TITLE: **Using Virus-Induced Gene Silencing and Gene Expression as Tools to Understand Geminivirus Infection in Cotton Plants**

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ABBREVIATIONS: *ChlI*, magnesium chelatase subunit I gene; CLCrV, *Cotton Leaf Crumple Virus*; dpi, days post-inoculation; GFP, green fluorescent protein; siRNA, small interfering RNA; VIGS, virus-induced gene silencing

Using Virus-Induced Gene Silencing and Gene Expression as Tools to Understand Geminivirus Infection in Cotton Plants

ABSTRACT

The pattern of infection of *Cotton leaf crumple virus* (CLCrV) carrying a host-gene-silencing-sequence or a green fluorescent protein (GFP) gene were compared in *Gossypium hirsutum* cv. Deltapine 5415. The host-gene-silencing-insert was derived from the cotton *ChlI* gene, required for chlorophyll stability. Although GFP fluorescence could be seen after 2-3 days in cotton plants inoculated by microprojectile bombardment, host gene silencing was not apparent until 11- 15 days. Host gene silencing was extensive in new growth and virtually every cell in a leaf could be seen to be actively silencing as there was no chlorophyll or only small areas with chlorophyll. In contrast, GFP fluorescence was confined to sections of vascular tissue in new growth and fluorescence lasted for only a few days. At late stages of plant growth, after some of the cotton bolls had matured, GFP fluorescence was restricted to the top part of the plant, in leaves less than 20% of full length. Host gene silencing in the ovary walls was variable and it was not possible to determine if silencing signals penetrated into ovule seed coats because they lack chlorophyll. However, GFP fluorescence could be seen in early-stage fiber cell development but only in one part of an ovule and only in 0-2 ovules per plant. Manipulation of environmental and inoculation conditions might result in increased host gene silencing in later stages of cotton development. The use of both GFP and a host-gene-silencing-insert for monitoring CLCrV interactions with cotton as the host plant provided interesting insights into the dynamics of geminivirus disease.

KEY WORDS:

VIGS, cotton, gene silencing, geminivirus, *Cotton Leaf Crumple Virus*, GFP

Members of the genus *Begomovirus* in the family *Geminiviridae* are one of the first groups of viruses to be used as vectors to study gene expression (Frischmuth, Frischmuth et al., 1993) and for gene silencing (Kjemtrup, Sampson et al. 1998; Robertson 2004). Geminiviruses are circular, single stranded DNA viruses that replicate through double stranded intermediates in plant nuclei (Gutierrez, 2000; Hanley-Bowdoin, Settlage et al., 2004). They encode approximately six to eight proteins, depending on the type of genome arrangement, required for transcription, replication, movement, and encapsidation. Replication and transcription require host factors including DNA polymerase, at least some cell cycle proteins for replication, RNA polymerase II, and polyadenylation machinery for transcription (Hanley-Bowdoin, Settlage et al., 2004). They may interfere with plant DNA methylation to escape host gene silencing (Wang, Buckley et al., 2005), and they encode proteins that have anti-silencing activity as measured by restoration of GFP fluorescence (Voinnet, Pinto et al., 1999; Bisaro, 2006).

Begomoviruses are considered one of the most successful groups among plant viruses in that they are diverse and infect a wide range of plant hosts (Brown and Bird, 1992; Bass, Nagar et al., 2000). Species in the genus *Begomovirus* are transmitted exclusively in nature by members (variants) of the polyphagous whitefly *Bemisia tabaci* (Gennadius) complex, which colonize herbaceous eudicots (Brown, 2007).

Cotton leaf crumple virus (CLCrV) is the only begomovirus of cotton known to occur in the New World. CLCrV causes a widespread disease of cotton in Arizona, California, Texas, and Sonora, Mexico (Brown and Nelson, 1984; Brown, Mihail et al., 1987; Brown and Nelson, 1987). The genome arrangement of CLCrV is typical of a bipartite begomovirus (Idris and Brown, 2004). The bipartite genome of CLCrV was cloned and shown to be infectious when biolistically inoculated to its natural hosts, cotton and bean (Idris and Brown, 2004).

