CRISPieR: Re-engineering CRISPR-Cas9 with functional applications in eukaryotic systems

Simple sgRNA Exchange

The Simple sgRNA Exchange system lets users use cost-efficient classical cloning to change the target sequence in a CRISPR-Cas9 system. This was achieved by flanking the target sequence of the sgRNA with the restriction sites *SphI* and *BamHI* (Figure 1).

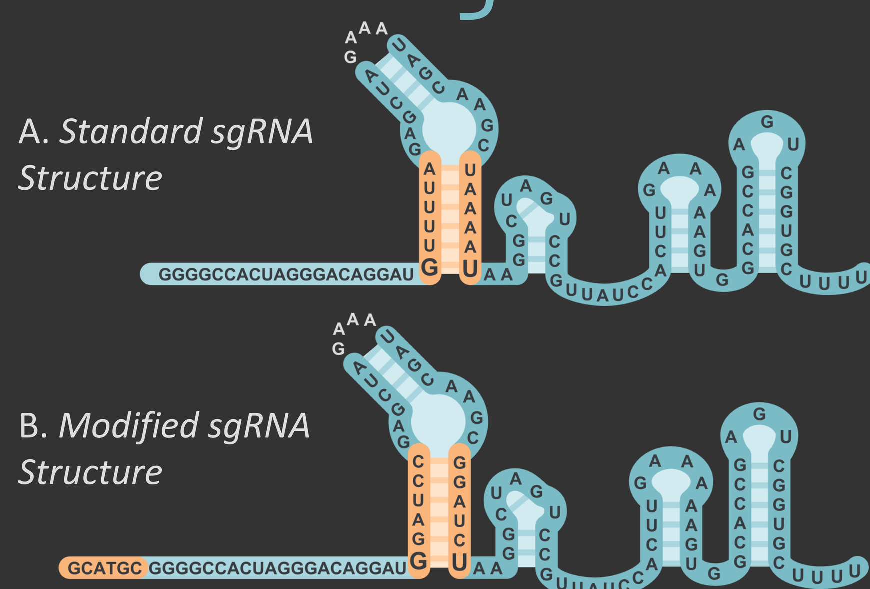


***Figure 1.** The standard sgRNA structure (A) was modified to incorporate a **BamHI** restriction site (B), maintaining the non-Watson-Crick G-U base pair in the lower stem.*

