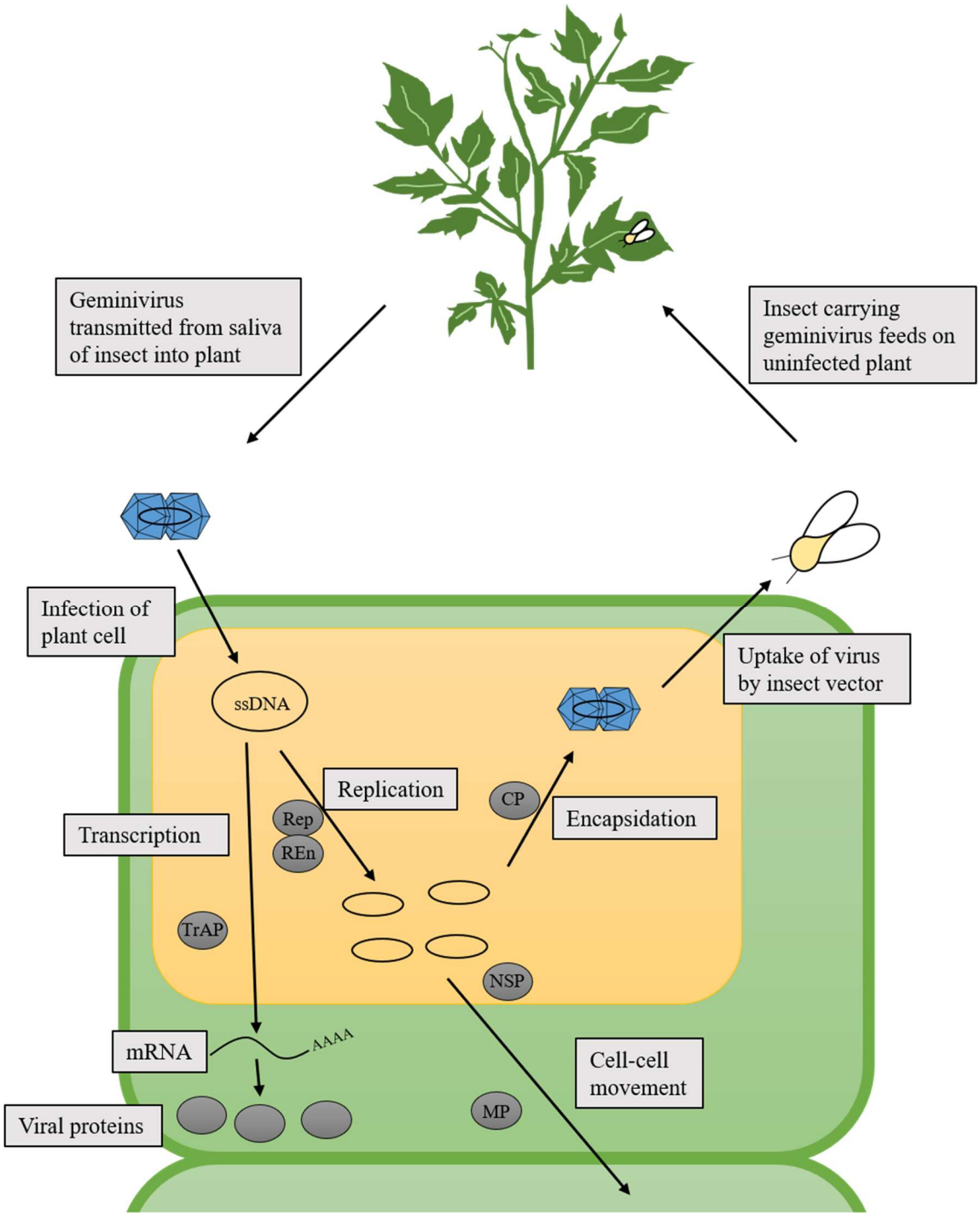


Figure 1.1: Overview of the lifecycle of the Geminivirus.

Geminiviruses genomes do not encode for any DNA synthesis proteins –their small genomes only encode five to seven viral proteins, some of which recruit the hosts' own replication machinery to replicate the viral DNA. In fact, the sole viral protein required for replication is the replication-associated protein (Rep), which plays an incredibly complex role in order to do so (the mechanisms of which is discussed below). Geminiviruses also alter the host's cell-cycle in order to change expression patterns of host replication genes in addition to the disruption of plant immune response. Unsurprisingly, all of the five to seven proteins encoded are multifunctional. Additionally, some begomoviruses have associated satellite DNA encoding proteins that enhance pathogenesis.

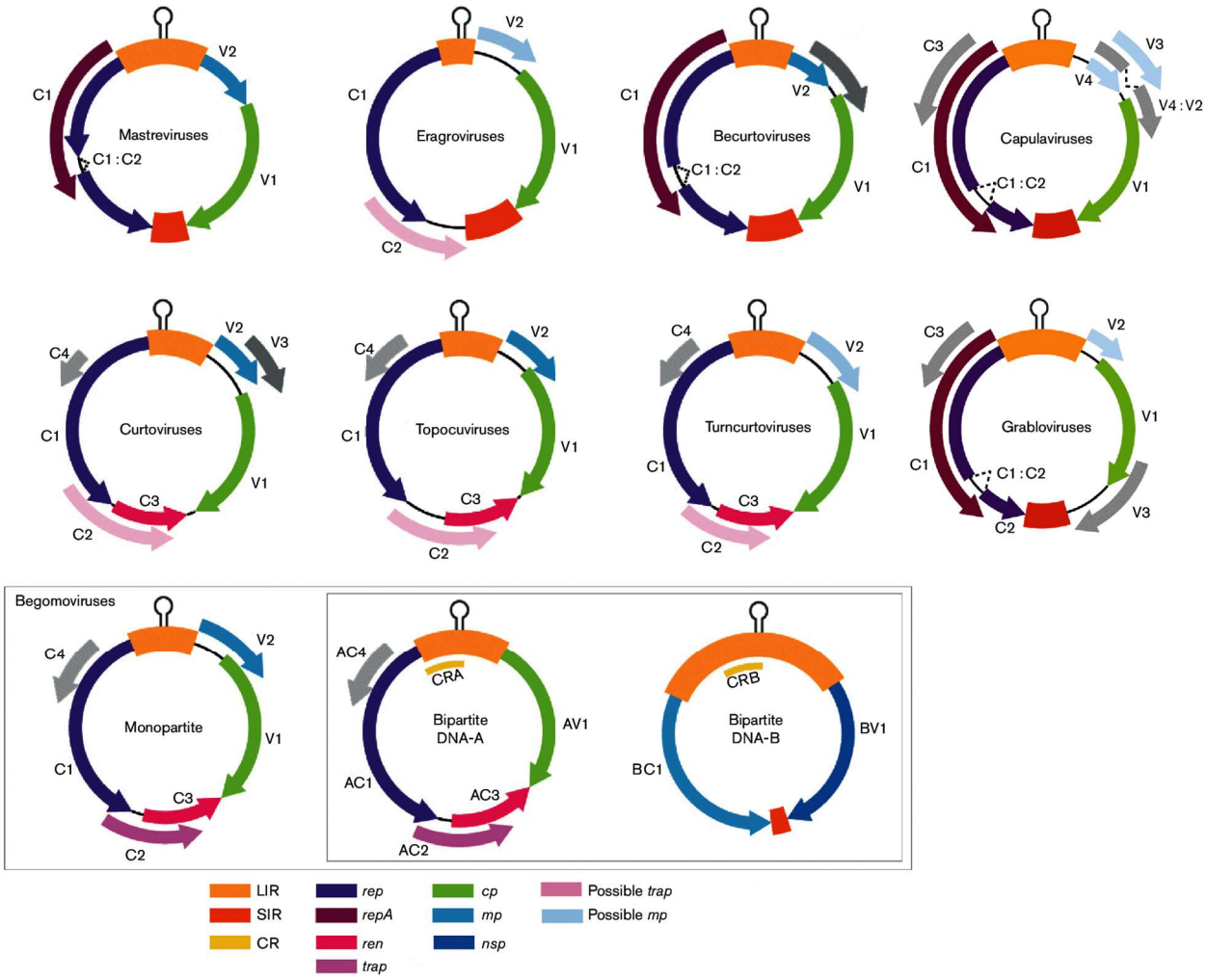


Figure 1.2: Genome organization of all nine different genera of Geminiviruses. Note that Mastreviruses, Becurtoviruses, Capulaviruses, and Grabloviruses all lack the REn protein but contain the RepA variant of the Rep protein, due to its ability to similarly bind PCNA. Taken from (Simmonds et al., 2017).

For the bipartite begomoviruses, the genomic components are designated DNA-A and DNA-B components. Satellite DNA components, which are associated with monopartite begomoviruses, are referred to as alpha- or beta-satellites. For bipartite begomoviruses, the DNA-A component encodes the proteins associated with viral DNA replication, vector

transmission, encapsulation, and suppression of RNA silencing; the DNA-B component encodes the intercellular and intracellular movement proteins. Monopartite begomoviruses have only one DNA component whose genome structure and layout is similar to the DNA-A component. Alpha-satellites encode their own replication initiator protein (Rep) which has anti-silencing properties. Beta-satellites encode the βC1 protein that also counters the transcriptional gene silencing (TGS) of plants.

## Geminivirus Genes

The geminivirus genome encodes a limited number of genes. Here we will briefly discuss their function.

**Replication Associated Protein (Rep/C1/AC1)**

The replication associated protein is the sole encoded protein in the virus required for replication of its genome. Rep interacts with a large number of host proteins and recruits the viral replisome as well as redirects plant signaling pathways during infection. In Mastreviruses, Becurtoviruses, Capulaviruses, and Grabloviruses, two versions of the protein exist –Rep and RepA, which are created by splicing (see Figure 1.2). Both variants are required for viral replication. RepA is like Rep in that it is a multifunctional protein that can bind many partners; it functions in many ways like the REn protein in the bipartite begomovirus. A more detailed discussion on Rep can be found in Chapter Two.

**Transcriptional Activator Protein (TrAP/C2/AC2)**

TrAP is another multifunctional, oligomeric protein that is involved in the activation of

genes, the suppression of silencing, and virus pathogenicity. TrAP contains a nuclear localization signal (NLS) and a zinc finger domain; the C-terminus contains an acidic region. The zinc finger and acidic region are both required for self-interaction of TrAP as well as its functions. TrAP is able to regulate the expression of the coat protein (CP) in phloem tissues by overriding the host repressor. TrAP activates transcription of the movement protein (MP) and coat protein (CP), but it itself does not bind to the DNA. Instead, TrAP is directed to the binding and mediation of host proteins that themselves target TrAP to the responsive promoters (Lacatus & Sunter, 2009).

TrAP has also been shown to suppress RNA silencing and decrease DNA methylation, which reduces the production of the siRNAs that are required for targeting of RNA silencing. Lastly, TrAP is able to counter a hypersensitive response in infected cells. The hypersensitive response is a form of programmed cell death that occurs at sites of pathogen infection. The death of cells in this way limits the pathogen spread, decreasing pathogenicity of the virus. However, TrAP interferes with the jasmonate signaling via the COP9 signalsome by interacting with the CSN5 protein and changing its expression; thus, it is able to regulate plant response to infection (Lozano-Duran et al., 2011).

**Replication Enhancer Protein (REn/C3/AC3)**

The REn protein is a small hydrophobic protein found in some geminivirus families. While it is not essential for viral replication, it does enhance viral DNA accumulation in the geminiviruses in which it is present (S. B. Settlage, See, & Hanley-Bowdoin, 2005; Sung & Coutts, 1995). Interestingly, there are no known homologs to the REn protein in other viruses, such as nanoviruses, that replicate via rolling-circle replication. The lack of REn homologs and the lack of REn in some geminivirus genera has meant the evolution of REn has remained a

mystery (Nawaz-ul-Rehman & Fauquet, 2009).

REn enhances the viral DNA accumulation through its interactions with itself, Rep, pRBR and PCNA, determined through mutational analysis of the REn protein. REn has pRBR-binding domains at both the N and C-terminus. These domains are similar to the pRBR-binding domains in Rep in that they lack the more common LXCXE motif (Sharon B. Settlage, Miller, Gruissem, & Hanley-Bowdoin, 2001). The interaction between pRBR and REn is, by itself, not sufficient to induce the expression of PCNA. However, it is possible that REn regulates the Rep-pRBR interaction through shared interactions that may enhance binding. This is further supported by the fact that geminiviruses that do not code for REn instead code for the RepA protein. RepA does contain the LXCX motif. In the model proposed, the REn/Rep complex is capable of binding pRBR, but in the genera that lack Ren, the RepA protein fulfills the same role of pRBR binding (Sharon B. Settlage et al., 2001).

REn also interacts with PCNA, and the dual interactions with Rep and PCNA likely mean that REn aids the recruitment of PCNA at the replication fork during viral DNA replication (Sharon B. Settlage et al., 2001).

The binding of REn to Rep has been shown to increase the ATPase activity of Rep (Pasumarthy, Choudhury, & Mukherjee, 2010).

**C4/AC4**

The C4 pathogenicity protein coding sequence is located within the Rep coding sequence, but it has a different frame from Rep. The functions of the C4 protein are diverse across different geminiviruses and the protein is the least conserved of the geminivirus proteins (Fondong, 2013). As such, the observed functions of this protein have varied from it being involved in movement

in TGMV (Pooma & Petty, 1996), to abnormal cell division that results in vein swelling in BCTV, possibly by inducing the expression of the cell-cycle regulator, receptor-like kinase (RPK) protein (Lai et al., 2009).

**Coat Protein (CP/V1/AV1)**

The CP is the only structural protein encoded by geminiviruses, and while it is a late expression gene mainly involved in packaging of the virus, it does play several other functions during infection. In some monopartite geminiviruses, the CP can shuttle the viral DNA between the nucleus and the cytoplasm. This is achieved by the single-stranded viral DNA binding to the N-terminus. There are three nuclear localization signals (NLSs) in the CP: on the N-terminus, the C-terminus, and in the proteins central region. The CP of TYLCV can bind to karyopherin α1, a transport protein that imports molecular cargo into the nucleus (Kunik, Mizrachy, Citovsky, & Gafni, 1999). There is also a leucine-rich nuclear export signal (NES) in the same central region as the central NLS motif. Together, these signals along with the ability to bind ssDNA on its N-terminus means that the CP can shuttle viral DNA to and from the nucleus (Ward & Lazarowitz, 1999).

Additionally, in monopartite begomoviruses and mastreviruses, the CPs are involved in the movement of viral DNA from cell-to-cell as well as the spread of the viral DNA during infection. This is likely produced through interactions between the CP and MP (see below), which have been shown in TYLCV (Priyadarshini, Ambika, Tippeswamy, & Savithri, 2011).

**V2/AV2**

The pre-coat V2 protein is involved in multiple diverse functions during geminivirus

infection including viral movement, suppression of gene silencing, and symptom induction. In TYLCV, V2 had originally been observed being involved in viral movement, but a recent study showed that it is not directly involved in movement (Hak et al., 2015). The V2 of TYLCV has also been shown to suppress the cytosine DNA methylation, part of TGS in plants (Wang et al., 2014).

**Nuclear Shuttle Protein (NSP/BV1)**

The NSP is required for viral ssDNA movement between the nucleus and the cytoplasm. The NSP binds to viral ssDNA, and this NSP-DNA complex then interacts with the movement protein (MP); the MP transports the complex between cells. Once in another cell, the NSP transports the bound viral DNA to the nucleus (Lazarowitz, 1999; Ward, Medville, Lazarowitz, & Turgeon, 1997). The transport into and out of the nucleus is achieved by the presence of two nuclear localization signals on the N-terminus and a C-terminal nuclear export signal (Fondong, 2013). Interestingly, the functions of NSP and CP are redundant, with NSP being shown to not be required for infectivity (Y.-C. Zhou, Garrido-Ramirez, Sudarshana, Yendluri, & Gilbertson, 2007).