Several begomoviruses have been used as silencing vectors including *Tomato golden mosaic virus* (Kjemtrup, Sampson et al., 1998), (Peele, Jordan et al., 2001), *Cabbage leaf curl virus* (Turnage, Muangsan et al., 2002), and *African cassava mosaic virus* (Fofana, Sangare et al., 2004). Gene silencing is a phenomenon that was first noticed in transgenic Petunia plants (Jorgensen, 1990). With hindsight, we now know that the silenced genes in Petunia were down-regulated by a very precise defense response designed to protect plants from foreign DNA (Xie, Johansen et al., 2004). Double stranded RNA (dsRNA), which is required to activate the defense response, does not occur naturally, with the exception of a second regulatory system in plants that also down-regulates gene expression (Silhavy, Molnar et al., 2002). Viruses are thought to produce dsRNA transiently, during replication or as a result of very high rates of transcription (Wang, Bian et al., 2004). This dsRNA is recognized by specific RNAses called dicers, which cleave the viral RNA into 21 – 24 nt pieces (small RNAs or siRNAs) (Bernstein, Caudy et al., 2001). Dicers deliver the siRNAs to a second protein complex that includes Argonaute (*Ago1*) in Arabidopsis (Xie, Johansen et al., 2004).

A key part of the virus-induced gene silencing (VIGS) defense is to send warning signals to adjacent cells that might be infected by the virus (Baulcombe, 2004). This is done by the plant upon geminivirus infection with the aid of an RNA-dependent RNA polymerase, the *RDR6* gene in Arabidopsis (Muangsan, Beclin et al., 2004). As VIGS proceeds, the infected cell silencing machinery replicates viral siRNA sequences, and an unknown process moves them to adjacent cells. The movement process may also move viral DNA genomes, or a separate cell-to-cell and long distance system may be used. Both viral nucleotides and siRNAs are found in systemically infected tissue. The first group of infected cells is “naive” because it has not seen viral nucleotides before. Systemically infected cells may or may not be naive, depending upon

whether a diffusible siRNA from the host defense system was able to get there before the virus. Both the virus and the siRNA defense molecules travel from source to sink through the phloem (Tournier, Tabler et al., 2006).

It is not known just how effective host-mediated silencing is as a defense system. For example, viruses do not infect all cells of a plant, or even all cells of the vascular tissue of source tissue. Is this because mobile viral nucleic acids are limited in number, or is it because viral nucleic acids are degraded in the systemically infected cells, due to a siRNA-primed defense system? This question is complicated by the existence of anti-silencing proteins in viruses (Dunoyer, Lecellier et al., 2004). Some of the antisilencing proteins bind siRNAs and others interfere with the action of Argonaute (Chapman, Prokhnevsky et al., 2004). Not all antisilencing proteins have been characterized or identified. These questions are important not only for understanding the underlying basis of viral disease, but also for using VIGS as a tool to determine host gene function (Dunoyer, Lecellier et al., 2004).

Because VIGS is thought to result from the host plant defending itself against viruses (Waterhouse, Wang et al., 2001), we reasoned that comparing VIGS of a host gene and expression of *GFP* from the same virus would provide information about the dynamics involved in geminivirus disease, with VIGS representing the “host defense response” and GFP, the “viral attack”. Here, we briefly describe the development of CLCrV into a vector capable of carrying and transcribing up to 800 nt of foreign DNA throughout tissues of the cotton plant (a more complete description will be published shortly). The pattern of host gene silencing is contrasted with the pattern of *GFP* expression to understand more about the virus-host interactions for CLCrV and to begin to understand the efficacy of CLCrV for use as a silencing vector.

METHODS

Constructs. The CLCrV genome is bipartite and was previously cloned and sequenced by Idris and Brown (Idris and Brown 2004). The CLCrVA component was re-cloned to contain a multiple cloning site that replaced the coat protein gene but retained the coat protein gene promoter and polyadenylation sequences (Fig. 1, Tuttle et al., in prep). Two clones were then made using the A component with the coat protein promoter driving expression of either a red-shifted *GFP* gene or the cotton *ChlI* gene (Tuttle et al., in prep).

