PennState
College of Agricultural Sciences

Program in the Molecular Biology of Cacao

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

Andrew S. Fister, Lena Landherr,
Siela N. Maximova, Mark J. Guiltinan


Photo Credit: M. Guiltinan

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=ouXrsr7U8WI
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

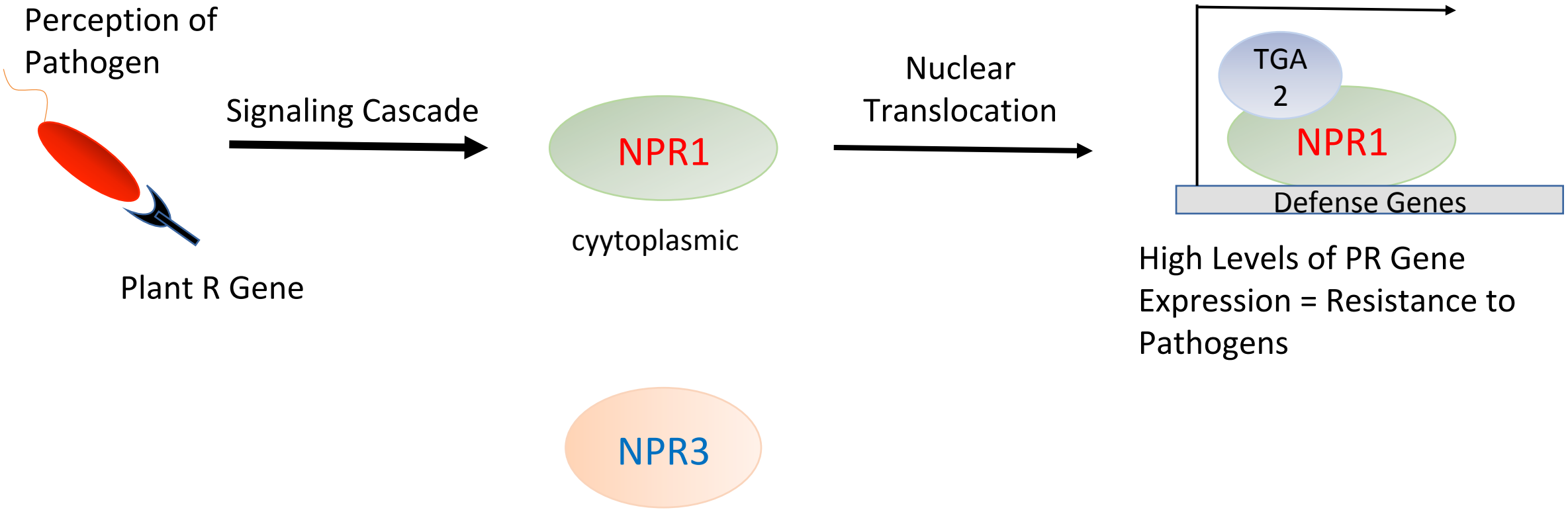
Reduced trans-fat soybean oil
Calyxt

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

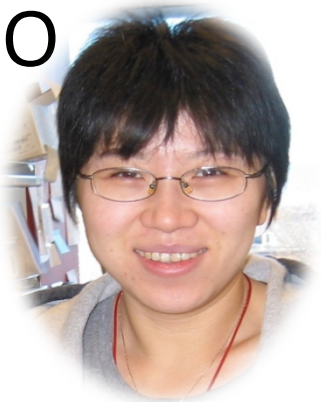
- Strategy
  - Design of CRISPR system components tailored for cacao
  - Testing with gene of known function: NPR3 a repressor of the plant defense system
    - Test ability of constructs to cleave NPR3 DNA in vitro
    - Test ability to cleave NPR3 in vivo via transient expression in leaves
    - Test phenotype of NPR3 editing on pathogen resistance using leaf bioassay with Phytopthora

NPR1 is the Master Regulator of the Defense Response



Fu and Dong, 2013 Annual Rev. Plant Biol.

