CRISPR-Cas9 mediated mutagenesis of a suppressor of defense in *T. cacao*

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CRISPR CAS9

• A molecular system in bacteria used to defend against virus infection
  • Viral sequences are integrated into host genome
  • CAS9 protein interacts with RNA guides derived from viral sequences
  • RNA-targeted CAS9 detects viral sequences, binds and breaks the DNA backbone inactivation the viral infection

• This system was engineered for use in animal and plants
  • Introduction of genes for CAS9 and guide RNAs
  • Guide RNAs can be designed to target any sequence in the genome, highly specific
  • After cleavage, incomplete repair of broken DNA often results in small deletions thus inactivating the targeted gene
  • Can also be used to introduce new DNA sequences to specific genomic locations via homology mediated end joining
  • Can be used to edit the genome without transgene insertion or transgene can be removed by breeding

• A breakthrough for many uses
  • Human gene therapy to cure diseases
  • Crop improvement
  • Animal improvement
  • Etc……

• Currently in the US, organisms edited with CRISPR CAS9 are not regulated as transgenic organisms
https://www.youtube.com/watch?v=ouXrsr7U8Wl
Video depiction of CRISPR CAS9
A powerful strategy for crop and food improvement

- Anti-browning mushroom
  Yinong Yang Lab

- High amylopectin content corn
  DuPont Pioneer

- Reduced acrylamide potato
  Calyxt

- Reduced trans-fat soybean oil
  Calyxt
Development of Gene Editing for Cacao

• **Goals**
  • Develop CRISPR system for cacao for use in functional genomics research
  • Explore utility for development of gene edited cacao for disease resistance or other traits of value

• **Strategy**
  • Design of CRISPR system components tailored for cacao
  • Testing with gene of known function: NPR3 a repressor of the plant defense system
    • Test ability of constructs to cleave NPR3 DNA in vitro
    • Test ability to cleave NPR3 in vivo via transient expression in leaves
    • Test phenotype of NPR3 editing on pathogen resistance using leaf bioassay with Phytophthora
NPR1 is the Master Regulator of the Defense Response

Perception of Pathogen → Signaling Cascade → NPR1 (cytoplasmic) → Nuclear Translocation → TGA2 → NPR1 (Defensive Genes)

High Levels of PR Gene Expression = Resistance to Pathogens

We previously demonstrated the function of NPR1 and NPR3 in Arabidopsis and Cacao.
Non-Expressor of Pathogenesis-Related 3 (NPR3) is a suppressor of the defense response.

Perception of Pathogen → Signaling Cascade → NPR1 → Nuclear Translocation → NPR1

No PR Gene Induction → Pathogen susceptibility

NPR3 → Ubiquination

Cullin mediated NPR1 degradation pathway
Will inactivation of the NPR3 gene lead to enhanced disease resistance?

Perception of Pathogen → Signaling Cascade → NPR1 → 1000+ gene activation → Defense Genes

Plant R Gene → Repressor function removed

CAS9 knockout → De-regulated Immune System Enhanced Resistance to Pathogens
Transient Transformation for Gene Functional Analysis

TcNPR3 Knockdown via AmiRNA results in enhanced disease resistance

Shi et al., 2013. BMC Plant Biology.

Relative Expression of TcNPR3 to TcActin

Vector Control

MiRNA_NPR3

0.25

0.20

0.15

0.10

0.05

Average Lesion Size (cm²)

1.0

0.9

0.8

0.7

0.6

0.5

0.4

0.3

0.2

0.1

Vector Control

MiRNA_NPR3

0.25

0.20

0.15

0.10

0.05

Ratio of PcActin to TcActin

Vector Control

MiRNA_NPR3

0.25

0.20

0.15

0.10

0.05

Shi et al., 2013. BMC Plant Biology.
But... this is a transgenic approach

• Can we get the same result using a non-transgenic CAS9 genome editing approach?
Dual Guide Genetic Construct to Introduce CRISPR Into Cacao Targeting NPR3 Gene
CRISPR-Cas9 Expression Vector in Ti Plasmid

AtU6 promoters drive gRNA expression.

CaMV 35S drives Cas9 expression

CaMV 35S promoter drives EGFP expression
Transformation of Cacao Leaves with CRISPR-Cas9 Vector

1. Collect Leaves
2. Cut into sections
3. Vacuum Agroinfiltrate
4. Screen for successful transformation
5. Extract DNA for Detection of Mutations
If CAS9 cleaves NPR3 at both targeted sites, we expect a 973 bp deletion and gene inactivation.

NPR3 Gene Model

If CAS9 cleaves NPR3 at both targeted sites, we expect a 973 bp deletion and gene inactivation.
Detection of edited cacao genomic DNA

Very high transformation success rate

Upper and lower bands were purified and cloned.
Precise 973 bp Deletion in NPR Gene Was Detected

32% of total DNA was cut (molar basis)

973 bp deletion
NPR3 Editing INCREASES Disease Resistance and Increases Expression of Specific PR Genes
Off Target Mutations NOT Detected at 5 Most Closely Related Sites in Cacao Genome

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<th>Sequence</th>
<th>Mismatches (#)</th>
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No mutations detected at a frequency higher then miseq error rate
Stably Transformed CRISPR Mutagenized Cacao Somatic Embryos
Next steps for evaluation of TcNPR3 mutation

- Recover TcNPR3 edited plants
- Test for disease resistance
- Study molecular effects of the editing

Broader goals for cacao CRISPR-Cas9

- Select and assay more targets
  - Flavor/metabolite pathways
- Develop multiplex vectors targeting multiple genes
- Use homology dependent repair to engineer precise insertions
- Looking for collaborators: knockout your favorite gene
- Development of knockout collections of all cacao genes for functional genomics?
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