Additionally, NSP has been shown to interact with histone H3, along with the movement protein (MP), and viral DNA. This suggests that histone H3 could play a role in the cell-to-cell movement by forming a movement-competent complex (Y. Zhou et al., 2011).

Furthermore, the NSP interacts with receptor kinases suppressing their activity (Fontes, Santos, Luz, Waclawovsky, & Chory, 2004). Additionally, NSP interacts with a proline-rich extension-like receptor protein kinase which may phosphorylate NSP to regulate its function (Mariano et al., 2004).

### Movement Protein (MP/BC1)

The MP of geminiviruses is required for the movement of geminiviruses between cells and long-distance movement along the plant. It has a central NSP interacting domain which binds to NSP-DNA complexes in order to transport viral DNA to different cells. Not all MPs can bind to viral DNA, however some can bind to both ssDNA and dsDNA with high affinity.

MPs can interact with some of the plant host proteins, such as synaptotagmin, a regulator of endocytosis. This interacts with the MP from CaLCuV at the plasma membrane in a way similar to the MP of RNA-based plant viruses (Fukuda, 2003).

### βC1

The βC1 protein is encoded by geminivirus betasatellites. βC1 counteracts transcriptional gene silencing (TGS), and it can bind to E2 ubiquitin-conjugating enzymes to reduce the accumulation of polyubiquitinated enzymes as well as interfere with the methyl cycle (Hanley-Bowdoin et al., 2013).

## Plant response to geminivirus infection

### Silencing

RNA silencing is one of the plant defense mechanisms in response to foreign nucleic acids, such as viruses and transposons. It utilizes small interfering RNAs (siRNAs) that inhibit either transcription (TGSs) or activate sequence-specific RNA degradation of viral mRNAs (PTGS). Silencing is one of the host plants main mechanisms of defense; the silencing reduces the transcription of the viral genome, either by targeting the mRNAs for degradation or by prevention of the initial transcription (Baulcombe, 2004; Pantaleo, 2011). One additional benefit

of PTGS is that as it is the mechanism by which the technique of viral-induced gene silencing is achieved, it can be utilized in the biomolecular technique known as virus-induced gene silencing (VIGS), a powerful tool used in the study of gene function in plants (Lu, 2003).

In order to counter the effects of silencing, geminiviruses have evolved viral suppressors of RNA silencing (VSRs). The TrAP protein has been shown to interfere with the RNA silencing activity of plants (van Wezel et al., 2002). TrAP likely achieves this through binding with the serine/threonine kinase (SnRK1) and adenosine kinase involved in the maintenance of the methyl cycle of the plant (Buchmann, Asad, Wolf, Mohannath, & Bisaro, 2009), methylation being a key epigenetic signal for silencing (Raja, Sanville, Buchmann, & Bisaro, 2008). The Rep protein also interferes with the methylation of the host, further detailed in Chapter Two below. The end result is an overall reduction of methylation.

**Post-translational modification**

During infection, the geminivirus alters the ubiquitination pathways in the plant. Ubiquitination is the post-translational modification of proteins by the linking of an ubiquitin protein to the target. The ubiquitin is linked by covalent attachment to a lysine residue, achieved through a series of enzymes: the E1 activating enzyme, the E2 conjugating enzyme, the linking E3 ligase (Pickart, 2001). Monoubiquitination of proteins will alter the proteins activity through the adding of a large group to the target that can block access to binding sites or change the conformation of the target to expose binding sites that were sterically blocked. This allows monoubiquitinated proteins to bind different substrates/partners. Monoubiquitination can additionally act as signaling group for determining subcellular localization. Polyubiquitination of proteins results in targeting to the proteasome for degradation (Alcaide-Loridan & Jupin, 2012).

The sumoylation of proteins is another post-translational modification that involves the linking of small ubiquitin-like modifier (SUMO) proteins to the target. Like ubiquitination, sumoylation has three E1, E2 and E3 enzymes involved in the cascade. Sumoylation of proteins has a similar effect to monoubiquitination: changing the binding properties and influencing subcellular localization (Wilkinson & Henley, 2012).

Geminiviruses interfere with both sumoylation and ubiquitination pathways. Some geminivirus proteins are subject to ubiquitination or sumoylation (Hanley-Bowdoin et al., 2013), but the main alteration to the host is to the protein degradation pathway (Sahu, Sharma, Puranik, Muthamilarasan, & Prasad, 2014).

**Gene expression**

Perhaps the most dramatic aspect of geminivirus infection of a host cell is the redirection of the host's gene expression. Although most geminivirus-encoded proteins are involved in some way in this process, the main contributor to cellular reprogramming is the Rep protein. The Rep protein recruits the host replicative machinery to the origin of replication but also up-regulates the expression of said machinery. As much the replication of the geminivirus occurs in differentiated cells which have exited the cell cycle, the virus induces the infected cell to re-enter the cell cycle, pushing the cells into S-phase. This is likely achieved through the binding of Rep and RepA/REn to the plant retinoblastoma homolog (pRBR), the key regulator of the cell-cycle. Binding of viral proteins to pRBR allows relief of the transcriptionally-repressed genes that encode for proteins required for replication (Egelkrout, Robertson, & Hanley-bowdoin, 2001). Additionally, several host protein kinases are targets for the binding of geminivirus proteins, including but not limited to Rep, altering signaling in host cells with both up-regulation and

down-regulation of different signaling pathways (Fontes et al., 2004; Hao, 2003; Kong & Hanley-Bowdoin, 2002; Shen & Hanley-Bowdoin, 2006). Further detail on Rep and its host binding partners can be found in Chapter Two below.

In summary, geminiviruses are able to effectively seize control of host plants cells, reprogramming them to produce the cellular machinery required for viral replication. They are able to do this with a limited number of viral proteins, and as such, many of these proteins are highly multifunctional, especially Rep. The study of the viral/host protein interactions and their downstream effects is of vital importance in order to find possible targets for conferring resistance to these viruses. Moreover, the study of how these viruses hijack the cell and reprogram them can give us insight into the developmental pathways of the cell itself.

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# Replication-Associated Protein (Rep), an overview

## Background

The Rep protein, also called either C1 or AC1, is the key to viral replication. It is the only viral protein required for replication, although its activity is greatly enhanced by the Replication Enhancing Protein (REn), and it acts as the main recruiter of the host replication machinery. Rep also recognizes the viral origin of replication using its three DNA recognition motifs to bind, cleave, and prime the DNA for replication (discussed later in this chapter).

Phylogenetic analysis of Rep proteins from geminiviruses suggests an evolutionary relationship with phytoplasmal plasmids and several other viral replication-associated proteins (Krupovic, Ravantti, & Bamford, 2009). Nanoviruses are another type of ssDNA virus that have similar replicative methods. Nanovirus DNA possess the inverted repeat sequences that form stem-loop structures. The Rep1 and Rep2 Faba Bean Necrotic Yellow Virus (FBNYV), a nanovirus, contains ATPase activity that is essential for DNA replication. The NTP-binding motif of the FBNYV Reps are GXXGXXGKT/S, similar to the GXXXXGKT/S (X – any amino acid) p-loop motif found in geminivirus Rep, the $K_m$ similar to that of the Rep of TYLCV (Timchenko et al., 1999).

The E1 proteins of papillomaviruses share many similar characteristics to the geminivirus Rep proteins. Papillomaviruses are the cause of warts, both cutaneous and genital, in both humans and animals. Papillomavirus E1 proteins form hexamers and dodecamers, made from double hexamers, at the origin of replication as does geminivirus Rep (Orozco, Kong, Batts, Elledge, & Hanley-Bowdoin, 2000; Wilson, West, Woytek, & Rangasamy, 2002). Like the C-terminus of Rep the, C-terminus of E1 proteins contain an ATPase domain and a P-loop that is

responsible for ATP binding. The E1 protein is a unidirectional helicase with 3' to 5' activity, as do geminivirus Rep (Clérot & Bernardi, 2006; Wilson et al., 2002).

## Rep – Structural domains

The structure of the Rep protein is highly modular –different domains impart different functions to the protein. Additionally, the sequences of these binding domains are highly conserved in Rep proteins across geminivirus species.

The N-terminus of Rep contains most of the binding sites for both DNA and protein partners. The use of OPTAL sequence alignment (Koonin & Ilyina, 1992) determined that Rep showed similarity to the RCR proteins in the pUB110 and pMV158 eubacterial plasmid families with the presence of motifs I, II, and III. An additional domain, called the Geminivirus Rep Sequence (GRS), is also found conserved across all Rep proteins. The DNA binding domain encompasses amino acids 1-130 and consists of three motifs; each motif mediates a particular process in the preparation of DNA for rolling-circle replication.

The plant retinoblastoma (pRb) homolog (pRBR) binding domain is more central and overlaps with the DNA binding domain, and it encompasses amino acids 101 to 180. This domain overlaps with the REn, oligomerization, GRIK, and GRIMP binding domains. This domain contains two conserved α-helices that are essential for binding the partners of Rep. This domain has also been shown to be required for the for the alteration of gene expression in host cells (Lucioli et al., 2016). The C-terminus of Rep is devoted to ATP binding and ligation. The ATP binding site is located ATPase domain including the Walker A and Walker B motifs.

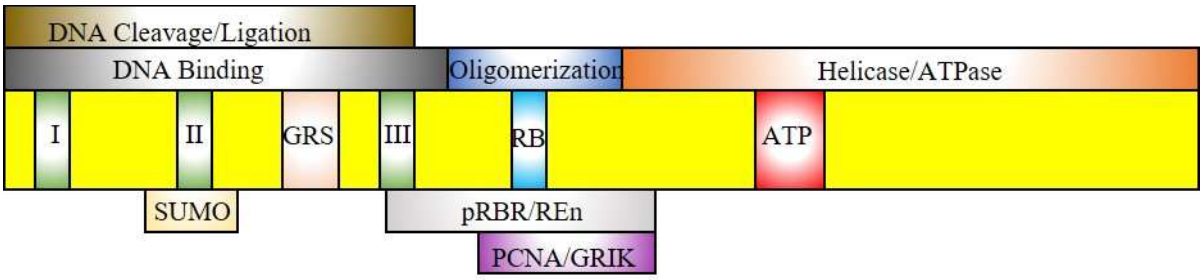


Figure 2.1: Schematic of the Rep protein of the geminivirus with the key binding and enzymatic domains highlighted. The conserved N-terminus sequences denoted motif I, II, and III are characteristic of many rolling-circle initiator proteins.

## Rep DNA recognition and binding

The binding of Rep to DNA is sequence-specific: it binds to a stem-loops structure and also recognizes a 60-bp sequence further upstream (Lazarowitz, Wu, Rogers, & Elmer, 1992). Geminiviruses are ssDNA viruses, but in order to form the stem-loop structure required for the initiation of rolling-circle replication, the replicative form of the genome is converted to double-stranded (ds) DNA. Analysis of the geminivirus origin of replication (ori) shows that there are repeating elements (iterons) upstream of the stem-loop structure. The exact sequence of these iterons varies from species to species, but all require the presence of iterons for binding (G Arguello-Astorga et al., 2004). For bipartite begomoviruses, both A and B components share common origins. This region is recognized by the Rep protein which binds cooperatively to it.

Two distinct amino acid sequences determine the Rep DNA-binding specificity. These so-called specificity determinants (SPDs) are found adjacent to the conserved RCR motifs. A group of SPDs are clustered on the N-terminus side of the RCR motif I, and the second group are on the C-terminus side of motif II (Londoño, Riego-Ruiz, & Argüello-Astorga, 2010).

**Self-regulation of expression**

One additional consequence of the ability of Rep to bind to DNA is that it is able to regulate its own expression. The location of the Rep-binding site is between the TATA box and the Rep transcription start site, and thus, binding to initiate replication will interfere with the expression of its own gene. This was shown by cotransfection experiments replacing the Rep ORF with a reporter gene –constitutive expression of Rep reduced the expression of the reporter gene (Sunter, Hartitz, & Bisaro, 1993). Moreover, as the initiation sites for both REn and TrAP are found within the coding region of Rep, the repression of the Rep gene up-regulates expression of the REn and TrAP genes (Shung & Sunter, 2007).

Furthermore, Rep has been shown to guide the post-translational monoubiquitination pathway for histone modifications. The epigenetic markers that are added to the histones were shown to recruit the host transcriptional machinery and up-regulate the transcription of the viral genome (discussed below) (Kushwaha, Bhardwaj, & Chakraborty, 2017).

## RNA Silencing Suppression by Rep

Rep of the Mastrevirus genus of the geminivirus family has been shown to suppress part of the RNA silencing defense mechanism in plants. One of the epigenetic markers that promotes gene silencing in plants is cytosine methylation. The viral DNA is subjected to siRNA-guided methylation leading to hypermethylation of the viral genome, which results in transcriptional gene silencing (TGS); this has been shown both *in vitro* and *in vivo. Arabidopsis* mutants that are deficient in cytosine methyltransferase are hypersusceptible to geminivirus infection, and hypermethylation of the viral DNA in promoter regions leads to host recovery (Rodriguez-

Negrete, Carrillo-Tripp, & Rivera-Bustamante, 2009). As previously discussed in Chapter One, geminiviruses are able to interfere with the silencing mechanism in plants. Rep encoded by alpha-satellites plays a part in the virus response to silencing by interfering with the cytosine methylation pathway (Rodríguez-Negrete et al., 2013).

Control of DNA methylation in plants is achieved by the activity of both methyltransferases and demethylases. Both Rep and C2 work in conjunction in order to reduce expression of the DNA methyltransferases: methyltransferase 1 (MET1) and Chromomethlyase 3 (CMT3). The reduction in expression of MET1 is dependent upon Rep's ability to bind to the plant retinoblastoma protein. The reduced expression of these proteins means that the hypermethylation of the geminivirus genome is not possible during infection, as hypermethylation is required for plant recovery from infection. The inhibition of this process benefits viral propagation (Rodríguez-Negrete et al., 2013).

**Rep impact on plant gene expression**

Comparison between transcriptional profiles of tomato plants infected with Tomato Yellow Leaf Curl Virus (TYLCV) and transgenic tomato plants expressing the N-terminal protein binding domain of Rep shows that it is responsible for a large amount of the redirected expression patterns brought about by geminivirus infection.

## Rep – Protein/protein interactions with the host cell

As Rep is the sole encoded protein in geminiviruses that is required for viral genome replication, it is thought to interact with plant proteins that are required for DNA replication.

In mature plants, the expression of the replisome is present only in the meristem, as this tissue as the "growing edge" of the plant containing duplicating cells. However, geminivirus infection is normally present in both leaf and vascular tissue, tissues that have both exited the cell-cycle. As the cells present in these mature tissues do not undergo division, it is thought that Rep also binds to, and acts upon, proteins involved in the regulation of the cell-cycle to express the proteins required for replication (Hanley-Bowdoin, Bejarano, Robertson, & Mansoor, 2013). A study into the transcriptomic profile of transgenic tomato plants expressing the N-terminal of TYLCV Rep showed that the central protein binding domain must be present for these changes in gene expression to occur –only expressing the first 130 amino acids of TYLCV Rep is not sufficient (Lucioli et al., 2016).

A recent study into the protein interactions between geminivirus and host using affinity purification and mass spectrometry analysis using GFP-tagged TYLCV infected *Nicotiana benthamiana* identified 284 proteins as part of Reps interactome, with the majority of interactions occurring at the N-terminus. This study indicates that we have only just begun to scratch the surface of elucidating all the host protein-Rep interactions and the further downstream effects on the host (Wang et al., 2017).