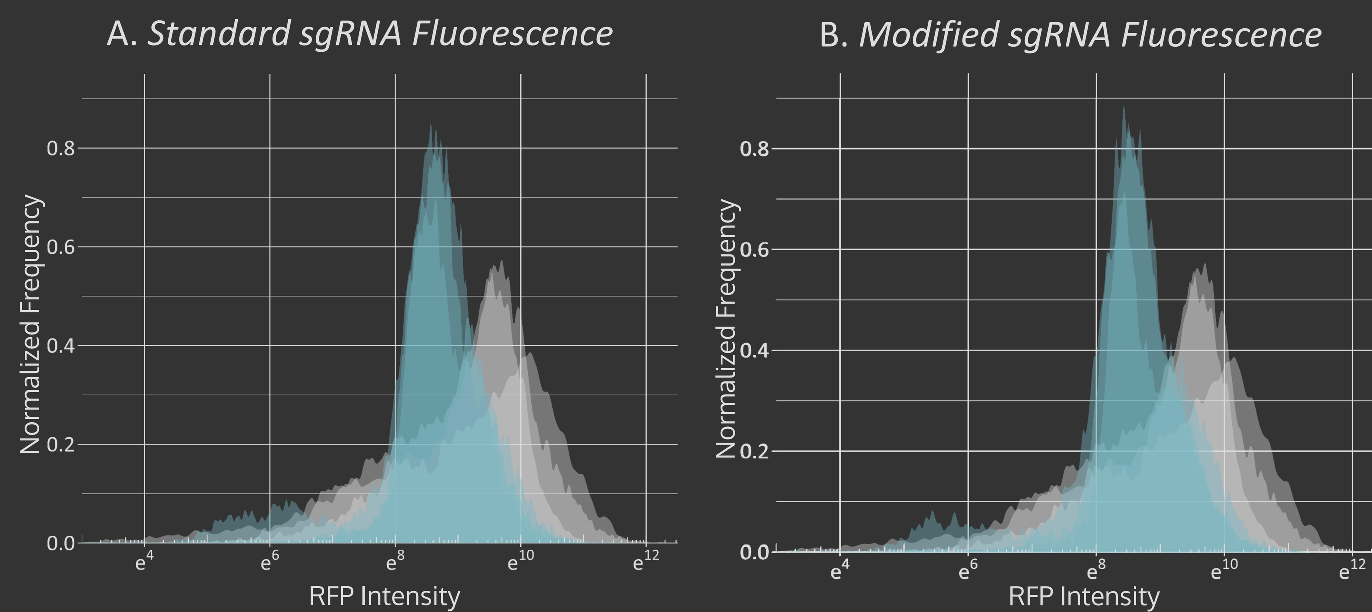


***Figure 2.** RFP Intensity after targeting with (A) dCas9 + standard sgRNA and (B) dCas9 + modified sgRNA in blue. Non-targeted RFP control measurements are shown in grey. Replicate measurements taken on three separate days are overlayed on the same axes.*