We previously demonstrated the function of NPR1 and NPR3 in Arabidopsis and Cacao



Zi Shi

Shi *et al. BMC Plant Biology* 2010, **10**:248
http://www.biomedcentral.com/1471-2229/10/248

BMC Plant Biology

RESEARCH ARTICLE Open Access

Functional analysis of the *Theobroma cacao NPR1* gene in *arabidopsis*

Zi Shi[1], Siela N Maximova[2], Yi Liu[1], Joseph Verica[2], Mark J Guiltinan[1,2*]

Molecular Plant RESEARCH ARTI

The Salicylic Acid Receptor NPR3 Is a Negative Regulator of the Transcriptional Defense Response during Early Flower Development in *Arabidopsis*

Zi Shi[a], Siela Maximova[b], Yi Liu[a], Joseph Verica[b] and Mark J. Guiltinan[a,b,1]

a The Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, USA
b Department of Plant Science, Pennsylvania State University, University Park, PA 16802, USA

BMC Plant Biology 2013, **13**:204
http://www.biomedcentral.com/1471-2229/13/204

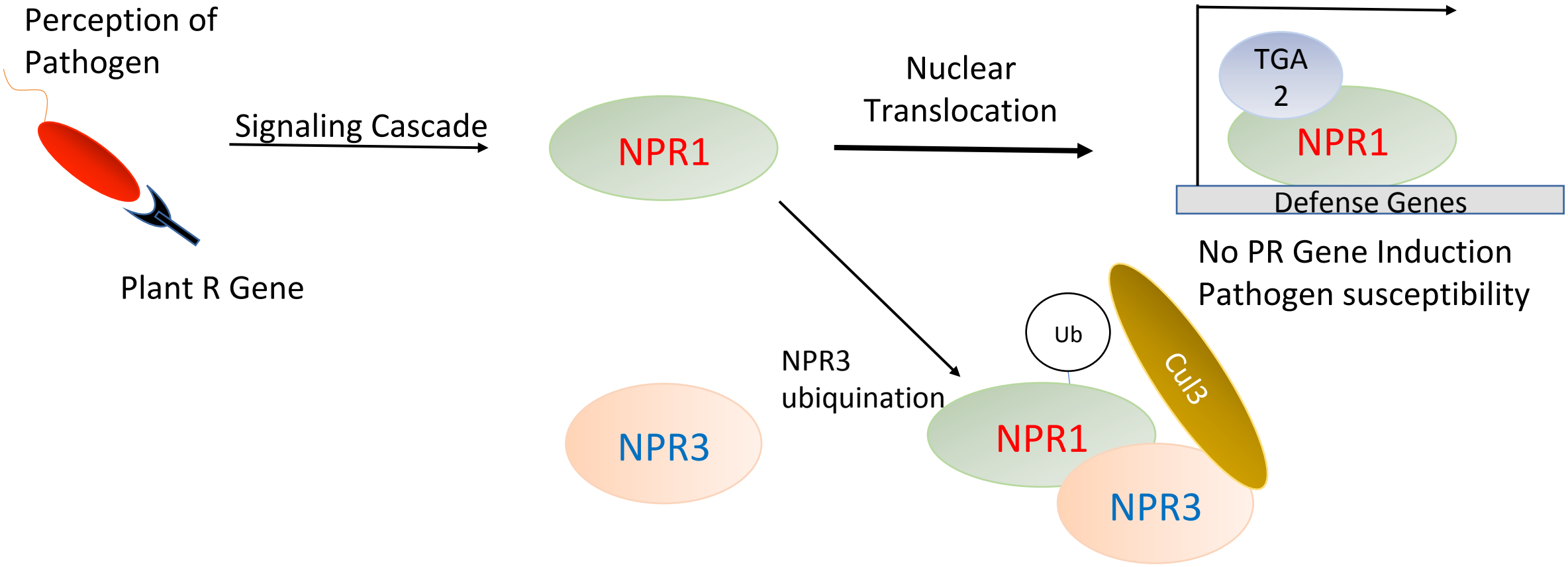
BMC Plant Biology

RESEARCH ARTICLE Open Access

TcNPR3 from *Theobroma cacao* functions as a repressor of the pathogen defense response

Zi Shi[1], Yufan Zhang[1], Siela N Maximova[2] and Mark J Guiltinan[1,2,3*]

Non-Expressor of Pathogenesis-Related 3 (NPR3) is a suppressor of the defense response



Will inactivation of the NPR3 gene lead to enhanced disease resistance?