**pRBR:**

The retinoblastoma protein (pRb) is a key regulator of the cell cycle, with evidence that it negatively regulates the progression through the cell cycle. In plants, the plant retinoblastoma-homologue (pRBR) seems to fulfil a similar role. The pRBR interacts with the E2F transcription factors, and the interaction between pRBR and E2F represses the expression of proteins responsible for the transition of cells into S-phase. During the regular cell-cycle, the interactions

between pRBR/E2F are disrupted in late G1-phase by the phosphorylation of pRBR. This removes the binding of the E2F transcription factors and allows the cell to enter the S-phase by inducing expression of the genes responsible for entry and DNA replicative machinery (De Jager & Murray, 1999; Egelkrout et al., 2002).

As the geminivirus infects cells in the leaf or vascular tissues that have exited the cell-cycle, the virus needs to activate genes associated with cell-cycle re-entry and S-phase. The virus also inhibits or down-regulates genes involved in early G1 and late G2 phases. As one of the key regulators of the cell cycle is pRBR, it is a target for geminivirus proteins –among them are Rep, RepA, and REn.

RepA, the Rep variant found in Mastreviruses, interacts with pRBR via a LXCXE (X any amino acid) motif. This motif is a common binding motif among RBR binding partners.

Mutational studies have shown that the interaction between CaLCuV, TGMV, and TYLCV Reps and pRBR are achieved through a helix 4 motif. This differs from the previously mentioned LXCXE motif that is present in Geminivirus RepA sequences; begomoviruses, curtoviruses, and topocuviruses all lack RepA. Disruption of the 4-helix motif significantly impacted the binding of the Rep proteins to the pRBR but, interestingly, in Mastreviruses, disruption of the LXCXE motif had no observable effect. Evolutionarily it is possible that the Rep protein in geminiviruses originally used the LXCXE motif to bind to the pRBR but evolved the 4-helix structure that eventually replaced the LXCXE motif (Gerardo Arguello-Astorga et al., 2004).

One proposed model is that the binding of Rep/RepA and REn to the pRBR up-regulates the expression of proteins by interfering with the binding of pRBR/E2F. The viral proteins bind to pRBR, displacing the E2F factors that are then free to up-regulate the genes that are involved

in DNA replication, cell-cycle entry and progression into S-phase, and also repress genes that keep the cell in G1-phase (Gerardo Arguello-Astorga et al., 2004). Included in the DNA replication proteins that are up-regulated is proliferating cell nuclear antigen (PCNA), another binding partner of Rep and a regulator of DNA replication.

**Proliferating cell nuclear antigen (PCNA)**

As discussed above, PCNA is up-regulated during geminivirus infection. PCNA is a heterotrimeric ring, and its positively charged interior sits close to the DNA, giving the PCNA a pseudo-six-fold symmetry. PCNA is located in the nuclei of eukaryotic cells that undergo cell division and is a highly conserved protein. PCNA plays a key role in DNA replication both as processivity factors and in regulation/recruitment of replication factors. Additionally, PCNA has been shown to play roles in different kinds of DNA repair. Post-translational modification of PCNA is a key regulator of its activity –both ubiquitination and sumoylation have effects on the activity of PCNA. PCNA also holds the DNA polymerase in place during replication.

As geminiviruses do not encode any replication proteins, relying instead on hijacking the plants replication machinery, it is unsurprising that it has been shown to interact with PCNA and that its transcription is activated by the interaction with Rep and pRBR. During geminivirus infection, cellular levels of PCNA increase drastically, accumulating in differentiated cells. This was shown in plants infected with *Tomato golden mosaic virus* (TGMV); this is believed to be achieved by the Rep/pRBR interactions as described above. The Rep of tomato yellow leaf curl Sardinia virus (TYLCSV) binds to the PCNA at the oligomerization domain, along with REn (Castillo, Collinet, Deret, Kashoggi, & Bejarano, 2003).

One observed effect of PCNA binding to Indian mung bean yellow mosaic virus (IMYMV) Rep is that it inhibits both the ATPase and nicking activity. This activity may possibly be a way that the virus controls the copy number of the DNA(Bagewadi, Chen, Lal, Choudhury, & Mukherjee, 2004).

Rep is one of the first proteins that bind to the viral origin of replication during the initial stages of replication. As Rep and REn bind together, during the nicking and joining event of viral replication, one hypothesis is that the binding of Rep/REn to PCNA could mediate the recruitment of PCNA and subsequent subunits to the origin of replication after the cleavage of the DNA. The additional ability of Rep to bind replication factor C that acts to load the PCNA onto the DNA would bring these component proteins together to set up the initial replisome. The presence of REn greatly increases the efficiency of DNA replication and it is possible that the dual interaction is partially responsible for observed increase in replication (Rizvi, Choudhury, & Tuteja, 2015).

**Replication Factor C, the Clamp Loader**

In order for the PCNA to "close" around the DNA during replication, it needs to be loaded onto the DNA –this is achieved by the activity of the so-called clamp loader (replication factor C). Binding of the clamp loader to the sliding clamp causes the sliding clamp to change conformation to an open position so it can be loaded to the DNA. Once bound to the DNA, the clamp loader uses ATP hydrolysis to close the PCNA sliding clamp around the DNA.
The eukaryotic clamp loader is a large multi-subunit protein composed of five subunits: RFCs1-5. The Rep of Wheat Dwarf Virus (WDV) has been shown to bind to the RFC-1 subunit (Luque, Sanz-Burgos, Ramirez-Parra, Castellano, & Gutierrez, 2002).

**Replication Protein A (RPA)**

The RPA is a ssDNA binding protein that is fairly conserved among eukaryotes. It is a heterotrimeric protein that is involved in replication, maintenance, and recombination of DNA. The three subunits of RPA are 14, 32, and 70kDa in size; all subunits contain ssDNA binding capability, with the 70kDa unit associating most strongly with the DNA. The 32kDa subunit plays a regulatory role during DNA binding. The subunit also has phosphorylation sites that are targets for cell cycle regulatory kinases. RPA binds to many other proteins that are involved in replication such as the clamp loader, helicases, and PCNA. During replication, RPA binds to the ssDNA to prevent the DNA from rewinding or the formation of unwanted secondary DNA structures.

The Rep of Mungbean yellow mosaic India virus (MYMIV) has been shown to be able to bind to the 32kDa subunit of the pea plant RPA (RPA32) and this binding up-regulates DNA replication of MYMIV. This binding occurs on the C-terminus of Rep, binding of RPA32 was shown to down-regulate Reps nicking and joining activity. Additionally, the binding of RPA32 up-regulates the ATPase activity of Rep; this up-regulation of ATPase could work to enhance the ATPase-dependent helicase activity of Rep for local unwinding of the DNA around the replication fork during the initial stages of replisome formation (Singh, Islam, Choudhury, Karjee, & Mukherjee, 2007).

**Histone (H3)**

During infection, the double-stranded, replicative form of the geminivirus is assembled with plant nucleosomes into so-called minichromosomes (Pilartz & Jeske, 1992) Confocal microscopy and chromatin immunoprecipitation (ChIP) has shown that Rep is involved in the

formation of the “beads on a string” around the viral genome; it is also possible that the detection of Rep in the viral nucleosome is due to the necessity of Rep binding for replication.

This assembly means that, like any DNA assembled into nucleosomes, that origins of replication may be blocked by being bound to histones, it is possible that Rep interacts with the plant H3 histone on order to displace the nucleosome. The displacement of the nucleosome, in addition with Rep-mediated post-translational modification of histones via the ubiquitination pathways (see below), allows additional Rep and plant replication machinery to access the origin of replication (Kong & Hanley-Bowdoin, 2002).

**Ubiquitination**

Ubiquitination is a post-translational modification that targets certain substrate proteins with ubiquitin. Ubiquitin is a small, 8.5kDa protein, 76 residues long and has several charged lysine residues on its surface. These charged residues help facilitate protein-protein interactions and can alter the structure of the target protein, changing its binding properties. As discussed above, the organization of the geminivirus genome into minichromosomes presents the same challenges to replication and transcription that the plant host has. It is therefore unsurprising to find that the geminivirus utilizes the same plant pathways for chromatin remodeling. The trimethylation of a H3 lysine (H3K4me3) is an epigenetic chromatin activation marker that promotes transcription of genes, and is among the most well-studied epigenetic markers. It is thought that the trimethylation is recognized by a variety of downstream effector proteins that remodel the chromatin in several different ways (Ruthenburg, Allis, & Wysocka, 2007). The monoubiquitination of the histone H2B, in eukaryotes, is also a key epigenetic chromatin marker for transcriptionally active genes and serves as a guide for other activation markers, such as the

H3K4me3. Indeed, the Chilli Leaf Curl Virus (ChiLCV) minichromosomes show both monoubiquitination of the H2B and trimethylation of the H3K4 on the chromatin that stimulate transcription of the genome (Kushwaha et al., 2017).

The monoubiquitination pathway for histones in *Nicotiana benthamina* involves the Ubiquitin-conjugating enzyme 2 (UBC2) and histone monoubiquitination 1 (HUB1). These two enzymes were shown to bind to the Rep protein from ChiLCV at its oligomerization domain. The ability of Rep to bind the viral DNA, H3, and the ubiquitination machinery is likely the way the geminivirus induces ubiquitination on the viral promoter (Kushwaha et al., 2017).

**Sumo-conjugating enzyme**

Sumoylation is the posttranslational modification of a protein by the covalent attachment of the SUMO protein to a Lysine residue of the target protein. SUMO is a ubiquitin-like protein that is approximately 12kDa in size and shares a mechanistically similar enzymatic cascade of attachment. Sumoylation of proteins alters the binding properties of the target protein by either influencing the conformation of the sumoylated protein, by blocking binding sites that would otherwise be accessible, or conversely by providing a new binding site on the SUMO protein itself (Wilkinson & Henley, 2012). In plants, studies have shown that sumoylation plays a key role in regulating cell proliferation, ABA signaling, and cellular response to pathogens (Park et al., 2011).

Rep of TGMV has been shown to interact with the SUMO-conjugating enzyme (SCE1) of *Nicotiana Benthamiana*, with the interaction occurring in the DNA-binding and cleavage/ligation domain of Rep. While Rep itself is not sumoylated, the interaction between Rep and SCE1 does modulate the pattern of sumoylation pattern of proteins in the host plant.

Geminiviruses interfere with the cell cycle and replicative machinery of infected cells to promote conditions supporting viral replication and accumulation, and many of Rep's other binding partners are involved in these processes. For example, PCNA and RBR are targets for sumoylation, and it is likely that Rep acts as a guide for SCE1 to help it target specific proteins with which Rep also interacts (Sánchez-Durán et al., 2011).

Although the exact role of sumoylation in geminivirus infection has yet to be elucidated, the interaction between Rep and SCE1 is essential for viral replication. Mutations to the K68 and K102 residues show that during infection there is a reduction in the accumulation of viral DNA and the severity of symptoms.

**Rad54/Rad51**

Geminiviruses replicate their genome by RCR and also recombination-dependent replication (RDR) (Richter, Ende, & Jeske, 2015). In RDR, the replicating DNA is not re-ligated by the Rep protein (see below) after one round of replication but instead keeps on re-replicating the DNA and displacing the newly synthesized DNA, forming concatemers. This has been observed in Africa Cassava Mosaic Virus (ACMV) (Saunders, Lucy, & Stanley, 1991). The Rad proteins are involved in homologous recombination and repair. Studies have shown that the Rad 54 and 51 proteins are up-regulated during geminivirus infection (Kaliappan, Choudhury, Suyal, & Mukherjee, 2012). The Rad proteins interact with Rep at their N-terminal domains on the oligomerization domain of Rep.

Yeast two-hybrid screenings have shown that the Rad51 protein interacts with the Rep of Mungbean Yellow Mosaic Virus (MYMIV). As the Rad 51 protein stabilizes the replication fork of DNA, it is hypothesized that the Rep helps recruit Rad 51 to the replisome in order to stabilize

replication at the fork (Suyal, Mukherjee, & Choudhury, 2013). Another yeast two-hybrid screen identified Rad 54 as another binding partner of Rep. It was initially thought that this binding was required for viral replication, possibly enhancing the nicking and joining or the ATPase activities of Rep (Kaliappan et al., 2012). However, a later study showed that the Rad 54 was not essential *in planta* and its role remains unknown (Richter et al., 2015).

**Geminivirus Rep-interacting Kinase (GRIK)**

Some *Arabidopsis* protein kinases are able to bind the Rep protein of *Tomato golden mosaic virus* (TGMV). Yeast two-hybrid studies identified two truncated versions of protein kinases that interacted with Rep -these kinases were termed GRIK1 and GRIK2. The GRIKs are part of a group of cytoplasmic protein kinases, and both of these kinases contain a calmodulin binding motifs, a messenger protein that is found in eukaryotes (Kong & Hanley-Bowdoin, 2002).

A later study confirmed that the full-length kinases also bind Rep. Additionally, two further protein kinases were identified using BLAST analysis in rice and the bacteria *Medicago truncatula.* The GRIKs are most similar to the yeast PAK1/TOS3/ELM1 kinases that activate the transcription of the SNF1/AMPK family of protein kinases found in eukaryotes, and the GRIKs likely mediate similar biochemical functions. Levels of expression of GRIK is high in young tissue, but it is not detected in mature, likely meaning that it is cell-cycle regulated. Furthermore, expression of both GRIK proteins is up-regulated in *Arabidopsis* during geminivirus infection, and it possibly plays a role in the geminivirus interference with the cell cycle (Shen & Hanley-Bowdoin, 2006).

**Geminivirus Rep-Interacting Motor Protein (GRIMP)**

Rep also interacts with a motor protein, termed GRIMP. This protein has a kinesin domain and is a member of the C-terminal kinesin subfamily. Members of this subfamily are important players during cell division –they localize to the spindle and regulate the microtubules during mitosis. Indeed, it was shown GRIMP localizes to the condensed chromosomes and the spindle during mitosis. Additionally, GRIMP is present in cell in mature tissue which would suggest that GRIMP also plays an as of yet unidentified role that is separate to that of its role in the cell-cycle. GRIMP is activated by a cyclin-dependent kinase (CDK) which phosphorylates it, allowing the binding to the spindle.

The reason behind Reps interactions with GRIMP are as of yet unknown. It has been hypothesized that the Rep binding to GRIMP somehow prevents phosphorylation, either by preventing binding of the CDK or blocking the phosphorylation site. The Rep-GRIMP interaction may be partially responsible for the halting of the cell-cycle in S-phase. Geminivirus infection arrests the cell-cycle; infected cells re-enter S-phase and prevent any further progression, allowing for the replication and accumulation of viral DNA. Infected cells are observed to contain condensed chromatin, which is a characteristic of the early prophase (Kong & Hanley-Bowdoin, 2002).

**Geminivirus Rep-A binding protein (GRAB)**

The GRAB proteins (GRAB1 and GRAB2) are members of the NAC family. The members of this protein family are transcription factors involved in plant development, defense, and abiotic stress among other functions (Olsen, Ernst, Leggio, & Skriver, 2005). The expression of GRAB proteins was shown to severely inhibit the replication of wheat dwarf virus (WDV), a

Mastrevirus, in wheat cell cultures. The C-terminus of the RepA protein of WDV is able to bind the N-terminus of the GRABs.