Bombardment. Microprojectile bombardment was done using standard procedures. DNA from plasmids encoding the A and B component were mixed and then precipitated. The equivalent of 5 µg DNA of each plasmid was used so that there was 10 µg total for each precipitation and each plant received approximately 1µg. In some experiments the amount of DNA was doubled. Seedlings of *Gossypium hirsutum* cv. Deltapine 5415 were bombarded approximately 1 week after germination, when cotyledons were fully extended and the first two leaves were less than 1 cm in length. Plants were grown at 25/23°C with approximately 1000 µEinsteins light for 16 hrs/day.

Assessment. Photographs were taken at various stages post bombardment with a Nikon digital camera or a Leica dissecting microscope and a Qimaging Micropublisher CCD camera. Observations were made and recorded on a semi-weekly basis.

RESULTS

GFP expression. GFP gene expression was detected in one case as early as 2 days post-inoculation (dpi), but this was only in one cell in one infected plant. However, by 4 dpi, GFP expression was detected at numerous sites throughout some of the bombarded leaves. Usually only one or two leaves per plant had significant numbers of GFP-expressing cells. At first, GFP

appeared to be expressed randomly throughout the mesophyll with no preference for vascular tissue. At high magnifications of the dissecting scope, gold particles were visible on the epidermis, confirming that GFP expression was associated with viral DNA inoculation.

Autofluorescence was a significant problem in cotton leaves, and it increased with plant age. Random and specific patterns of autofluorescence prevented an accurate assessment of GFP-positive cells using the excitation and blocking filter set used for the red-shifted GFP. To get around this problem, a second filter was used. This filter blocked all light that had wavelengths longer than that needed to excite YFP (yellow fluorescent protein). Autofluorescence was seen with both filter sets but only GFP fluorescence was seen with the first set of filters. Photodocumentation of cotton leaves under both filter sets followed by overlays in Adobe Photoshop permitted unambiguous detection of red-shifted GFP expression in cotton. Mock-inoculated leaves used as a control showed the same pattern of fluorescence when the second filter set was used.

The second round of infected cells was deduced to be immediately after systemic infection occurred for the following reasons. Geminivirus DNA can move in plants without the coat protein and shows a strong tropism for phloem as shown by in situ hybridization of wild type virus and replacement of the coat protein gene with GFP (Sudarshana, Wang et al., 1998; Morra and Petty, 2000). Unencapsidated DNA requires two genes to move out of cells, a nuclear shuttle protein and a plasma membrane associated protein (Sanderfoot, Ingham et al., 1996; Ward, Medville et al., 1997). The source tissue is actively exporting carbohydrates into phloem tissues and hence the driving force is toward phloem. Whiteflies normally feed on plants by inserting their stylus into the phloem and may deposit viral particles into sieve tubes. Because the virus has a tropism for phloem and can be seen in new growth after 12 – 15 days, it is logical that

at least most viral molecules complete the first round of DNA replication, enter phloem and travel in a pattern of source to sink. In bombarded cotton, the inoculated cotyledons are theoretically acting as a source and rapidly expanding upper leaves as a sink (Yoshimoto, Inoue et al., 2003).

Surprisingly, GFP expression was confined to vascular tissues in systemically infected tissues. Bundle sheath cells, xylem and phloem parenchyma and other nucleated cells have been shown to contain viral DNA or viral particles (Esau, 1933; Morra and Petty, 2000). Similar patterns of GFP expression were reported by Bob Gilbertson's group when bean dwarf mosaic geminivirus (BDMV) was used to carry the GFP gene in bean (Sudarshana, Wang et al., 1998). Although many geminiviruses are phloem-limited (confined to vascular tissue), the two other geminivirus silencing vectors we made, from tomato golden mosaic virus and cabbage leaf curl virus, were not (Peele, Jordan et al., 2001; Turnage, Muangsan et al., 2002).

