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

Simple sgRNA Exchange

The Simple sgRNA Exchange cloning system allows users to cost-effectively exchange target sequences in a CRISPR-Cas9 system. This was achieved by flanking the target sequence of the sgRNA with restriction sites SpeI and BamHI (Figure 1).

Design and Experiments

The same target sequence was used in both standard and modified sgRNAs to target the Laci promoter upstream of RFP on pBba_K1645999. The two sgRNA structures were cloned and co-transformed with dCas9 into E. coli strain BL21 DE3 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 in Society

Our public attitude survey indicates that not many people are aware of CRISPR-Cas9 gene editing, but they are 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 our plant defense application.

CRISPR Plant Defense

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

Cas9 mutants are used to target the genome (Figure 3). The CRISPR effect parameter (t1/2) is derived from an exponential fit to the fraction of CaMV genomes active at each timepoint across 1000 stochastic genome simulations, which deactivate genes.

PCR
sgRNAs
Cas9
pcCAMBIA A1305.1

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.

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).

Cas9 PAM Flexibility

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.

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

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 (t1/2) is captured in the ODE model.

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

Figure 13. Replication model shows total CaMV virions within a cell over time with and without a CRISPR/Cas9 defense system.

Cas9 protein binds to a PAM site (NGG), attempts to match its sgRNA target sequence to the DNA if it finds a match.