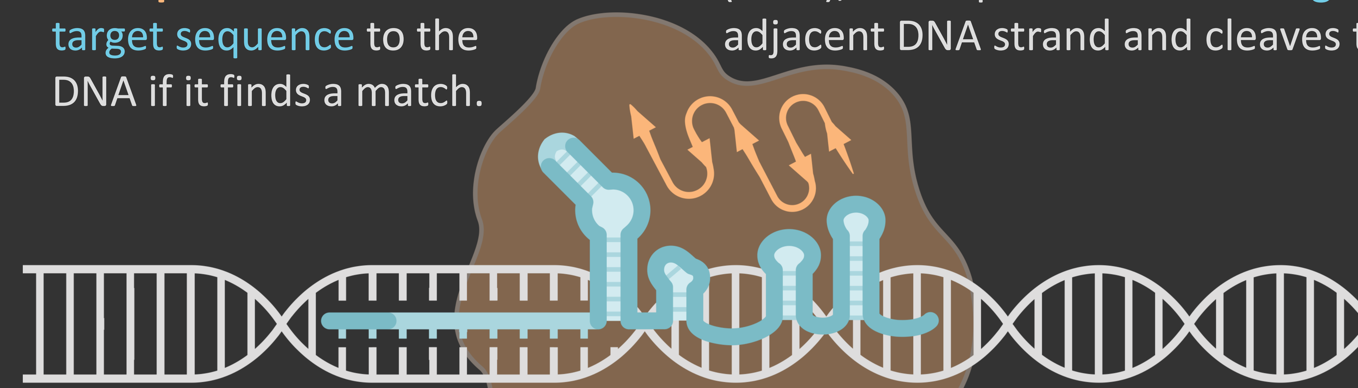
Design and Experiments

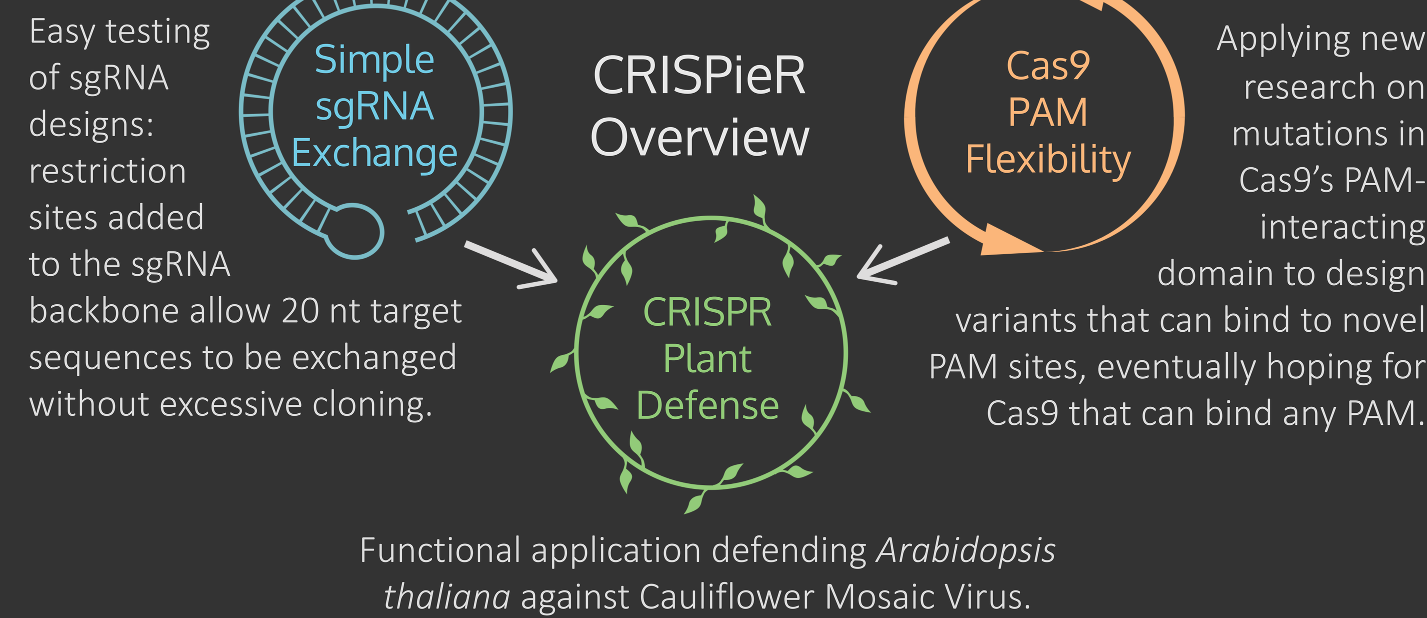
The same target sequence was used in both standard and modified sgRNA to target the *LacI* promoter upstream of RFP on BBa_K1645999. The two sgRNA structures were cloned and co-transformed with dCas9 into *E. coli* strain BL21DE3 and RFP and dCas9 expression was induced by IPTG.

Results and Discussion

Imaging flow cytometry (Amnis ®) was used to measure RFP intensity. Similar drops in intensity were observed when targeted using standard and modified sgRNAs (Figure 2). This result shows that the modification to the sgRNA structure did not change dCas9 targeting functionality.

CRISPR-Cas9 is an exciting tool for synthetic biologists because it can target and edit genomes with unprecedented specificity. The CRISPR **Cas9 protein** binds to a **PAM site** (NGG), attempts to match its sgRNA target sequence to the adjacent DNA strand and cleaves the DNA if it finds a match.





Functional application defending *Arabidopsis thaliana* against Cauliflower Mosaic Virus.

CRISPR in Society

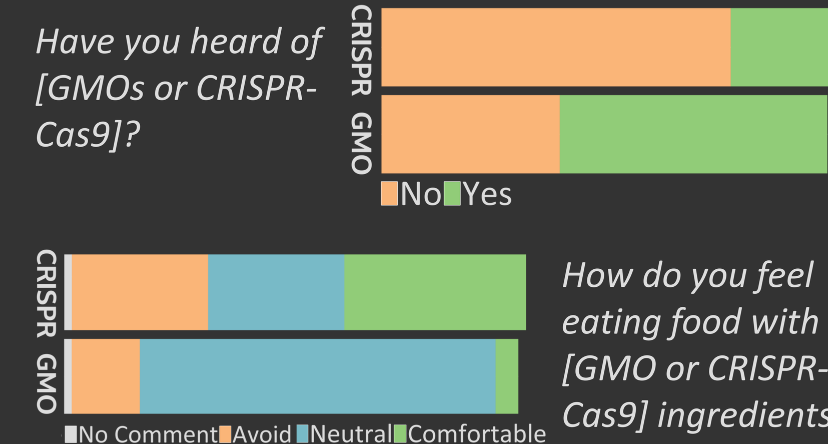
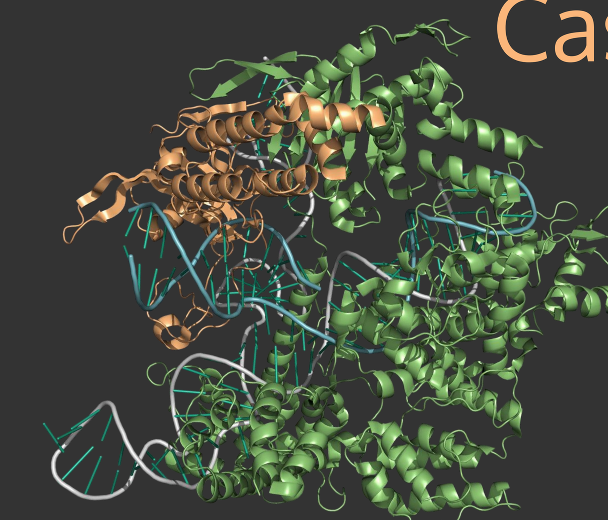