One possible explanation for this binding is that expression of GRAB is part of the plant defense mechanism to geminivirus infection, but the mechanism by which it interferes with virus replication is unknown. The fact that GRABs are part of the NAC family of transcription factors means that it is possible that GRABs activate downstream genes that are part of the response to infection or for a cell differentiation pathway. Thus, the binding and sequestering by RepA could prevent them from activating these pathways (Xie, Sanz-Burgos, Guo, García, & Gutiérrez, 1999).

**Minichromasome maintenance protein 2 (MCM2)**

MCM2 is a replicative helicase that is involved in eukaryotic replication initiation. It is a highly conserved protein among eukaryotes. MCM2 forms a replicative helicase along with MCM3-7; MCM2 has been shown to regulate the helicase activity of the complex. This complex is a key regulator during the formation of the pre-replication complex –it helps recruit the other replication proteins to the complex.

**Additional possible Rep binding partners**

Although several Rep binding proteins from the host have been identified, and their interactions somewhat characterized, both mass spectrometry analysis and other screening methods indicate that there are many others still out there (Wang et al., 2017).

# Rep – Protein/protein interactions with other viral proteins

## Rep

The Rep protein forms oligomers that can vary in size. The amino acids required for oligomerization are 157-159. Structural studies predict the presence of two α-helices at amino acids 132-154 (Orozco, Miller, Settlage, & Hanley-Bowdoin, 1997). Many types of DNA-binding proteins use α-helices for oligomerization. Additionally, there is a high degree of sequence and structural conservation in the surrounding amino acids between the Rep proteins of different geminiviruses. It is also likely that other contacts between Rep oligomers occur outside of this region.

Different oligomerization states have different properties. The DNA binding capability of Rep is dependent upon its oligomerization state, with the Rep being in either hexameric, or dodecameric states. This is similar to how other helicases of the superfamily 3 (SF3), of which Rep is a member (Clérot & Bernardi, 2006), are loaded onto DNA. A 26-mer form of the Rep protein binds to the origin of replication and is responsible for the nicking and joining activity that occurs at the origin.

## REn

Whilst Rep is the only encoded protein in geminiviruses that is required for replication, the accessory protein REn greatly enhances replicative activity, resulting in higher levels of viral DNA accumulation. The exact method by which REn enhances replication is not yet fully understood, but its binding properties do give us insight into the role it plays. REn is a 16kDa accessory protein 134 amino acids long, it is also highly hydrophobic (Castillo et al., 2003). REn

is capable of binding to host proteins as well as itself and Rep, one of the main host proteins that REn binds to PCNA.

REn binds to Rep through nonpolar, aromatic, contacts. In order for REn to bind Rep the Rep must be in a multimeric state –this is possibly because of the need for multiple aromatic amino acids in order for the binding to be stable. The ability of REn to interact with itself, Rep, and PCNA means that it is highly likely that the way REn enhances the activity of Rep is by aiding in the recruitment of PCNA to the initial replisome (Settlage, Miller, Gruissem, & Hanley-Bowdoin, 2001).

Additionally, studies have shown that the binding of an oligomeric form of REn does increase the ATPase activity of Rep *in vitro*. This was done without the presence of DNA showing that the ATPase enhancement is not related to the enhanced recruitment of replication machinery (Pasumarthy, Choudhury, & Mukherjee, 2010).

The last important interaction that REn has is with pRBR. REn contains pRBR binding domains at both the N and C-termini. These domains are similar to the pRBR binding domain of Rep, but not that of RepA. The dual capabilities of Rep and REn to bind to each other and the fact that they contain similar pRBR-binding domains has led to a model that the REn/Rep complex acts together to bind efficiently to pRBR. Individually, REn and Rep bind weakly to pRBR, but the Rep/REn complex binds well. It is thought that the binding activity and efficiency of the Rep/REn complex is similar to the of the activity of RepA (Settlage et al., 2001).

## Enzymatic Activities of Rep

### Helicase Activity

A truncated version of the N-terminal domain, consisting of the amino acids 122 to 359, of the Rep protein of the geminivirus Tomato Yellow Leaf Curl Virus (TYLCV) was shown to have helicase activity *in vitro*.

Helicase activity of Rep may help serve the initiation of RCR. The nicking and joining activity of Rep, the first step in RCR, is effective only on ssDNA (in the stem-loop structure). Thus, local unwinding of the double-stranded replicative form of the DNA at the origin of replication by the Rep protein could be required for formation of the initial structure during RCR.

Rep helicase activity is polar, showing 3' to 5' unidirectional activity. This would fit with the helicase activity being involved in replication during RCR. The leading strand synthesis occurs in the 5` to 3` direction, and there is no need for lagging strand synthesis due to the fact that geminiviruses are single-stranded.

This helicase activity is dependent on the oligomerization state of the protein. It has been observed that the helicase activity of Rep occurs when it forms a dodecamer. Many other helicase activity has been observed in conjunction with dodecamers consisting of double hexamers, and associated with bi-directional unwinding of DNA (Clérot & Bernardi, 2006). However, RCR is mainly unidirectional. This discrepancy can be possibly explained by one of the hexamers being inactive. This hypothesis has been proposed as an explanation of the rolling-hairpin replication of Minute Virus of Mice (MVM), a parvovirus, by Christensen and Tattersall (Cotmore, Christensen, & Tattersall, 2000). Rolling hairpin replication is similar to RCR in many respects. The binding of a second, inactive, oligomer acts as a guide of the active oligomer

at the origin of replication. This is a possible explanation for the observed data in the Clérot paper.

Comparative sequence analysis of the Rep protein shows that it belongs to the SF3 helicase family (Clérot & Bernardi, 2006). These helicases possess three conserved motifs: the Walker A, Walker B, and motif C. The Walker A motif is the ATP binding, phosphate-loop (P-loop). The motif is composed of the amino acid sequence pattern GXXXXGK[T/S] (X, any residue). The Walker B motif is the ATP hydrolysis catalytic site and has either the DXXD or XXXXDD (X, any residue) amino acid sequence (further detail on ATP hydrolysis is discussed below).

Helicase activity is also dependent on the ATP binding and ATPase activities of Rep – removal of the ATPase activity results in total loss of the helicase activity, but not formation of oligomers. This indicates that ATP hydrolysis only affects the helicase activity, not the formation of the required oligomer.

**ATPase Activity**

The ATPase activity of Rep was determined in 1995 (Desbiez, David, Mettouchi, Laufs, & Gronenborn, 1995). As discussed above, the Walker A motifs P-loop is the ATP binding domain. The conserved lysine residue in the P-loop is required for *in vitro* ATPase activity and the *in vivo* replication of the viral DNA. The ATPase activity is required for the helicase activity of the Rep protein.

The ATPase activity of Rep is up-regulated by the presence of ssDNA, but also the abolishment of the ATP binding by mutation of the conserved lysine residue in the Walker A motif means a loss of the ssDNA binding capability. It is worth noting that functional studies

have shown that binding of ATPγS, a nonhydrolyzable ATP analog capable of binding to ATPases, is sufficient for the formation of the Rep-ssDNA complex. This indicates that it is ATP binding, not hydrolysis, that is required for helicase activity.

The hydrolysis of bound ATP is done by the Walker B motif. The conserved aspartic acid residues in the motif coordinate with magnesium ions ($Mg^{2+}$). In the *Tomato leaf curl Kerala virus* (ToLCKeV), ATPase activity is enhanced by the binding of an oligomeric form of the protein REn (Kittelmann, Rau, Gronenborn, & Jeske, 2009).

**Nicking and Joining Activity**

The Rep protein has both cleavage and ligation activities. The cleavage and ligation occurs at a common recognizable sequence found within the origin of replication, specifically in the stem-loop structure that is formed during the initiation of rolling circle replication. The tyrosine residue in motif III is the catalytically active residue that nicks the DNA at the origin of replication between the thymine and adenine bases at position 7 and 8 in the binding site. The nicking activity covalently links the tyrosine to the thymine via a tyrosyl-ester bond. The nicking activity produces a free 3` hydroxyl group that primes the plus strand for synthesis. This free 3` group remains during replication and further acts as an acceptor for the 5` end, which is still linked to Rep. The ligation event, occurring after one round of replication, releases the newly synthesized strand (Laufs et al., 1995).

This nicking and joining activity of Rep at the same site is typical of type-I topoisomerase. *In vitro* Rep has been shown that it will relax supercoiled DNA, provided that it has the Rep binding site. Also the topoisomerase activity of Rep is reduced by the presence of

camptothecin, a specific inhibitor of type-I topoisomerases, thus Rep can be considered a site-specific type I topoisomerase (Pant et al., 2001).

## Steps in RCR and the role of Rep

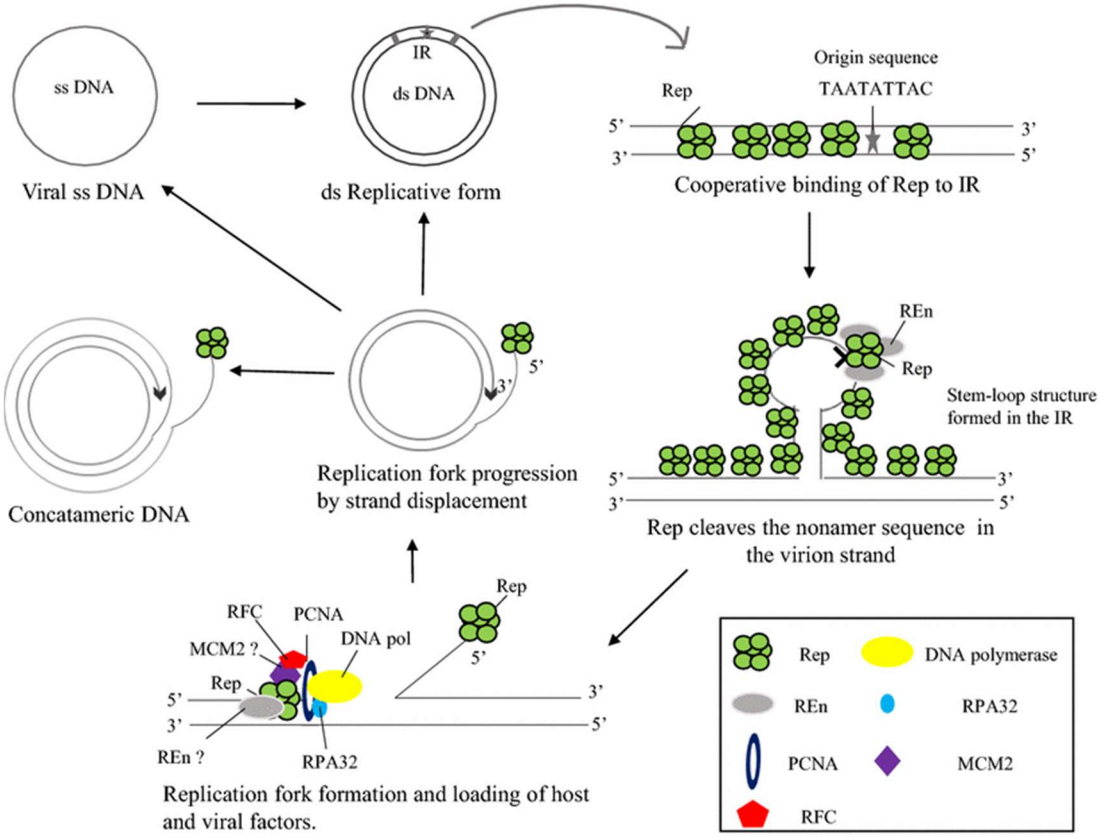


Figure 2.2: The model for RCR of the Geminivirus genome. This model is likely incomplete.

The first step in rolling circle replication is the formation of the replicative form of the DNA by converting the single-stranded viral DNA into dsDNA. The conversion of ssDNA to dsDNA is achieved by extension of an RNA primer to form the minus strand. The primer binding location and nature of minus strand synthesis can vary between species (Rizvi et al., 2015). An

oligomeric form, possibly a hexamer, of Rep is then able to bind to the IR region around the origin of replication using the three recognition helices on the proteins N-terminus. The binding of Rep to the DNA is cooperative.

Cooperative binding of Rep to the IR region leads to the formation of the stem-loop structure. The formation of this structure causes the origin sequence (TAATATTAC) to be single-stranded as it is in the loop. At some point, Rep binds to both ATP and the REn protein, though the exact order of the binding steps is as yet unknown, the ATP binds the Rep at the Walker A and B sites at the C-terminus; Rep is then able to cleave the origin site between nucleotides 7 and 8 (TAATATT//AC). This nicking is achieved by the catalytic tyrosine in motif III. This Rep oligomer then remains bound to the 5` end of the nick (Rizvi et al., 2015), the form of Rep that binds to and nicks the DNA is a 26-mer.

Another Rep oligomer that is associated with the 3` end of the nicked DNA is required for the recruitment of host factors and local unwinding of DNA in the 3` to 5` direction. It has been observed that the oligomeric state required for the helicase activity is a dodecamer (Clérot & Bernardi, 2006). Again, the exact binding order and procession of the replication fork is unknown. Rep can unwind up to 200 bp and it is possible that it unwinds some of the DNA around the replication fork to allow space for the other factors to bind.

## Summary

The Rep protein is a highly versatile protein that is able to bind to a wide variety of proteins, both host and viral, in order to replicate the viral genome. The multifunctional nature of this protein is necessary as geminiviruses do not encode a large number of proteins. This does,

however, make the study and understanding of Rep important, as interference with its binding capabilities would greatly inhibit its function, making it a prime target for conferring resistance to the virus in plants.

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# Aptamer Project

## Background

Geminivirus infection inflicts a high economic cost on the agricultural industry, on both vegetable (Inoue-Nagata, Lima, & Gilbertson, 2016; Legg & Fauquet, 2004) and commercial crops (Saeed & Samad, 2017), and over the last several decades, it has emerged as a major threat to farmers around the world, especially in Africa and South America (Inoue-Nagata et al., 2016; Legg & Fauquet, 2004; Moffat, 1999) and with emergences in Asia (De Bruyn et al., 2012; Saeed & Samad, 2017). Further complicating the situation, multiple infections are also common, with several different strains of geminivirus being present in an infected crop. This often leads to worse symptoms and increased viral accumulation in plants (Harrison & Robinson, 1999). Because of this research into strategies of infection control, resistance, and symptom severity are of great importance.

Avenues of investigation include both transgenic plants and breeding plants for resistance. The breeding approach has had some limited success, but is hampered by the few occurrences of natural resistance to the virus (Morales, 2001). Additionally, breeding for resistance can be overcome by the virus if a recombinant strain were to emerge (Harrison & Robinson, 1999). Other possibilities include using RNAi to confer resistance. However, this approach may be limited at the level of broad spectrum resistance as it is reliant on sequence similarity between geminivirus strains (Briddon, 2015)

The use of peptide aptamers in the targeting of specific proteins has been used to inactivate specific viral proteins (Colombo, Mizzotti, Masiero, Kater, & Pesaresi, 2015). Peptide

aptamers are often artificially constructed recombinant proteins that bind and inactivate a target protein. They are able to interfere with both protein-protein and, protein-DNA binding, allowing them to target viral proteins, which often rely on these interactions for replication. Peptide aptamers bind in a similar manner to antibodies, but can be identified using a yeast two-hybrid screening against a library of aptamers (Real et al., 2004).

As previously discussed, the Rep protein of the geminivirus is a multifunctional protein that is the sole encoded viral protein required for replication. It recruits both the host plants replication machinery to replicate the viral DNA and other viral proteins such as REn to enhance virus replication. Due to the multifunctional nature of Rep and its reliance on both protein-protein and protein-DNA interactions for its function, it is an excellent candidate for peptide aptamers. It is also a highly conserved protein across geminivirus strains, both in sequence and structure, meaning that there is greater potential for this method to yield a more broad-spectrum resistance.