GFP expression was detected only in portions of leaf tissues; not a whole section of the cotton leaf or an entire midvein. Small, irregular areas of GFP fluorescence were observed at various places in different leaves including the basal regions, midveins, and outer edges of leaf vasculature (Fig. 2). GFP expression during the first two months of growth at relatively low temperatures (25°C) could be found in fully expanded leaves. Curiously, at 60 – 70 dpi, the expression of GFP was restricted to young leaves that were less than 20% as long as lower leaves. No GFP expression was found elsewhere in the plant.

***ChlI* silencing.** Silencing of *ChlI* became visible as loss of chlorophyll at approximately 17 – 21 dpi. The extent of silencing varied somewhat from plant to plant and from experiment to experiment. Patterns of silencing ranged from almost the entire leaf to isolated or connected sectors and areas with diffuse borders to large areas of vein-associated speckles. Fig. 3 shows

some examples of the different patterns of silencing. As time progressed, leaves had a lower probability of showing extensive silencing. Flowering began at approximately 60 dpi while mock-inoculated plants showed flowering about 10 days earlier. The ovary wall is normally green but after VIGS often showed sectors of yellow due to silencing.

The *ChlI* silencing marker was not effective for detecting silencing within the boll due to the lack of chlorophyll. Ovaries could be examined for GFP fluorescence, however, and if the virus is there, silencing should be possible. In fact, there were a few ovules that showed GFP fluorescence in their outer seed coat. In one ovule, fluorescence was particularly bright and could have been caused by viral infection in two or more adjacent or nearby cells. Because slicing of ovaries was destructive, we were not able to continue observations of GFP fluorescence over time. It is possible that more ovules were infected but the expression was not stable and the time of sampling may not have been coincident with expression. At the time of GFP fluorescence, fibers were just beginning to elongate. We can conclude from these experiments that viral DNA is present in the seed coat and that GFP expression occurs early in fiber cell elongation. The use of more sensitive silencing markers, such as the *INO* gene required for development of the outer wall of the integument (Villanueva, Broadhvest et al. 1999), will be informative for determining the stage in fiber development we can expect VIGS-mediated silencing to begin.

DISCUSSION

We used two modifications to the CLCrV genome to track its progress in the cotton plant. One modification used the GFP gene to identify cells containing actively replicating and infectious viral DNA. Because only the double stranded DNA form of the virus is capable of being transcribed into mRNA and the GFP protein does not move from cell to cell, GFP expression could be correlated with active virus. It should be pointed out that the actively

transcribing viral DNA is also the target of the siRNAs and therefore net GFP fluorescence observed was determined by both viral gene transcription and host GFP silencing by siRNAs. Both the duration of GFP expression as well as the pattern of expression could be influenced by host gene silencing. The second modification of the CLCrV genome was to insert a *ChlI* fragment that could be used to measure the activity of the host gene silencing defense response to the virus. While GFP expression was cell autonomous (only cells containing actively replicating DNA expressed the GFP protein), *ChlI* silencing showed extensive cell-to-cell spread. By inoculating plants at the same time, under the same conditions, comparisons between GFP expression in one plant could be made with *ChlI* silencing in a second plant. Five plants were used for each treatment, including empty vector and mock-inoculated plants, and the experiment was repeated twice with similar results.

Viral gene expression (GFP) was seen a few days after inoculation by microprojectile bombardment while gene silencing (loss of chlorophyll) required over two weeks to detect (17 – 21 dpi). This suggests that the host does not initially recognize the virus as foreign until there is systemic movement, or that the host silencing machinery is not sufficiently activated to be effective. Host gene silencing (loss of chlorophyll) was detected in leaves of systemic infection as large areas, sometimes the entire leaf. Host gene silencing was much more extensive than GFP gene expression in terms of numbers of cells, cell types, and tissue types. On the other hand, viral mediated GFP expression was evident for relatively short periods of time in individual cells of systemically infected leaves (days) while silencing persisted for weeks.