***Figure 3.** Results from two public attitude surveys. Participants in each survey answered equivalent questions about their perceptions of "GMOs" and "CRISPR-Cas9".*

Our public attitude survey indicates that not many people are aware of CRISPR-Cas9 gene editing, but the public was more willing to consume food with CRISPR-Cas9 ingredients than with GMO ingredients. These findings show the potential for gene-editing to be used in agriculture as in our plant defense application.

Cas9 PAM Flexibility



The wild type Cas9 protein only binds to the 3 nt PAM site NGG. In June of this year Kleinstiver et al. (Nature 2015, Vol 523) published a paper describing two novel Cas9 variants able to bind to NGAG and NGAN PAM sequences, respectively.

Computational Model

Using PyRosetta we aimed to computationally determine the PAM sequences that Cas9 mutants can bind to.

The simulations were successful in reproducing the wild type Cas9 PAM specificity (see Figure 4). Our simulation of the Kleinstiver mutants failed to reproduce the specificity of the third PAM nucleotide in the NGAG variants (see Figure 5) and NGAN variants (not shown).

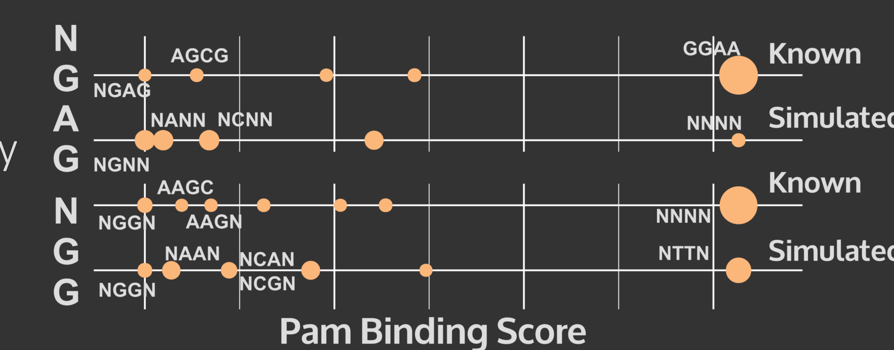


***Figure 4.** PAM binding scores for wild type and NGAG-binding Cas9. Known scores were derived from literature and simulated from PyRosetta. All scores were hierarchically clustered to show PAM binding consensus.*

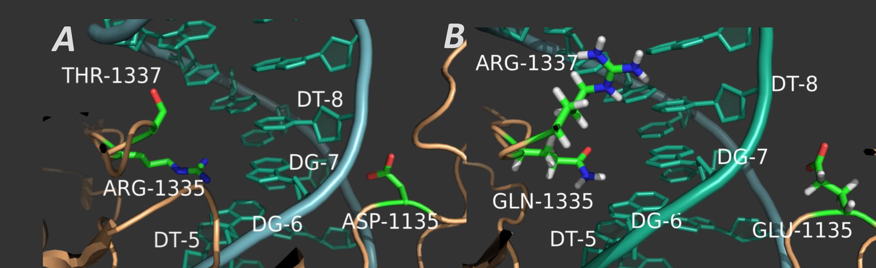


***Figure 5.** PAM binding site of wild type Cas9 (A) and NGAG-binding mutant Cas9 (B) visualized in PyMOL. The three mutated amino acids are labeled.*