Lopez et al (Lopez-Ochoa, Ramirez-Prado, & Hanley-Bowdoin, 2006) performed a yeast two-hybrid screen against the N-terminus of the Rep protein from *Tomato Golden Mosaic Virus* (TGMV) that identified several potential aptamers that would bind. These aptamers were from a library that consisted of random 20-mer peptide sequences constrained within the active site of *E.coli* thioredoxin (TrxA). The TrxA "scaffolding" is a well-established fusion partner for small peptide inserts (Canali et al., 2014; Lynn E Murry, Laurie G Elliott, Sherry A Capitant, Joanne A West, Kimberly K Hanson, Liliana Scarafia, Scott Johnston, Camille DeLuca-Flaherty, Scott Nichols, Dolores Cunanan, Paul S Dietrich, Irvin J Mettler, Steve Dewald, Debra A Warnick, Carol Rhodes, 1993) and allows for easier manipulation of the aptamer. This screening identified sixteen aptamers that were able to bind to the N-terminus of the TGMV Rep protein, the N-

terminus of Rep containing its protein binding domains, and they identified seven binding motifs. These aptamers were able to reduce viral DNA accumulation in plant cell cultures of tobacco cells. They also established that the aptamers were able to bind to the Rep protein of *Cabbage Leaf Curl Virus* (CaLCuV).

A follow up study showed that the 16 peptide aptamers were able to interact with all or most of the Rep proteins from the three *Geminivirdae* genera. They were able to identify two aptamers, A22 and A64 that had strong interactions in different regions of the N-terminus of Rep. Lastly, they showed that transgenic tomato lines that expressed the two aptamers had delayed viral accumulation, lower viral DNA levels, and showed no, or very little symptoms of infection.(Reyes, Nash, Dallas, Trinidad Ascencio-Ibáñez, & Hanley-Bowdoin, n.d.)

## Aims

The aims of this project are to take 16 peptide aptamers and see if they are able to retain their binding and interference activity without the presence of the protein scaffold in *Arabidopsis thaliana.* We also wish to test the binding affinity of a truncated version of the A40 aptamer and a version of the A64 aptamer where the alanine at position 9 has been substituted for aspartic acid.

## Materials and Methods

### *Design of Aptamer Genes and Primers*

For the design of the aptamer genes, we utilized the codon bias for *Solanum lycopersicum* (tomato) so that follow-up studies in tomatos are able to use the materials and design of our aptamers. From the codon bias table, we were able to construct complete sequences of the genes

for each of our aptamers of study. We included restriction sites for the enzymes XhoI and BglII on the respective 5` and 3` ends of the nucleotide oligomer for use in molecular cloning. The complete oligomers ended up being approximately 85 nucleotides in length. This allowed us to design primers that each contained half of the aptamer sequence and had overlap on their 3` ends for amplification. Successful amplification of the aptamer was confirmed by gel electrophoresis.

Table 3.1: Sequence of Rep-binding peptide aptamers. Sequences marked with an * are mutant versions of the aptamers.

| Aptamer | Sequence |
|---|---|
| A22 | CRTRGCGCHLCRMLSQFTGG |
| A40 | LQYSWNLYSVASFKTRRVSS |
| A40Δ* | LQYSWNLYSVASF |
| A59 | AKDVERGAGGKIKACELCRL |
| A64 | TELWWADFCKMHMEGGKGMC |
| A64D9* | TELWWADFDKMHMEGGKGMC |
| A84 | GGRQTEPSLTLLADLTLLLS |
| A99 | RERGGDDYRRMMHPGAASGP |
| A116 | SCDEAFDAASVASELFCQPY |
| A127 | TWGLVCTGTGWGLLDTVVRA |
| A147 | GRGGCMLCDVDGSSAWLHTEGRLTGPITSQQCLSFQYLGNGEFIDG |
| A160 | GMSGRIPEPDDWVVLFITGC |
| A177 | TGCHYKGARCCRLTWDVLIL |

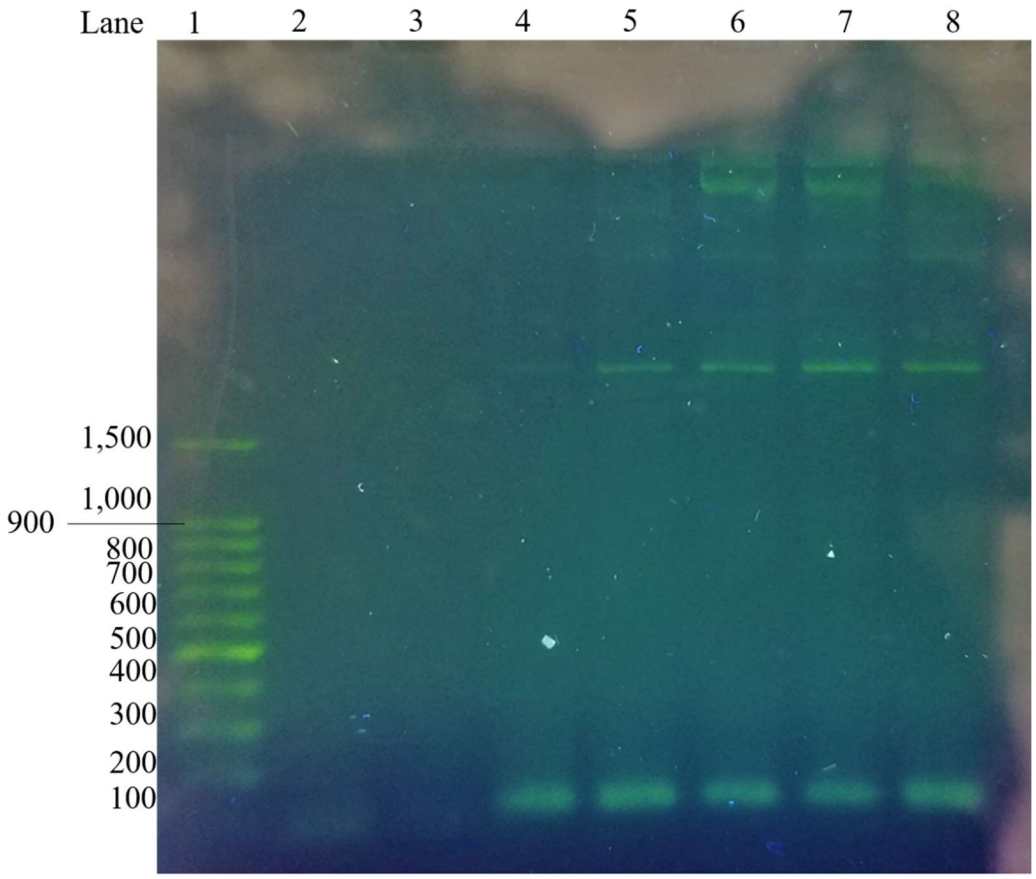


Figure 3.1: Amplification of cloned regions of the 007 plasmid extracted from transformed, replated DH5α colonies. Lane 1 contains a 100bp marker, lane 2 shows the A40 oligonucleotide sequence, lane 3 contains the In-Fusion primers as a control. The remaining lanes contain the A22 In-Fusion sequence (lane 4), A40 In-Fusion sequence (lane 5) A160 In-Fusion sequence (lanes 6 and 7) and the A177 In-Fusion sequence (lane 8).

Cloned regions were amplified using the In-Fusion primers. The control in lane three is the In-Fusion primers to ensure there is no amplification from the sets of primers alone. Confirmation of successful cloning was determined by Sanger sequencing

*VIGS Vector*

For our purposes, the VIGS vector 007 was chosen as the vector for cloning the aptamer genes. Whilst the common use of VIGS is to test silencing in plants, because of the presence of the viral genes, it was chosen here as a way to directly infect Arabidopsis with a version of the virus that codes for the peptide aptamer as part of its genome. This delivery mechanism allows us to quickly test for effectiveness of the aptamer without generating transgenic lines of Arabidopsis that contain a gene for the aptamer sequence.

*Cloning into VIGS vector*

We used In-Fusion cloning system from Takara Biosciences to clone our aptamer gene into the VIGS vector. In-Fusion cloning was used due to difficulties that were had in cloning of the aptamer sequence directly into the vector, likely due to the small size of the insert. The In-Fusion system is capable of cloning short synthetic oligonucleotide inserts of 50-150bp (Canali et al., 2014) and so was best suited for our purpose. The vector was linearized by digesting with the restriction enzymes XhoI and BglII before purifying using a spin columns from Qiagen. As per the user's manual we generated gene-specific primers with extensions homologous to the VIGS vector ends using the sequences around the restriction sites; the primer sequences were designed using SnapGene software. Again, proper amplification was confirmed using gel electrophoresis of the PCR reaction. The In-Fusion PCR product was then cleaned using spin column purification by Qiagen. The In-Fusion reaction was set up according to the specifications in the user's manual with appropriate controls, and the reaction was carried out under the manufacturer's guidelines. 5µl of the reaction mix was then used to transform *E.coli* DH5α competent cells by electroporation. Electroporation was carried out at 2.5kV, 200Ω with a time constant >4.1. Transformed DH5α cells were then screened by growing on agarose plates containing the antibiotic carbenicillin. Selected colonies were re-plated, then re-plated colonies

were harvested and screened for the vector. Once the presence of the vector had been determined, the vector was checked for presence of the insert. Due to the small size of the insert in comparison to the vector, we were unable to verify by double digestion using XhoI and BglII, as the amount of insert was too small to be visualized on a gel. Instead the vector was checked by amplification of the cloning site using the In-Fusion primers. Once possible successful clones had been identified, the presence of the insert was verified by Sanger sequencing.

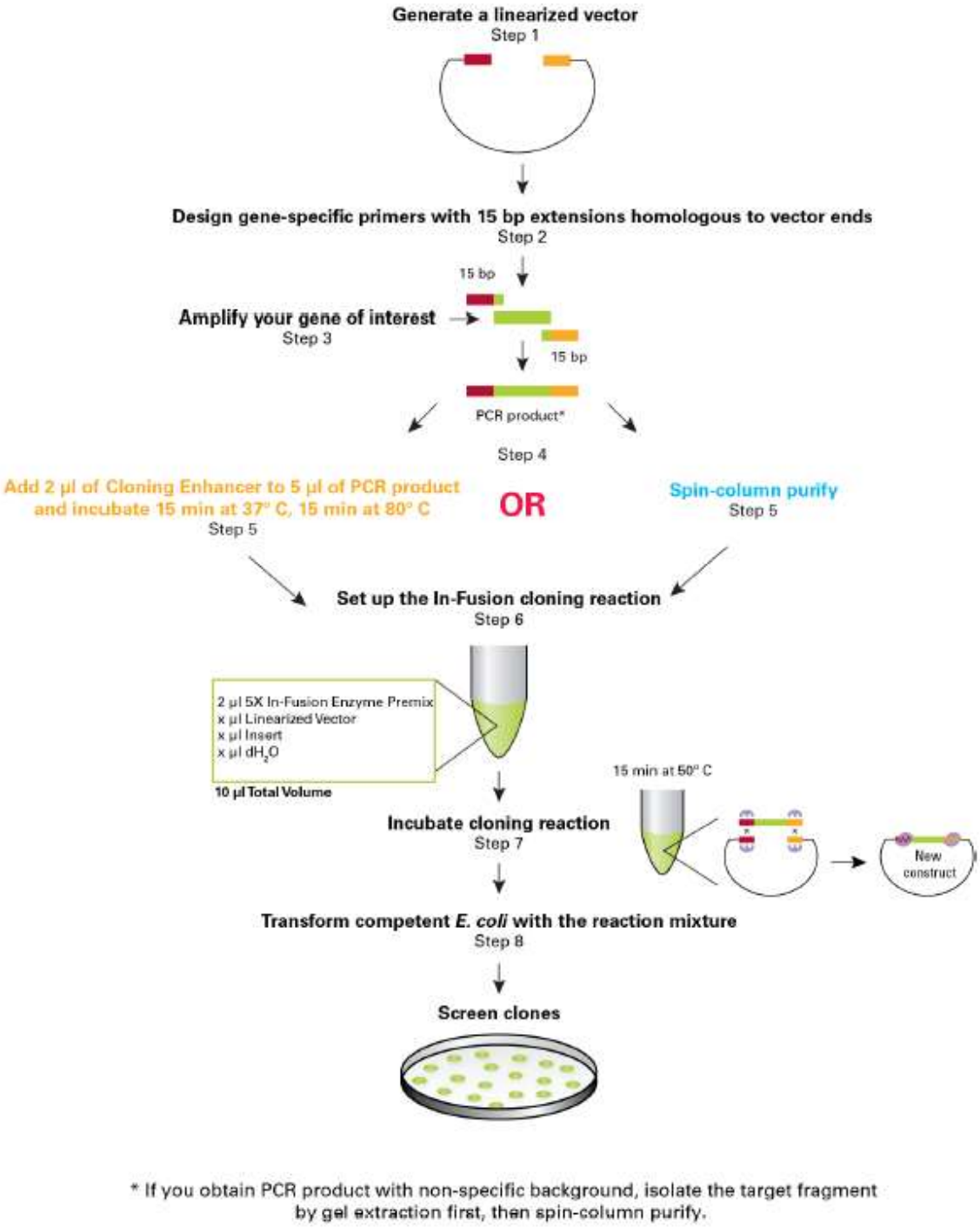


Figure 3.2: An overview of the In-Fusion cloning protocol taken from the user's manual(Canali et al., 2014). In step 4 we used a spin column to purify our secondary PCR product.

*Infecting Col-0 by biolistics*

The Col-0 ecotype of *Arabidopsis thaliana* was chosen for this experiment because it shows complete symptoms of geminivirus infection.

*Col-0 growth conditions*

Col-0 plants were grown on Sungro professional growth mix, seedlings were transplanted two weeks after germination onto single trays. After a further two weeks growth, plants were infected with transformed CaLCuV 007 and wild type B components.

*Particle Bombardment*

Particle bombardment was carried out using a Venganza Inc. gene gun. 5µl each of both transformed CaLCuV 007 and B components were added to a 50µl aliquot of gold particles at a concentration of 15µg/µl. 50µl CaCl2 (2.5M) and 20µl spermidine (0.1M) were added while agitating. The mixture was then vortexed for 5mins and then spun down at 10000rpm for 10 seconds and the supernatant discarded. The pellet was resuspended in 200 proof ethanol and spun down again. The supernatant was discarded and the pellet resuspended in 65µl of 200 proof ethanol. 10µl were then added to swinnex filter holders and used to bombard Col-0, with each mix yielding 6 bombardments.

*Symptom Monitoring*

Symptoms were visually observed over the course of infection and the plants that showed the most severe symptoms were chosen.

# Results


Figure 3.3: Plant response at 26dpi. Plants with the most severe symptoms were selected. (A) Arabidopsis infected with the 007+A22 aptamer, (B) A40 aptamer, (C) A177 aptamer. The infection of the 007 vector without any aptamer insert (D), wild-type virus (E) and mock infection (F).

All plants infected with the VIGS vector, either containing the aptamer sequence or not, and the wild type virus showed some degree of symptoms. At 21dpi symptoms, plants were at their most pronounced, and images were taken at this stage.

## Discussion

All of the cloned aptamer sequences showed some reduction in symptoms to a greater or lesser extent. The A22 aptamer (Figure 3.3.A) appears to have the most symptom reduction; plants showed few symptoms with only a small degree of leaf curling present in visibly infected plants. No yellowing of leaves was present at 21 days post-infection. This would appear to correspond to the data that was presented in the Reyes paper, which indicated that the A22 aptamer had the highest interference score that strongly interfered with the TGMV replication. The lack of significant symptoms on the A22 expressing VIGS vector could indicate that the aptamer is still able to bind and interfere with the Rep protein without the thioredoxin scaffold.