Our previous experiments with geminiviruses showed that although silencing of host genes was not cell autonomous, viral movement was still required for silencing in systemic or upper tissues. The pattern of gene silencing was consistent with it being a defense mechanism

because it occurred in every single cell (mesophyll and palisade) outwards from were the virus was detected to a distance of over 15 cells. The boundary between silenced and non-silenced cells was often discrete, suggesting that either the process of silencing was on or it was off. In systemically silenced tissue, the whole leaf was sometimes silenced. This suggests that gene silencing is an active and potent host defense against the virus. In spite of the widespread gene silencing, viral DNA persisted throughout the life of the plant and could be seen in the seed coat of developing ovules.

It should be noted that modifications to the CLCrV vector reduce its effectiveness as a pathogen. When the coat protein is replaced with foreign DNA, single stranded DNA accumulates at much lower levels and the total amount of viral DNA accumulation is reduced. Symptoms are attenuated but the virus is still infectious, and continues to move and replicate throughout the life cycle of the plant. Although ideally we should use wild type virus, it is not possible to “tag” or mark the virus without compromising its ability to infect. Virus-host interactions are a dynamic and continuously evolving manifestation of geminivirus disease. Direct interactions between viral anti-silencing proteins and host silencing machinery have only recently been determined to play a role in disease. The deployment of visual markers for studying virus-host interactions can increase our understanding of the processes that limit or enhance disease interactions. The variety of viral anti-silencing proteins underscores the importance of controlling silencing in determining successful infections.

There is much to learn about the process of viral infection and gene silencing. For example, we do not know how small RNAs travel from cell to cell and apparently through cells to achieve extensive silencing in all directions of a leaf. Also, the role of antisilencing proteins known to be encoded by geminiviruses is not understood (Bisaro, 2006). How can VIGS of

endogenous genes be efficient if the geminivirus vector itself is the object of plant gene silencing, and similarly – how can stable silencing be achieved in the presence of antisilencing proteins encoded by the virus? Viral proteins that function to prevent silencing (antisilencing proteins) and their mechanism of action is currently an active area of research. The tools we describe here can be used to understand basic factors affecting silencing spread, for example using different temperatures and humidity, or induced drought conditions. If we can begin to understand why virus infections change in response to environmental variables and why drought aggravates virus infection, we can pursue methods for increasing the health of the plant. We look forward to an exciting time in the virology of the cotton plant, and the possibility of eradicating some diseases while at the same time being able to use viruses as research tools to understand the cotton plant itself in more detail.

CONCLUSIONS

Little is known about the precise relationship between viral gene expression and the host gene silencing defense response. Our studies using both viral gene expression and viral gene silencing markers suggest that the initial infection of viruses goes undetected by the host and proceeds in the absence of gene silencing. Once the host gene silencing machinery is activated, it begins to overlap with viral gene expression in systemically infected tissues. Delivery into new growth depends upon phloem transport, and, if the timing of gene silencing varied, the virus might escape. Although we used *ChlI* to visualize gene silencing, previous studies show that siRNAs are made to other viral genes in infected plants. We presume that siRNAs homologous to viral replication genes are also moving with the *ChlI* siRNAs. To overcome gene silencing directly, viral DNA must become double stranded and begin transcription of antisilencing proteins or be able to replicate using low levels of mRNA. We found that CLCrV expressing

GFP was limited to vascular tissue and was not found in mesophyll or epidermal cells. Although gene silencing was wide spread in systemically infected tissues, viral DNA persisted and could even be found in the seed coat of developing ovules. Viral gene expression is transient while host gene silencing appears to be maintained throughout the life of the tissue as shown by the lack of healthy yellow tissue reverting to green. The persistence of gene silencing activity may reflect an adaptation by the host to continuous insect pressure under normal conditions.

