



# Episomal Down-regulation of Gene Expression in Plants and Fungi



Carrie Jacobus<sup>1</sup>, Gary Payne<sup>2</sup>, Niki Robertson<sup>1,3</sup>

Department of Genetics<sup>1</sup>, Department of Plant Pathology<sup>2</sup>, Department of Botany<sup>3</sup>

North Carolina State University, Raleigh, NC, USA, 27606

## Overview

There are two kinds of episomes, RNA and DNA. RNA episomes are viruses while DNA episomes can be viruses or plasmids. Episomes do not integrate into the host's genome when transformed into plants and fungi and therefore they are useful silencing tools. Geminiviruses have been shown to be effective inducers of silencing in plants while autonomously replicating plasmids have been shown to initiate silencing in the fungus *Mucor circinelloides*. To learn more about episomal gene silencing, two systems were chosen for study: *Arabidopsis* and Cabbage Leaf Curl Virus (CaLCuV); and *Aspergillus* and an autonomously replicating DNA plasmid. One goal of this research is to determine if an episomal silencing vector can be developed for *Aspergillus*, which is a genus that contains many agronomically important fungi that infect and contaminate crop plants with mycotoxins. Another silencing trigger, in the form of hair-pin RNA, has also been used to induce silencing in the fungus *Magnaporthe oryzae* and will be used in studying *Aspergillus*. By using GFP transgenes in both *Arabidopsis* and *Aspergillus*, silencing by DNA episomes can be directly compared.

## Gemini Viruses as Silencing Vectors

The Cabbage Leaf Curl Virus (CaLCuV) is a DNA virus used to induce PTGS in *Arabidopsis*. The A component of this geminivirus carries genes needed for replication while genes in the B component are needed for viral movement in the host. To use this virus for gene silencing, the sequence encoding the coat protein in component A is deleted and the coding sequence of the gene to be silenced is inserted in its place behind the coding protein promoter. Both A and B components of the virus are bombarded into an *Arabidopsis* plant using a gene gun. Once inside the plant, the virus replicates and a high copy number of RNA transcripts are produced corresponding to the gene to be silenced. This high level of transcript causes the plant to turn on its silencing machinery resulting in degradation of mRNA transcripts and therefore turning off gene expression.

## GFP ORF Silencing in *Arabidopsis*

The sequence encoding GFP was inserted into the A component of CaLCuV.007. A GFP transgenic line of *Arabidopsis* was bombarded at the twelve leaves stage. UV light was used to excite the GFP and a GFP filter was used to remove any other fluorescence at that wavelength. GFP silencing in the bombarded leaves was detected at 15 dpi. Once the silencing signals reached the vasculature of the bombarded leaves, the silencing signals moved down the petiole leading to systemic silencing. New tissue emerging after this stage was silenced for GFP. GFP silencing was detected in leaves, inflorescences, siliques, and flowers.

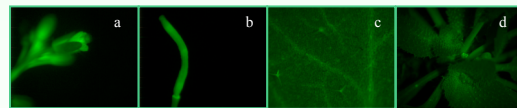


Figure 1 - Transgenic GFP *Arabidopsis* structures a) flower, b) silique c) leaf, d) rosette center

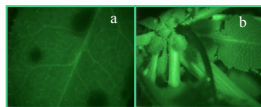


Figure 2 - GFP ORF Silencing a) silencing spots on leaf, b) silenced inflorescence

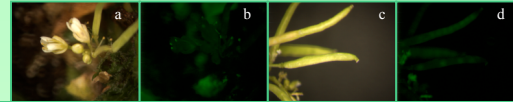


Figure 3 - GFP ORF Silencing. Silenced flowers a) brightfield, b) GFP. Silenced siliques c) brightfield, d) GFP

Silencing correlates with virus infected regions of the plant. Genomic extractions on silenced terminal siliques from eight plants were performed at 57dpi. Viral primers were used to run a PCR on the genomic extractions. PCR products corresponding to the viral GFP sequence were present for all eight silenced terminal siliques. These data demonstrate that the host silencing machinery does not eliminate DNA viruses.

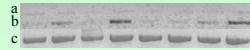


Figure 4 - Viral Load a) genomic DNA, b) 20 cycles of PCR, c) 40 cycles of PCR

## GFP Promoter Silencing in *Arabidopsis*

There is evidence that RNA produced from a transcribed promoter sequence can induce TGS.