007+A40-infected plants (Figure 3.3.B) showed the most symptoms, possibly indicating the least amount of interference with the virus. However, it did show overall fewer and less severe symptoms than the 007 VIGS vector and that of the WT CaLCuV infected plants. There were still fairly visible symptoms such as leaf curling and yellowing. Again, this may be consistent with the data in the previous Reyes paper which gave A40 a low interference score. The plants infected with the 007+A177 (Figure 3.3.C) aptamer insert did show leaf curling but no yellowing after 21 days; no interference data is available for this aptamer.

In summary, the results obtained from this trial are promising. The A22 aptamer, which had been identified previously as binding strongly to the Rep of TGMV, and it showed symptom reduction whilst constrained within the thioredoxin scaffold. The results here are in line with what would be expected if the aptamer were to work outside of the scaffold. Further testing for the additional aptamers in table 3.1, specifically the A64 aptamer, also showed strong symptom reduction like A40. Additionally, quantification of viral DNA accumulation during infection

should be carried out in order to determine the amount of virus present. The previous study showed that while symptoms were reduced, viral accumulation was affected to a lesser extent. Testing with other plants, such as the creation of transgenic tomato lines that express aptamers that show symptom reduction in Arabidopsis screenings, using another bipartite geminivirus in the future to test in tomato TMV (Tomato mottle virus) would, long-term, be the ultimate goal of these tests.

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# Strigolactones

## Background

Strigolactones are a new, emerging class of plant hormone. They are part of the semiochemicals, a group of biologically active molecules that are used as signal messengers between individuals in a species (Zwanenburg, Pospíšil, & Ćavar Zeljković, 2016). They were first identified in 1966 in the root exudates of cotton as a germination stimulant for the weed *Striga.* The elucidation of the structure came in 1972 when the stereochemistry was determined using X-ray crystallography 10 years later (Brooks, Bevinakatti, & Powell, 1985). They were initially thought of as secondary metabolites of plants and were considered harmful (Pandey, Sharma, & Pandey, 2016). More than 20 strigolactone and strigolactone-like compounds have been identified so far.

### Strigolactone structure

Naturally occurring strigolactones fall into two groups depending on their structure: canonical and non-canonical. In canonical strigolactones, the butenolide (D) ring connects to the "core" ABC ring structure through an enol-ether bridge. Variation to the side groups on the ABC rings are then responsible for the different varieties of canonical strigolactones. Non-canonical strigolactones retain the D-ring, but it is connected to a variety of possible structures. There are a large number of branching structures that have been determined on the ABC rings. The determination of these structures from samples of root exudes has allowed for further insight into the biosynthetic pathways that assemble different strigolactones. Some of these inferred pathways have been confirmed by the treatment of plants with carotenoid inhibitors. Plants

treated with these have been shown to exude fewer strigolactones from the roots. From the treatment of plants with these inhibitors, a hypothesized biosynthetic pathway involving carotene-cleavage dioxygenases (CCDs), specifically CCD7 and CCD8, was proposed (Matusova, 2005). Moreover, the proposed pathway had CCD7 and CCD8 operating consecutively in the pathway. This pathway was later confirmed by subsequent studies (Booker et al., 2004; Snowden et al., 2005). The action of CCD7 and CCD8 in the biosynthetic pathway is discussed below.

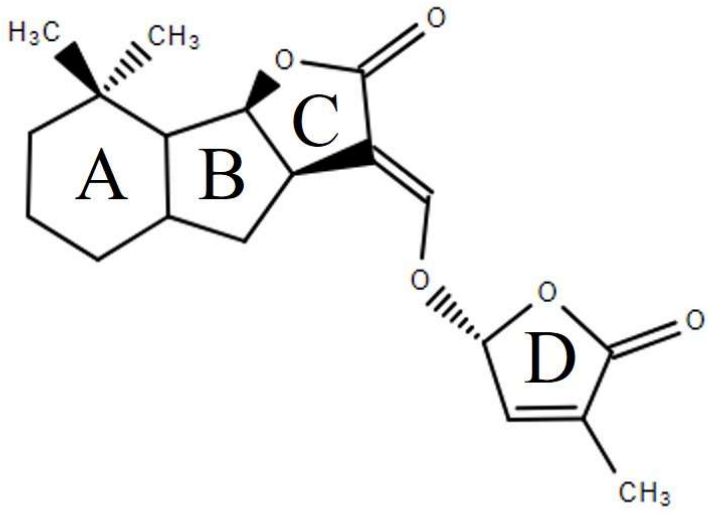


Figure 4.1: The basic "core" structure of a canonical strigolactone. Various side groups can be attached to the ABC ring structure, but all variants retain the same structural core.

**Biosynthesis and regulation**

The biosynthesis of strigolactones is derived from a carotenoid predecessor. Some of the enzymes and steps involved in the synthesis have been identified. All-*trans*-*β*-carotene is isomerized into 9`-*cis*-*β*-carotene by the chloroplastic enzyme Beta-carotene isomerase D27. This is then converted by CCD7(MAX3) into 9`-*cis*-*β*-apo-carotenal (and *β*-ionone). CCD8(MAX4) then converts the 9`-*cis*-*β*-apo-carotenal into carlactone (Prandi & Cardinale, 2014). In *Arabidopsis,* the CCD7 and CCD8 are encoded by the More Axillary Growth (MAX) MAX3 and MAX4 genes respectively. Further downstream in *Arabidopsis,* the MAX1 gene, encoding a cytochrome P450, converts the carlactone into carlactonic acid which is then methylated to make methyl carlactonate. This has been confirmed *in vitro* in a yeast microsomal system, and *in vivo* by LC-MS/MS analysis of *Arabidopsis* root extracts, additionally the addition of carlactonic acid and methyl carlactonate to a max1 mutant restored the wt *Arabidopsis* phenotype (Abe et al., 2014). Another protein that possibly acts downstream in the pathway, in *Arabidopsis*, is a lateral branching oxidoreductase (LBO). It is reported that LBO acts upon the methyl calactonate and carlactonic acid, converting them into further products (Brewer et al., 2016).

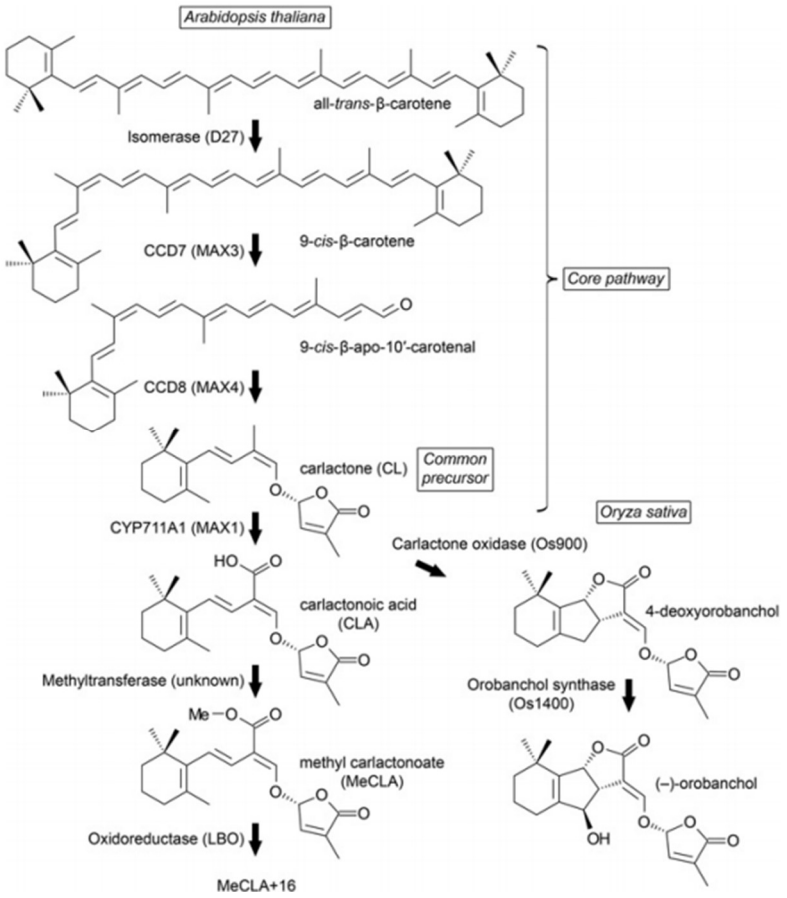


Figure 4.2: Overview of the strigolactone biosynthesis pathway in Arabidopsis (taken from Brewer, P. B. et al)(Brewer et al., 2016).

Control of the biosynthesis of strigolactones is regulated upstream by the control of transcription of the D27 enzyme by the transcription factors NSP1 and NSP2. These two transcription factors are GRAS-type transcription factors (W. Liu et al., 2011). CCD7 is regulated by auxin expression in pea, it is also likely that this is similar to *Arabidopsis* (Brewer, Koltai, & Beveridge, 2013). Increased auxin levels up-regulate expression of CCD7; auxin localization is controlled by the localization of PIN protein. In turn, PIN is negatively controlled by strigolactones. Strigolactone signaling depletes PIN1 from the plasma membrane of plant cells. The interaction between auxin and strigolactone signaling is thought to organize the shooting of buds in the meristem. Budding activation occurs when PIN1 insertion into the plasma membrane outstrips depletion, and budding inhibition occurs when strigolactone-mediated depletion outstrips PIN1 insertion (Hayward, Stirnberg, Beveridge, & Leyser, 2009; Shinohara, Taylor, & Leyser, 2013).

**Strigolactones in response to plant stresses**

Strigolactones have been observed to have several roles in plant responses to both biotic and abiotic stress. In *Arabidopsis,* studies show that strigolactones are involved in the drought stress response, alongside the plant hormone abscisic acid (ABA). ABA-deficient mutants showed reduced levels of ABA and strigolactones. Additionally, transcription levels of the CCD7 and CCD8 were reduced (López-Ráez et al., 2010). ABA and strigolactones have a great deal of crosstalk in plant response to environmental stresses (Saeed, Naseem, & Ali, 2017), and strigolactone-deficient mutants of *Arabidopsis* showed greater stress sensitivity at different levels of development. This hypersensitivity to stress was demonstrated for salinity and dehydration (Ha et al., 2014), but the exact role that strigolactones play is still in question, with both

subsequent studies showing conflicting (Bu et al., 2014) and supporting (J. Liu et al., 2015; Visentin et al., 2016) data in other species as to the nature of the interaction.

Nutrient deficiency in plants is often a result of low levels of phosphate or nitrogen (or both) in the soil and is a common source of abiotic stress. The uptake of these nutrients is achieved mainly through the root system. The response of plants to low levels of ground nutrients is a change in the structure of the root system. Strigolactones have been shown to have effects on the root architecture, and so it is unsurprising that low levels of phosphates in the soil trigger the production of strigolactones in the root. It was shown that the levels of the strigolactone orobanchol in red clover increased by 20 times, and the level of strigolactones increased in sorghum under low phosphate and nitrogen levels. This increase in strigolactones has also been observed in *Arabidopsis* when starved of phosphates (Mayzlish-Gati et al., 2012). This is important, as *Arabidopsis* is not host to arbuscular mycorrhiza (AM) fungi. Previous studies have been in AM-hosting plants, and AM fungi are recruited by red clover in response to low phosphate levels. This indicates that the role of strigolactones in nutrient deficiency for *Arabidopsis* is separate from the recruitment of AM fungi seen in other plants. Thus, it is hypothesized that this response is in altering root architecture in *Arabidopsis* (Saeed et al., 2017).

## Aims

As previously discussed, there is considerable interplay between strigolactones and ABA during stress responses. Moreover, strigolactones and auxin regulate each other's expression and transport in the meristem. During geminivirus infection, auxin levels in plant cells and shoot tips increase (Park et al., 2004). Due to previously characterized interactions between auxin and strigolactones in shoot branching (Hayward et al., 2009) and symptoms of geminivirus infection

including abnormal shoot and leaf development, we are interested if patterns of strigolactone signaling differ between uninfected and geminivirus-infected Arabidopsis.

## Materials and methods

*Growth of SL lines*

The transgenic *Arabidopsis* lines SLT3 21.1.1. and SLT3 24.5.3, containing a putative strigolactone sensitive promoter in front of a GUS reporter gene, were grown on Sungro professional growth mix and grown under eight hour light conditions. Seedlings were transplanted two weeks after germination into single pots. After a further two weeks, growth lines were infected with the VIGS vector CaLCuV 008 and wild type B components, wild type A and B components, or left uninfected as a control.

*Particle Bombardment*

Particle bombardment was carried out using a Venganza Inc. microdrop sprayer. 5µl each of both CaLCuV 008 and wild type B components, or wild-type A and B components were added to a 50µl aliquot of gold particles at a concentration of 15µg/µl. 50µl CaCl2 (2.5M) and 20µl spermidine (0.1M) were added while agitating. The mixture was then vortexed for 5 minutes and then spun down at 10,000rpm for 10 seconds and the supernatant discarded. The pellet was resuspended in 200 proof ethanol and spun down again; the supernatant was discarded and the pellet resuspended in 65µl of 200 proof ethanol. 10µl were then added to Swinnex filter holders and used to bombard the lines, with each mix yielding 6 bombardments.

*Tissue Harvesting and sectioning*

Two weeks post-infection, both leaf and apical meristem tissue samples were obtained. Tissue samples were submerged in a 1% Formaldehyde PBS (PBS-F) solution and then vacuum-infiltrated for 15 minutes. After vacuum-infiltration, the samples were set in blocks of 5% agarose in PBS.

100µm sections were then taken of the tissue using a Vibratome (Leica); thereafter, collected sections were stored in PBS buffer prior to staining.

*GUS-staining*

The B-glucuronidase (GUS) reporter was tagged to the DWARF14-LIKE2 (DLK2) gene. DLK2 is up-regulated by KARRIKIN INSENSITIVE2 (KAI2), and is structurally similar. KAI2 has been identified as being part of the strigolactone signaling pathway, and the tagging of reporter genes, luciferase (LUC) and GUS, to DLK2 has been previously used to detect responses to strigolactones (Sun, Flematti, Smith, & Waters, 2016; Végh et al., 2017).

Tissue samples were prefixed with cold 90% acetone for 1 hour at -20ºC. The samples were then washed twice with 100mM sodium phosphate buffer at pH 7.4. The GUS substrate was a solution of 0.5mg/ml of X-Gluc, 0.5mM potassium ferricyanide, and 0.5mM potassium ferrocyanide in a 100mM sodium phosphate buffer at pH 7.4. The samples were immersed in the substrate and incubated for 12 hours, whilst rocking, in the dark.

Stained samples were then mounted on microscope slides in 90% glucose in PBS, and slides were viewed under an E800 Nikon Eclipse microscope and images recorded.

# Results

The transgenic SLT 21.1.1 line showed a more pronounced GUS signal than the stains from the SLT 24.5.3 line. The GUS stain of the meristem of infected transgenic line SLT 21.1.1 indicated that strigolactone expression was found to be focused in the shoot apical meristem in Arabidopsis. The stain was focused around the developing leaf tissue and the base of trichomes; no stain was present in the root tissue at the meristem (see Figure 4.3 below). Additionally, no stain was found to be in the developed leaf tissue of the infected transgenic line SLT 21.1.1.