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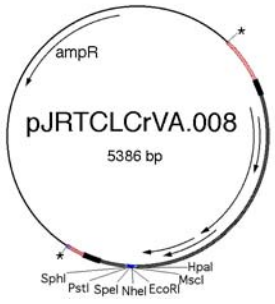
FIGURE CAPTIONS

Figure 1. Plasmid used for VIGS or for expression of small proteins such as GFP. Overlapping genes, shown as arrows on the right, encode proteins needed for viral DNA replication. The short black sequence present on both sides of the overlapping arrows contains the "common region;" the viral origin of replication which must be duplicated in the same orientation for replicational release in plant cells. Following bombardment, viral DNA replication proceeds from one viral origin to the next and the molecule is then ligated into a circular, mobile, viral vector. The cluster of restriction enzyme sites at the end of the arrows is the multiple cloning site for inserting foreign DNA of up to 800 bp. Geminiviruses have two promoters per component, each driving transcription away from the common region. Not shown is the coat protein promoter, which drives expression of sequences cloned into in the multiple cloning site in the opposite direction to the replication genes' transcription. The single arrow in the plasmid backbone is the ampicillin resistance gene of the *E. coli* plasmid, shown as a thin black line. The asterisks show the boundary between viral DNA and plasmid DNA. The red (or gray) sequence is left behind because it lies outside of the common regions. Once this plasmid is co-bombarded with the B component (not shown, contains genes for movement) into plant cells, viral DNA is released that begins transcription, replication, and movement throughout the plant.

Figure 2. GFP expression in DP 5415 leaves shows that viral gene expression is limited to vascular tissue. Left, UV fluorescence image of leaf showing GFP expressing from the virus. Right, normal light image showing that leaf veination coincides with the pattern of GFP fluorescence.

Figure 3. DP5415 plants inoculated with VIGS vector for *ChlI* (left 2 plants), no-insert VIGS vector control, and mock-inoculated plant. The upper right arrow shows a leaf with no chlorophyll while other leaves show chlorophyll in small regions or flecks (3 uppermost left arrows) or as diffuse regions bounded on one or more sides by a leaf vein (central lower arrows). Due to an irregularity, the mock-inoculated plant is two weeks younger than other plants.



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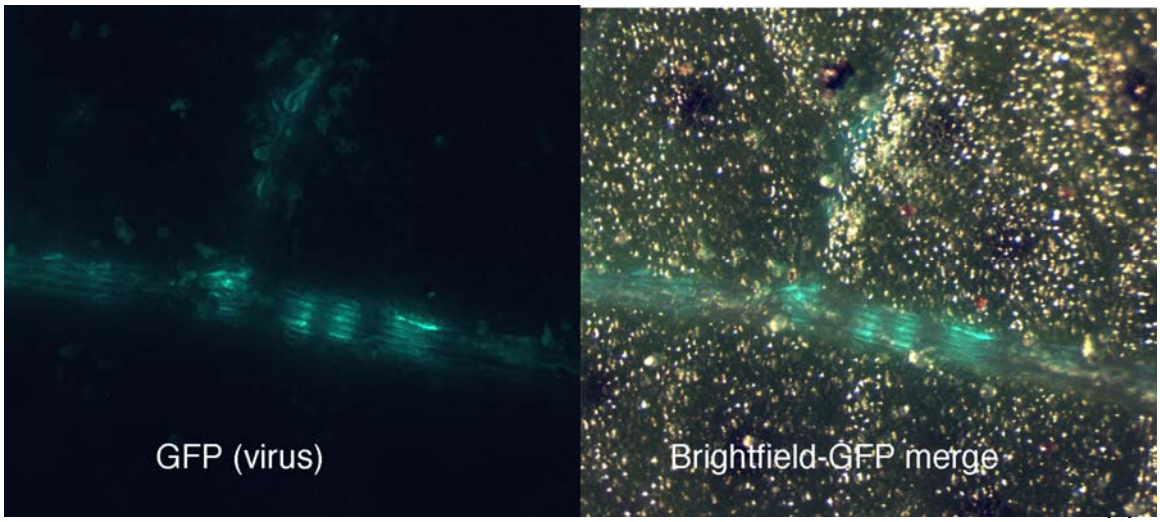

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