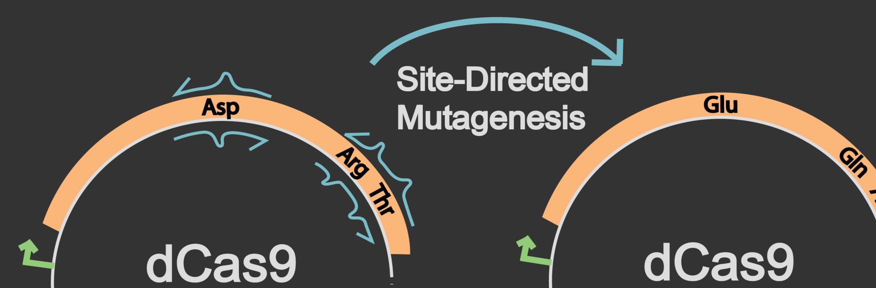


***Figure 6.** To validate our use of a dCas9 rather than Cas9 structure in PyRosetta, three mutations in dCas9 were induced by site-directed mutagenesis to make it bind to the alternate NGAG PAM site. Mutations were sequence confirmed. Flow cytometry results for GFP expression are being analysed.*

Recommendations

Further computational work with the PyRosetta docking procedure is needed to better simulate the protein-DNA binding. Analysis of the scoring function may identify a method to better predict PAM specificity.

CRISPR Plant Defense

To explore *in vivo* application of the sgRNA Exchange and flexible PAM systems, we designed a construct to defend *Arabidopsis thaliana* against infection by Cauliflower Mosaic Virus (CaMV) using CRISPR-Cas9.

Adding CRISPR to Plants

Our CRISPR-Cas9 cassette, containing Cas9 and sgRNAs, can be integrated into the germline genome of *Arabidopsis* via the *E.coli* – *Agrobacterium* shuttle vector pCAMBIA.

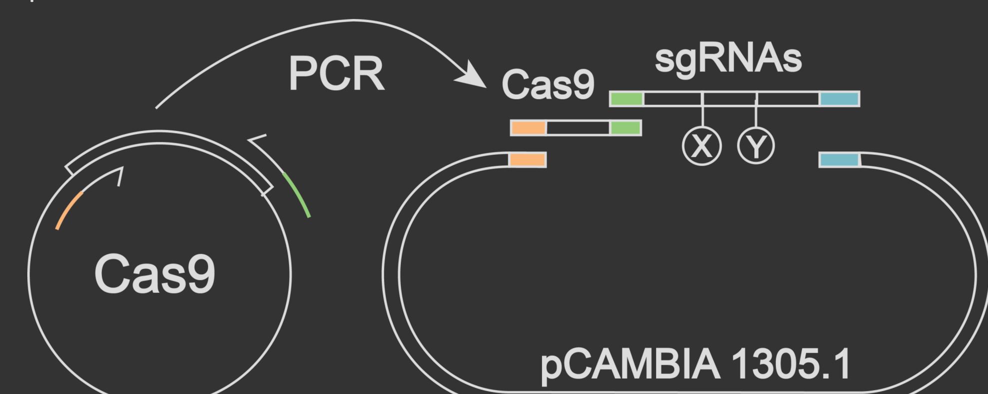


***Figure 11.** Procedure to clone CRISPR-Cas9 cassette into pCAMBIA with Gibson assembly and PCR-overlap extension.*

Models of genomes affected by CRISPR-Cas9, CaMV replication and intercellular infection spread: Model-Based Design

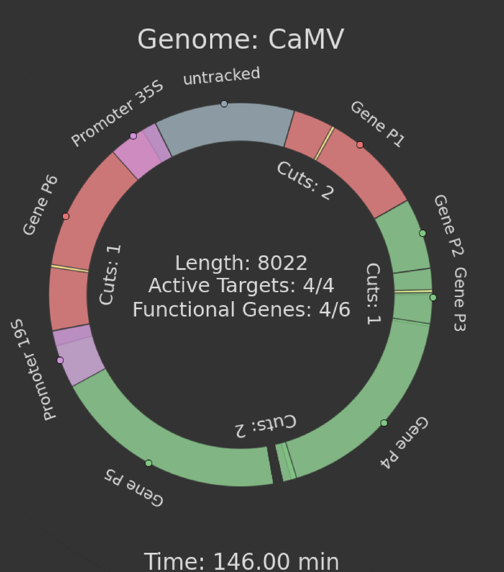


***Figure 7.** Stochastic simulation of Cas9 cutting multiple targets in a CaMV genome. The model tracks the accumulation of frameshift mutations, which deactivate genes.*