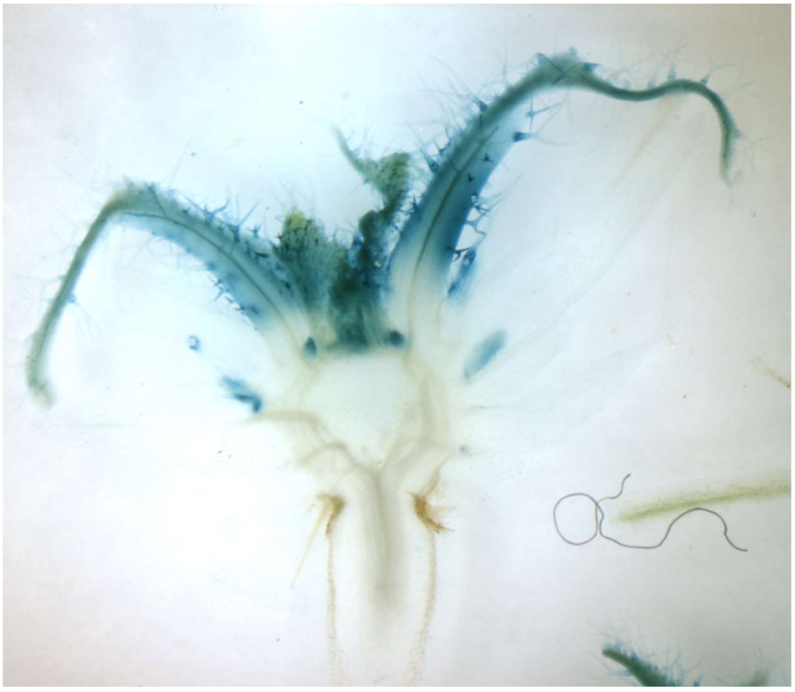

Figure 4.3: Section of meristem of infected transgenic line SLT 21.1.1. The GUS stain is focused around the developing stems and trichomes.

The stain of the transgenic line STL 24.5.3 showed a differing GUS reporting to SLT 21.1.1. The GUS stain indicated that the expression of DLK2 was limited to the developing

trichomes (see Figure 4.4) and not located elsewhere in the apical meristem. Leaf section stains did not reveal any GUS presence.

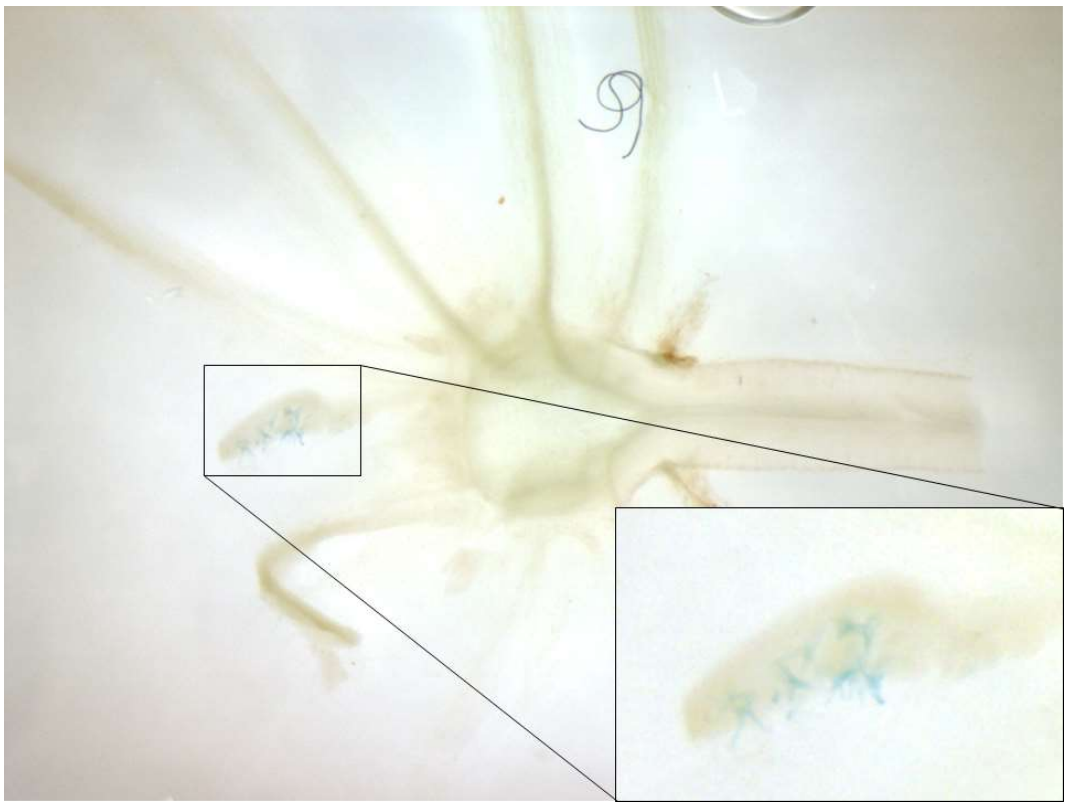

Figure 4.4: Section of the meristem of infected transgenic line SLT 24.5.3 with focus on the developing trichome in young leaf tissue. Strigolactone reporting is present only in the trichomes of the leaf.

Infected transgenic lines showed no GUS signal in the leaf tissue. More interestingly, the transgenic line SLT 24.5.3 showed no GUS signal in the meristem (Figure 4.5 B.-C.), like uninfected tissue. There was no GUS signal present either in the trichomes of SLT 24.5.3 in uninfected lines (Figure 5. B-D.)

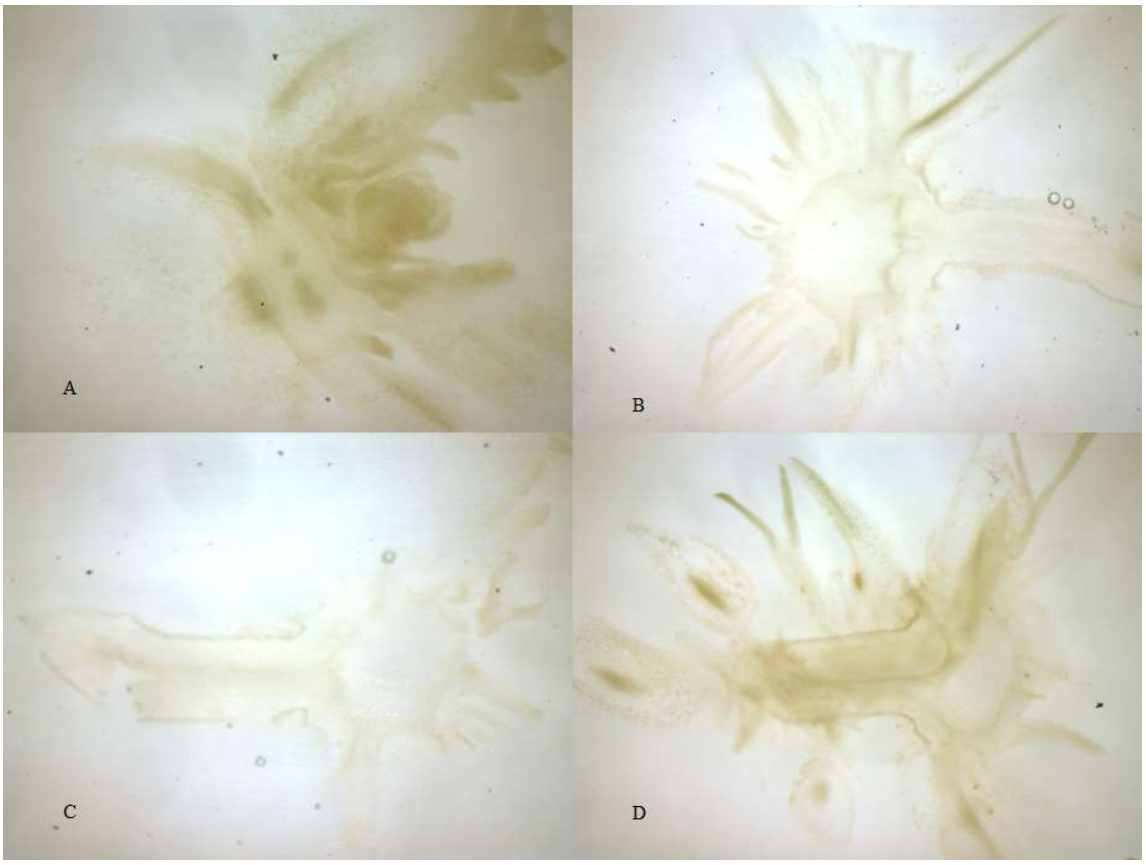


Figure 4.5: Sections of uninfected meristem tissue. A: SLT 21.1.1, B-D: SLT 24.5.3.

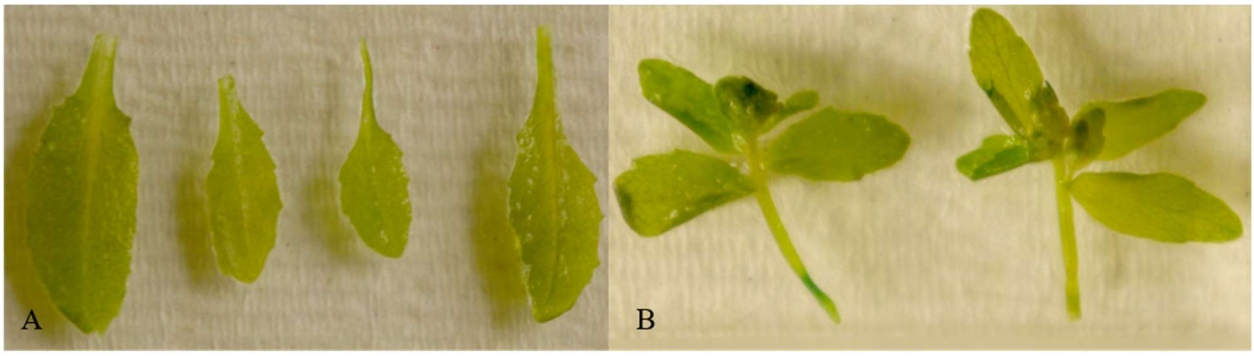


Figure 4.6: GUS stain of leaf (A), and floral tissue (B) from uninfected 24.5.3 line.

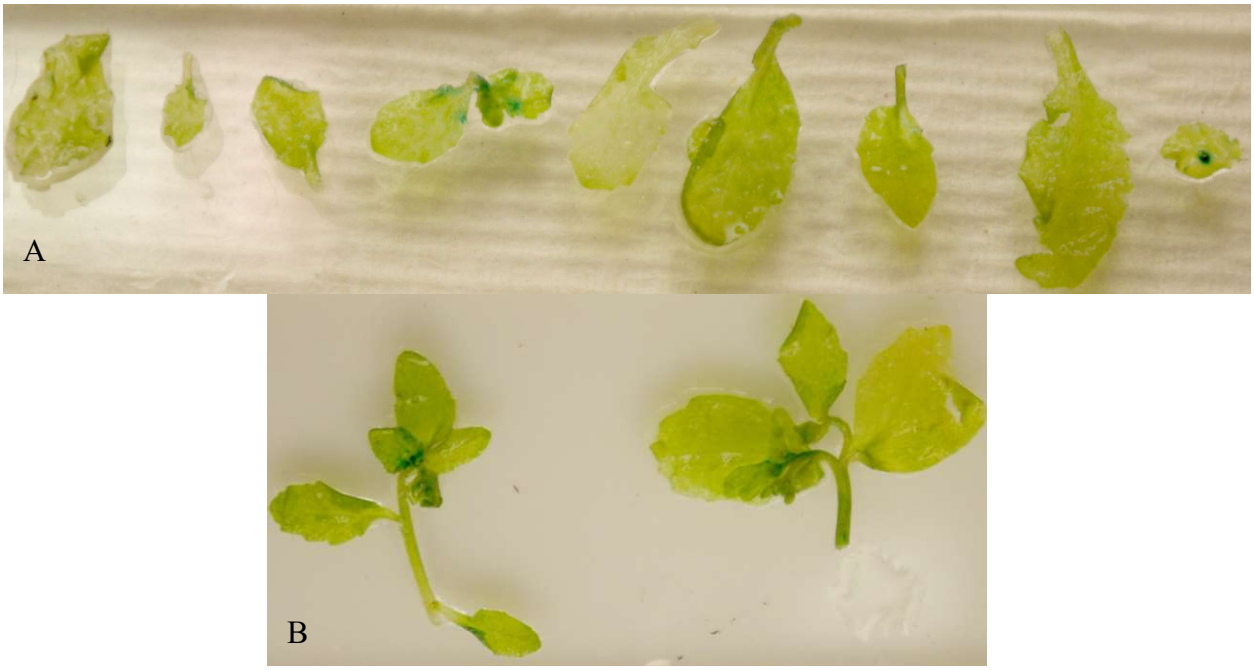


Figure 4.7: GUS stain of leaf (A), and floral tissue (B) from infected 24.5.3 line.

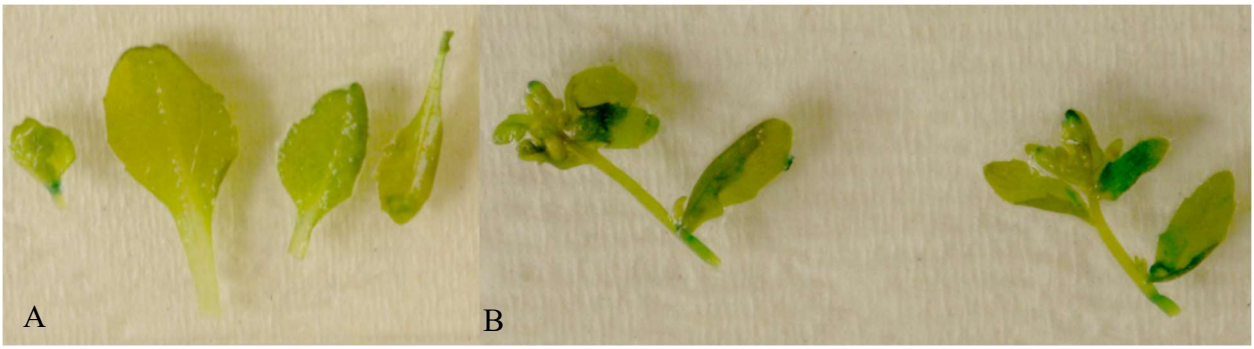


Figure 4.8: GUS stain of leaf (A), and floral tissue (B) from uninfected 21.1.1 lines.

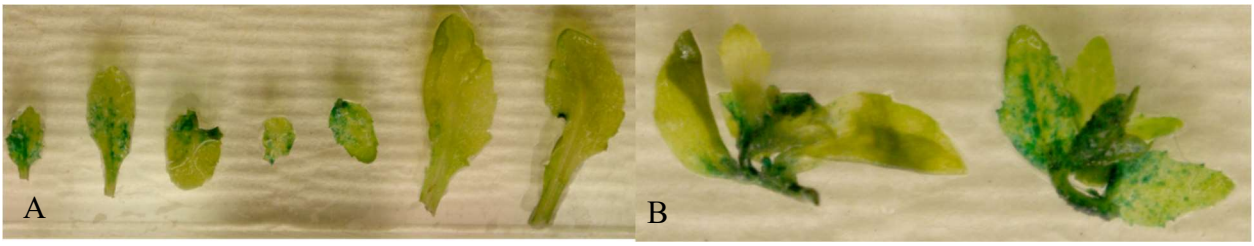


Figure 4.9: GUS stain of leaf (A), and floral tissue (B) from infected 21.1.1 lines.

Floral tissue showed GUS signal in uninfected lines. This is likely due to the role that strigolactones play in development of shooting buds (Figures 4.6.B and 4.8.B). The infected floral tissue shows a higher level of GUS signal (Figures 4.7.B and 4.9.B), possibly indicating increase in levels of strigolactones. Leaf tissue from uninfected lines (Figures 4.6.A and 4.8.A) showed either no, or minimal GUS signal, and the leaf tissue from infected lines (Figures 4.7.A and 4.9.A) showed a greater degree of GUS signal.