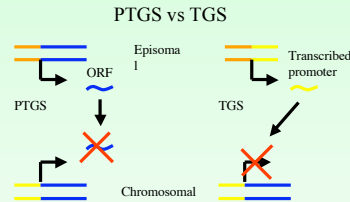


Figure 5 - Silencing targets of PTGS vs. TGS

To examine TGS silencing in *Arabidopsis* a GFP transgenic line was used in which GFP expression is driven by the 35S promoter. A CaLCuVA.007 construct containing the 35S promoter sequence transcribed by the viral *ARI* promoter was bombarded into the GFP transgenic line. When these plants were examined, no distinct silencing spots were seen in the leaves of the bombarded plants. However, new tissue from some of these plants showed down regulation of GFP expression.

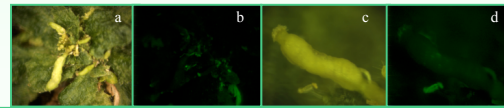


Figure 6 - GFP Promoter Silencing. Silenced inflorescence and leaves a) brightfield, b) GFP. Silenced silique c) brightfield, d) GFP

The plants that were bombarded with the 35S construct showed severe viral symptoms when compared with plants bombarded with either the GFP construct or a control construct. It is unclear why the 35S construct is behaving this way.



Figure 7 - Viral symptoms a) 1011 with CaLCuVA.007::35S b) 1011 with CaLCuVA.007::GFP

## Silencing *Aspergillus*

Transgenic GFP strains of *Aspergillus flavus* and *Aspergillus nidulans* were created through cotransformation using a PEG protocol. The RJW3 *A. nidulans* strain was cotransformed with pHY03 carrying a tryptophan auxotrophic marker along with pNUC'EM2 carrying a GFP transgene driven by the human cytomegalovirus promoter. Transformants were selected for their ability to grow without tryptophan and for fluorescence under long wavelength UV light. The 86-10 *A. flavus* strain was constructed in a similar manner using the same pNUC'EM2 GFP plasmid.

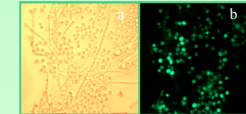


Figure 8 - Transgenic *Aspergillus flavus* 40X a) brightfield, b) GFP

Two silencing strategies will be used to silence GFP expression in these lines: inverted repeat (IR) constructs and autonomously replicating episomes. IRs have been shown to be effective inducers of silencing in many organisms including the fungus *Magnaporthe*. There is a naturally occurring autonomously replicating element in *Aspergillus* that is called AMA1. When this element is introduced into a plasmid and transformed into *Aspergillus*, it causes the plasmid to autonomously replicate. This episome can be used like the geminivirus system in *Arabidopsis* to produce a high number of transcripts to induce gene silencing. A similar system has been used to silence the fungus *Mucor circinelloides*. Both PTGS and TGS mechanisms will be studied using the two silencing strategies.

## Conclusions

- Geminiviruses are capable of inducing silencing using ORF and promoter sequences.
- The CaLCuVA.007::35S construct causes severe symptoms in *Arabidopsis*.
- Geminivirus episomes are stably propagated in tissue showing active silencing, even when they contain regions of homology to the silenced transgene.
- With the construction of a 35S GFP equivalent in *Aspergillus* along with the autonomously replicating plasmid as an episome, it will be possible to compare silencing mechanisms in plants and fungi.

	<i>Arabidopsis</i>		<i>Aspergillus</i>	
	ORF	Promoter	ORF	Promoter
Silencing	Yes	Yes	?	?

## References

Jones, L., Hamilton, A.J., Voimnet, O., Thomas, C.L., Maule, A.J., and Baulcombe, D.C. (1999) RNA-DNA interactions and DNA Methylation in Post-Transcriptional Gene Silencing. *Plant Cell* 11, 2291-2301.

Kadotani, N., Nakayashiki, H., Tosa, Y., and Mayama, S. (2003) RNA Silencing in the Phytopathogenic Fungus *Magnaporthe oryzae*. *MPMI* 16, 769-776.

Nicolas, F.E., Torres-Martinez, S., and Ruiz-Vazquez, R.M. (2003) Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. *EMBO* 22, 3983-3991.

Ruix, M.T., Voimnet, O., and Baulcombe, D.C. (1998) Initiation and Maintenance of Virus-Induced Gene Silencing. *Plant Cell* 10, 937-946.