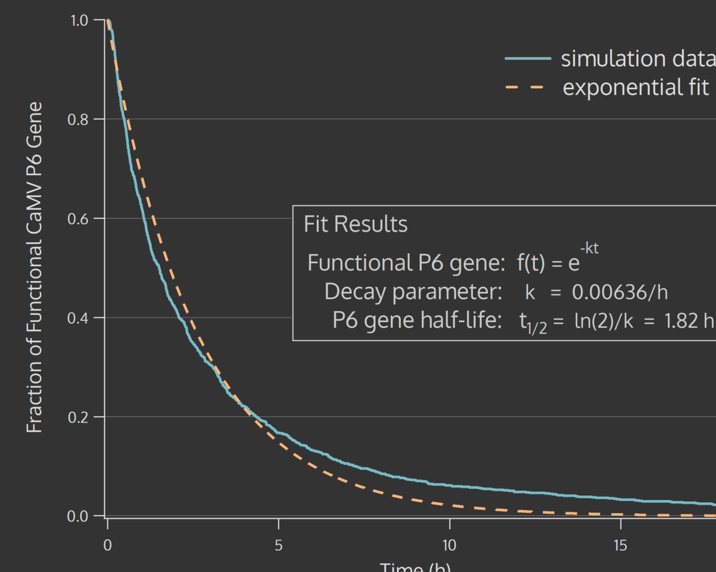


***Figure 8.** A CRISPR effect parameter ($t_{1/2}$) is derived from an exponential fit to the fraction of CaMV genomes active at each timestep across 1000 stochastic genome simulations.*

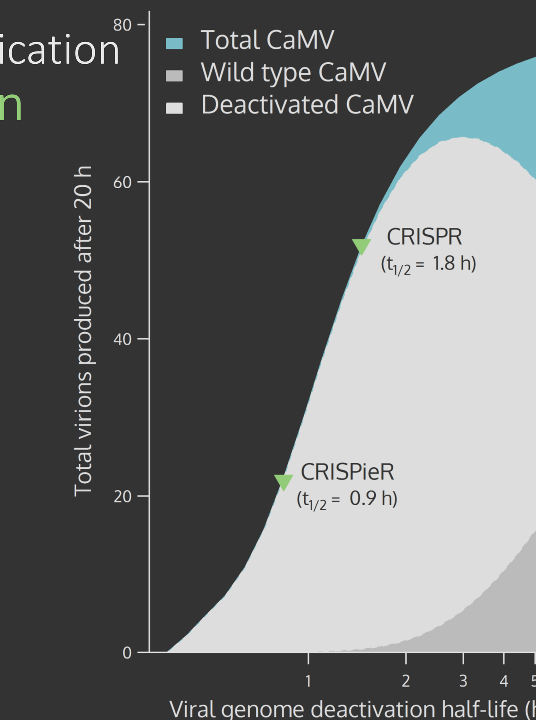


***Figure 9.** Dose-response of CaMV virion levels to the CRISPR effect parameter ($t_{1/2}$) given at 20 hours post infection by an ODE replication model.*

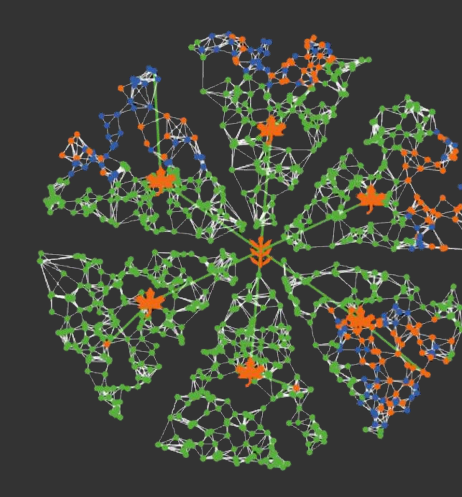


***Figure 10.** Agent-based Netlogo model shows the spread of infection (red) and acquired resistance (blue) in a leaf simulating an ODE replication model. The CRISPR effect parameter ($t_{1/2}$) is captured in the ODE model.*

Modeling Results

Our modeling predicts that Cas9 targeting of CaMV provides robust cell defense ($t_{1/2}$=1.82 hours), dramatically slowing infection across cells. Theoretical upper bounds on Cas9 activity with CRISPieR showed nearly complete inhibition of viral spread.