# Discussion

The observation of strigolactone up-regulation in infected plant tissue is likely due to the up-regulation of auxin in infected plants (Park et al., 2004). As detailed above, the synthesis of strigolactones from their precursors is reliant upon the expression of the MAX3 and MAX4 (see Figure 2 above). The expression of these two enzymes is up-regulated by auxin in an auxin-response factor 1 (AXR1)-dependent manner. The AXR1 inhibits the expression of auxin-responsive protein 2 (IAA2), which itself is an inhibitor of the expression of MAX3 and MAX4.

Microarray analysis of strigolactones have been found to affect the transcription of genes in Arabidopsis both by up-regulating 31 genes and down-regulating 33 others (Mashiguchi et al., 2009). Additionally, of the 33 genes down-regulated by strigolactones, 75% of them are up-regulated by auxin (see Supplemental Tables 6.1 and 6.2).

Additional testing using qPCR may elucidate whether the up-regulation of strigolactones observed in this testing translates to a change in the transcription of strigolactone-affected genes. There may be additional effects that the up-regulation of strigolactones in infected Arabidopsis have upon strigolactone-induced or repressed genes. It would not be unexpected that the increase or decrease in expression may not reflect strigolactone levels due to plant hormone cross-talk. Exploration of strigolactone-regulated pathways may reveal additional insights into how geminiviruses hijack the cells' biomechanical pathways in order to propagate the infection.

Another area of particular interest are genes that have been reported to be up-regulated by auxin but repressed by strigolactones (see Table 6.2 in supplemental material) due to the elevated levels of both of these hormones during infection by geminiviruses.

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# Ecotype project

## Background

Virus induced gene silencing (VIGS) is part of the plant defense mechanism against invading viruses. Both DNA and RNA virus sequences are recognized by the plant defenses, triggering silencing pathways that target viral mRNAs in the case of post-transcriptional gene silencing (PTGS) (Pantaleo 2011). This pathway is used in VIGS to induce silencing of host genes (Robertson 2004). By introducing a modified virus that contains a part of an endogenous gene's sequence, the plants PTGS pathway targets its own mRNA for that gene, and it is degraded. This means that VIGS provides a versatile method for the study of the function of plant genes, and importantly, for the study of genes in which silencing or mutation causes plant lethality (Robertson 2004).

The sequencing of the Arabidopsis genome, the development of online resources such as The Arabidopsis Information Resource (TAIR), and the fact that Arabidopsis has been used as a source for virus-resistant genes means that VIGS is a powerful tool for identifying genes that confer resistance. Arabidopsis has had thousands of different accessions collected from around the world, and the ability of Arabidopsis to self-pollinate means that the collected accessions can be used to create inbred lines. These lines can then be used to study the natural variation between different lines to determine any naturally occurring resistances. Thus, it is important to identify Arabidopsis accessions that could be used as possible VIGS targets. Using a visible phenotype produced by silencing allows us to test possible accessions for the suitability of becoming VIGS hosts.

A 2017 study screened many different Arabidopsis (Reyes et al. 2017) accessions using a 008 VIGS construct containing a fragment of the CH-42 gene. The CH-42 gene encodes magnesium chelatase subunit I, a subunit of magnesium chelatase which is an enzyme that catalyzes one of the first steps in the synthesis of chlorophyll (Beale 1999). The silencing of this gene causes the plant to develop yellow-white areas on leaves due to chlorophyll loss.

The study scored the extent of both symptoms and silencing and then placed each infected accession into four categorie: Class A, significant symptoms and silencing, and those with a weak VIGS response, Class B, low symptoms and high silencing – a class which is a good candidate for being a VIGS host, Class C, low silencing, but severe symptoms, and Class D, low symptoms and low silencing (Reyes et al. 2017). Of the 166 accessions that were tested, 144 fell into Class A, Class B had 10 accessions, Class C had three accessions, and Class D had 6 accessions.

## Aims

Here we add to that work by screening more of the accessions, helping to add to a library of responses by testing the responses to infection with the 008 VIGS vector containing a fragment of the CH-42 gene. For consistency, we used the same setup and conditions as the Reyes study and will be using the same classification of symptoms and silencing.

## Methods

We screened the Arabidopsis accessions CS1266, CS1308, CS1516, CS22570, and CS28306. The seeds were stratified by storing at 4°C for 24 hours before being planted on SUNGRO professional growing mix. Seedlings were grown for two weeks with an eight hour

light cycle before being transplanted into individual pots. Accessions were then left to grow for a further two weeks before being infected with the VIGS vector using particle bombardment.

Mature Arabidopsis plants were infected with the VIGS vector, 2.5µg each of 008 VIGS vector and the B component of wild-type CaLCuV, were added to 50µl gold particles. This was followed by 50µl of 2.5M $CaCl_2$ then 20µl of 0.1M spermidine, coating the gold with the DNA. The gold particles were then washed twice with 200 proof ethanol before being resuspended in 65µl 200 proof ethanol. Each mix can be used for 6 bombardments using 10µl to load into a swinnex filter holder and plants bombarded at 40-50 psi. Infected plants were observed throughout the course of infection, and after 4-5 weeks post-infection, symptoms were recorded.

## Results

Responses to the VIGS vector were diverse; silencing was observed on the Arabidopsis line CS28306. This line showed silencing along with some symptoms. However, symptoms were not as pronounced as those in Col-0 lines.

Interestingly, the Arabidopsis line CS22970 showed reduced symptoms or lack of symptoms. Furthermore, infected plants with reduced symptoms showed signs of recovery after 3 weeks.


Figure 5.1: Silencing response in Col-0 (A) and accession CS28306 (B). Whilst both have a clear silencing response as seen in the leaf yellowing the CS28306, symptoms are not as severe as those in Col-0. Leaves in CS28306 are curled, but not to the same extent as Col-0, and neither do they have the mottling and discoloration present in Col-0. CS28306 is either in Class B or A.


Figure 5.2: Arabidopsis line CS22570 at (B) 28dpi and (C) 35dpi from two different repeats of the experiment. (A) is a mock-infected control, while C does show some slight symptoms. B is noticeably symptom free; this accession is part of Class D.

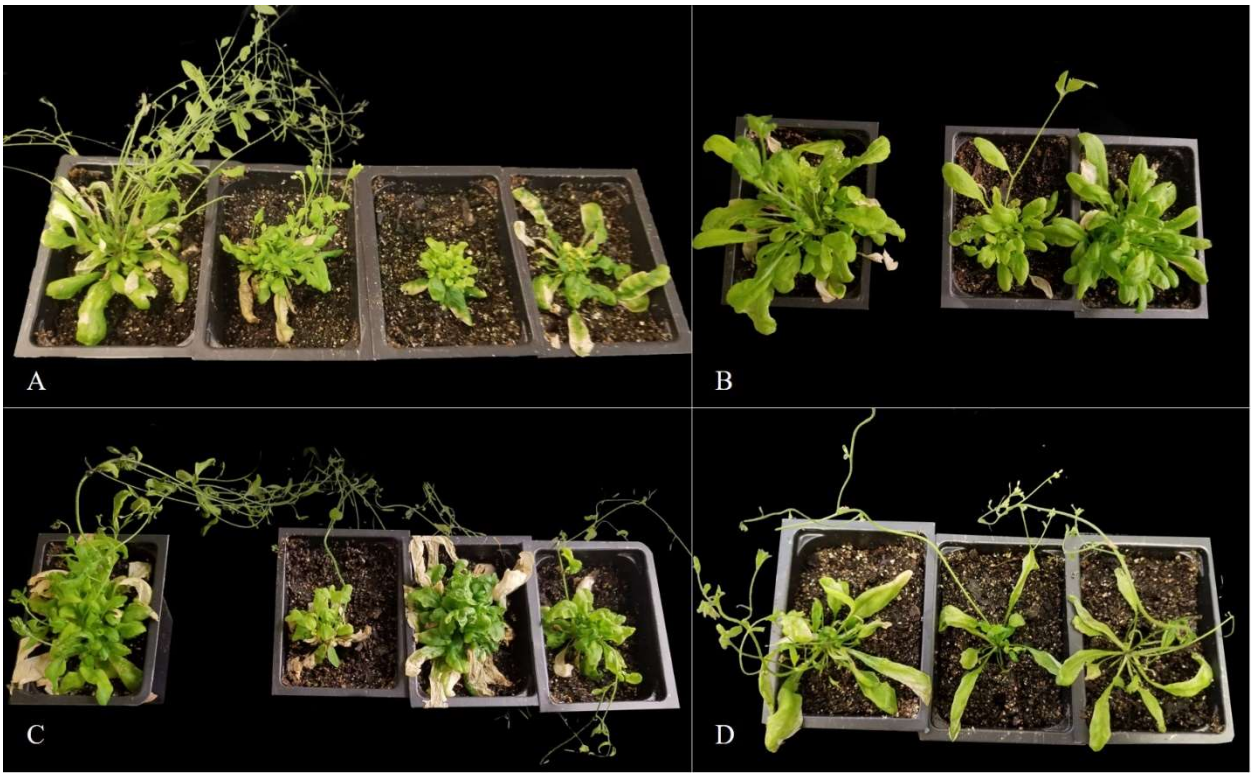


Figure 5.3: Arabidopsis accession lines CS1266 (A), CS1516 (B), CS1308 (C), and CS28386 (D) at 25dpi. The uninfected controls are the leftmost plants on each image. These accessions are classified as Class A.

Most of the accessions are in the Class A category. Four of the six tested lines fall into this classification (Figure 5.3), showing a mix of weak VIGS responses or significant symptoms.

## Discussion

This project is a continuation of previous work to identify Arabidopsis accessions which could be suitable as VIGS hosts in order to create a library of accessions that can be used in viral studies. The key phenotypic responses desired are chlorosis of leaves, which would indicate a strong VIGS response and low symptoms.

The majority of the Arabidopsis that have been tested in this screening (Figure 5.3) fall into the Class A category. This is not unexpected, as most Arabidopsis that have been tested have fallen into this category.

The accession CS28306 has shown both symptoms and silencing, and it appears to fall into Class B, the class suitable for use in VIGS-based trials. However, the observed symptoms of leaf curling may place it in Class A. It is possible that the CS28306 may be difficult to confidently categorize in either class without further testing.

What may be of particular note is the CS22570 line did not show any symptoms nor any silencing. The Pla-1 ecotype tested in the previous study was also asymptomatic and had no signs of silencing. It will be worth carrying out further tests on viral DNA accumulation, possibly using a qPCR-based system on leaves infected with CaLuV. Additional research could also include testing for susceptibility to other geminiviruses such as *Curtoviruses* or more severe strains of *Begomoviruses*. If the CS22570 does prove to be immune to DNA viruses, then a test with an RNA virus should be carried out.

In summary, these initial results have indicated one accession that can possibly be used for VIGS-based study and a possible accession that shows resistance to the virus. Both of these Arabidopsis lines show interesting phenotypic responses that warrant further study.

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# Supplemental Material

Table 6.1: List of genes induced by strigolactones, adapted from (Mashiguchi et al., 2009).

Strigolactone-Induced Genes

| AGI | Description |
|---|---|
| AT1G64380 | AP2 domain-containing transcription factor |
| AT1G31350 | F-box family protein |
| AT2G40670 | ARR16 (response regulator 16) |
| AT3G60290 | oxidoreductase |
| AT2G34140 | Dof-type zinc finger domain-containing protein |
| AT4G30350 | heat shock protein-related |
| AT4G37240 | similar to unknown protein (AT2G23690) |
| AT3G16330 | similar to unknown protein (AT1G52140) |
| AT5G64620 | C/VIF2 (CELL WALL / VACUOLAR INHIBITOR OF FRUCTOSIDASE 2 |
| AT1G65390 | ATPP2-A5; carbohydrate binding |
| AT5G02890 | transferase family protein |
| AT5G45340 | CYP707A3 |
| AT1G65390 | ATPP2-A5; carbohydrate binding |
| AT5G53420 | similar to unknown protein (AT4G27900) |
| AT2G36590 | ProT3 (PROLINE TRANSPORTER 3) |
| AT4G39070 | zinc finger (B-box type) family protein |
| AT4G14400 | ACD6 (ACCELERATED CELL DEATH 6) |
| AT3G16360 | AHP4 (HPT PHOSPHOTRANSMITTER 4) |
| AT5G64550 | loricrin-related |
| AT1G18710 | AtMYB47 |
| AT5G57710 | heat shock protein |
| AT5G25160 | ZFP3 (ZINC FINGER PROTEIN 3) |
| AT1G29160 | Dof-type zinc finger domain-containing protein |
| AT1G22810 | AP2 domain-containing transcription factor |
| AT2G29970 | heat shock protein |
| AT5G39610 | AtNAC6 (AtNAC2) |
| AT1G30040 | AtGA2OX2 (gibberellin 2-beta-dioxygenase) |
| AT5G06530 | ABC transporter family protein |
| AT4G18170 | WRKY28 (WRKY DNA-binding protein 28) |
| AT3G50650 | scarecrow-like transcription factor 7 (SCL7) |
| AT5G07580 | AP2 domain-containing transcription factor |

Table 6.2: List of strigolactone-repressed genes, adapted from (Mashiguchi et al., 2009).

Strigolactone-Repressed Genes

| AGI | Description | Auxin-Inducable? |
|---|---|---|
| AT3G03830 | auxin-responsive protein, putative | Yes |
| AT1G29510 | SAUR68 (SMALL AUXIN UPREGULATED 68) | Yes |
| AT1G29490 | auxin-responsive family protein | Yes |
| AT1G29440 | auxin-responsive family protein | Yes |
| AT1G29460 | auxin-responsive protein, putative | Yes |
| AT2G37030 | auxin-responsive family protein | Yes |
| AT3G03840 | auxin-responsive protein, putative | Yes |
| AT1G29500 | auxin-responsive protein, putative | Yes |
| AT1G15580 | IAA5 | Yes |
| AT5G18060 | auxin-responsive protein, putative | Yes |
| AT3G50340 | unknown protein | Yes |
| AT1G29430 | auxin-responsive family protein | Yes |
| AT2G22810 | ACS4 (1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 4) | Yes |
| AT4G38850 | SAUR_AC1 | Yes |
| AT1G29450 | auxin-responsive protein, putative | Yes |
| AT2G18010 | auxin-responsive family protein | Yes |
| AT4G14560 | IAA1 | Yes |
| AT5G51810 | AT2353/ATGA20OX2/GA20OX2 (GIBBERELLIN 20 OXIDASE 2) | Yes |
| AT1G47540 | trypsin inhibitor, putative | No |
| AT3G28740 | cytochrome P450 | Yes |
| AT3G42800 | unknown protein | Yes |
| AT1G53870 | unknown protein | No |
| AT5G12050 | unnamed protein product | Yes |
| AT4G38840 | auxin-responsive protein, putative | Yes |
| AT5G05290 | ATEXPA2 (EXPANSIN A2) | No |
| AT1G52830 | IAA6 | Yes |
| AT5G16980 | NADP-dependent oxidoreductase, putative | No |
| AT3G56710 | SIB1 (SIGMA FACTOR BINDING PROTEIN 1) | No |
| AT4G28520 | CRU3 (CRUCIFERIN 3) | Yes |
| AT4G38860 | auxin-responsive protein, putative | Yes |
| AT2G32100 | ATOFP16/OFP16 (ovate family protein 16) | No |
| AT4G27654 | unknown protein | No |
| AT5G50915 | bHLH family protein | No |

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