Experimental Results

We validated our construct by transfecting protoplast plant cells and inducing transient expression of Cas9 (Figure 12). Genome integration in *Arabidopsis* of the pCAMBIA construct was not achieved given the time constraints of growing the plants.

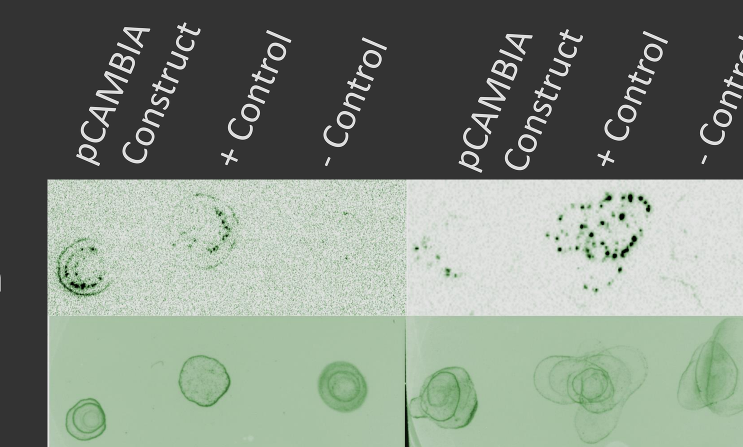


***Figure 12**. Two dot blots using anti-Cas9 antibody demonstrate protoplast expression. Top row is chemiluminescence, bottom row is visible light.*

Future Work

CRISPieR still requires validation of the functionality of the NGAG PAM binding in our mutated dCas9 and classical cloning confirmation of the sgRNA Exchange restriction site.

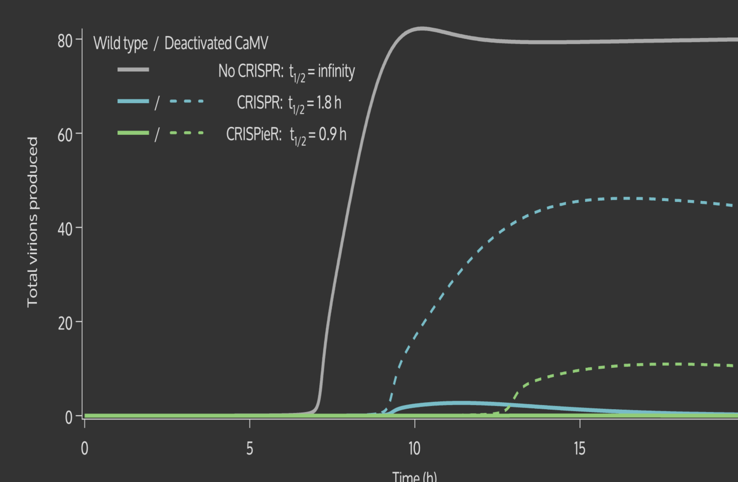


***Figure 13.** Replication model shows total CaMV virions within a cell over time with and without a CRISPR/CRISPieR defense system.*

The pCAMBIA CRISPR defense construct sequence needs validation. Plants may be engineered to contain the CRISPR-Cas9 cassette using a floral dip, then infected by CaMV and tested by qPCR.

Mathematical modeling (Figure 13) shows the CRISPR plant defense system would function even better with CRISPieR improvements. The legality and potential marketing of the system was analysed with a mind to scaling up the project. The Broad Institute owns a patent for the CRISPR-Cas9 system, but its scope is vague. Future steps are to obtain an independent patent or collaborate with the Institute for a license. CRISPieR is a foundational advance that combines a wide range of synthetic biology techniques to improve an exciting technology.

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