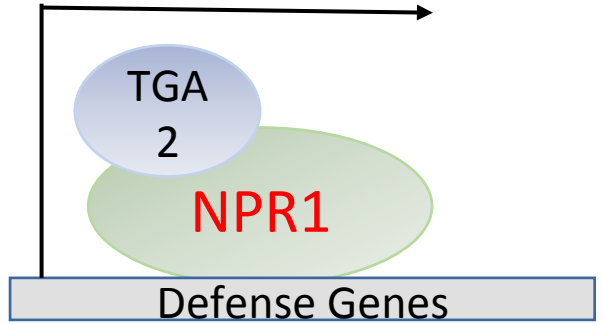
Perception of Pathogen

Plant R Gene

Signaling Cascade

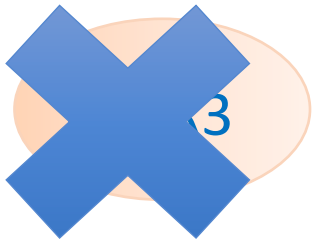
NPR1

1000+ gene activation



Repressor function removed

CAS9 knockout



De-regulated Immune System
Enhanced Resistance to Pathogens

Transient Transformation for Gene Functional Analysis

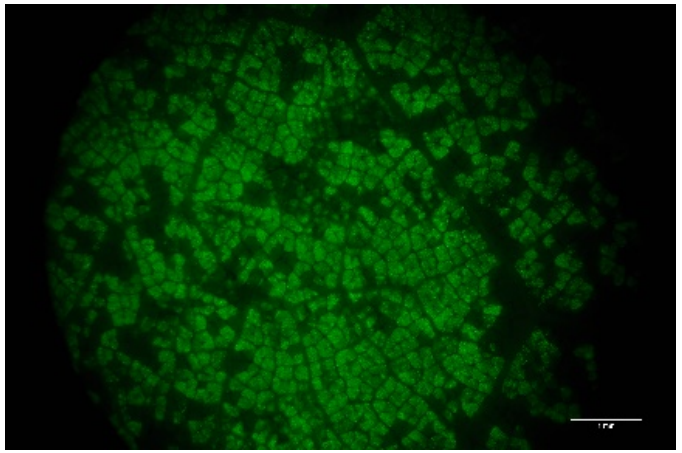
Collect Leaves

Cut into sections

Vector Control

MiRNA TcNPR3
Or
CAS9-NPR3

Vacuum Agroinfiltrate

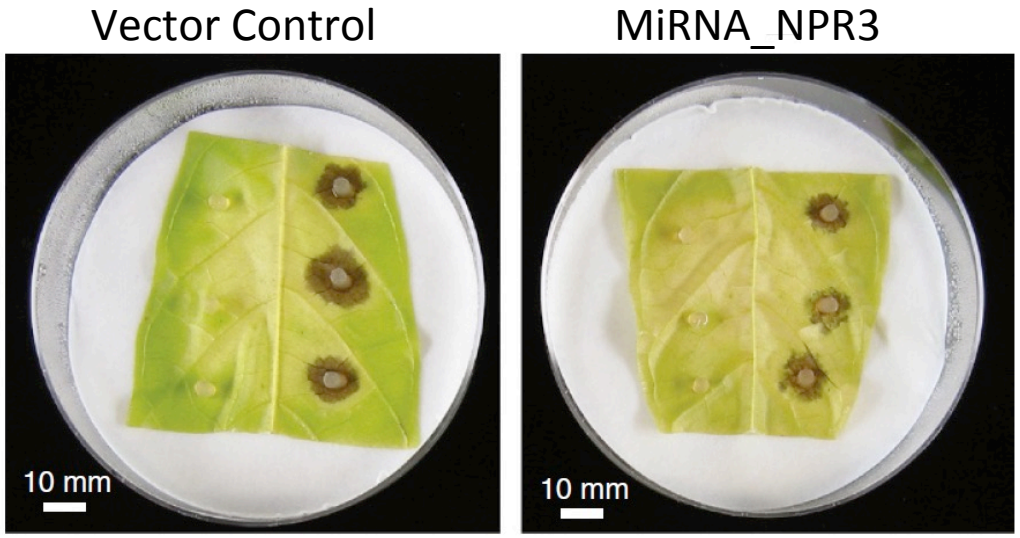


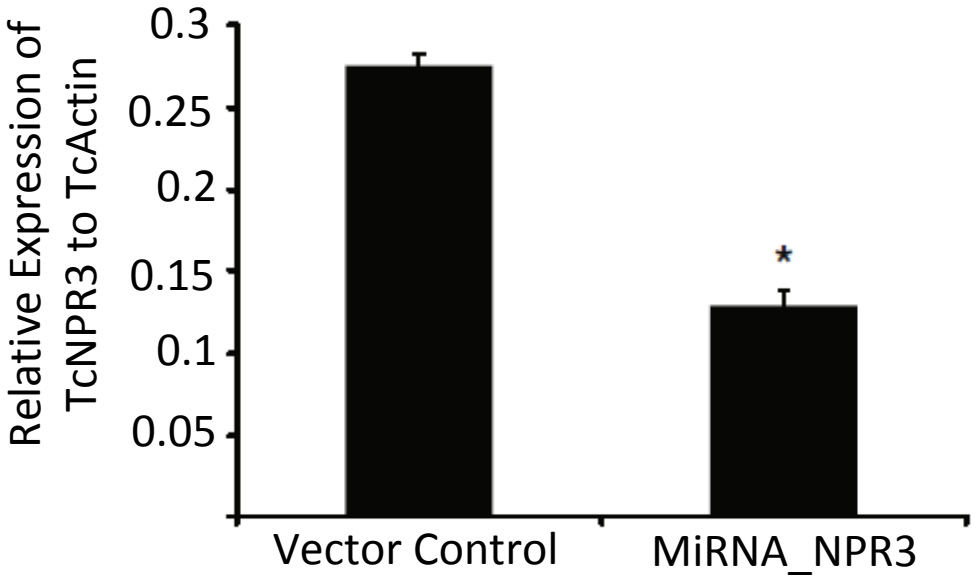
Screen for successful transformation

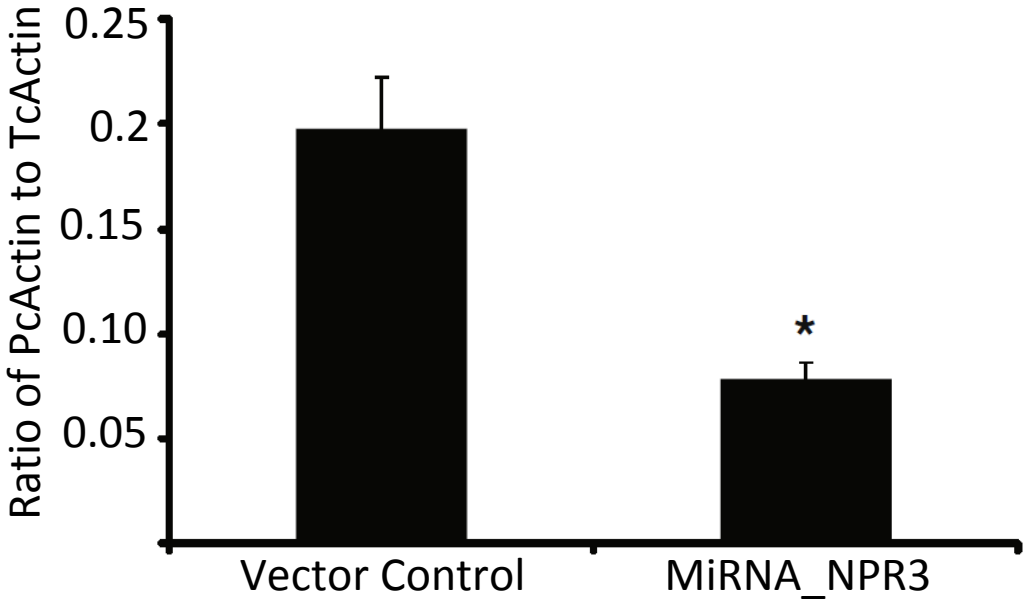
Disease Resistance Bioasay

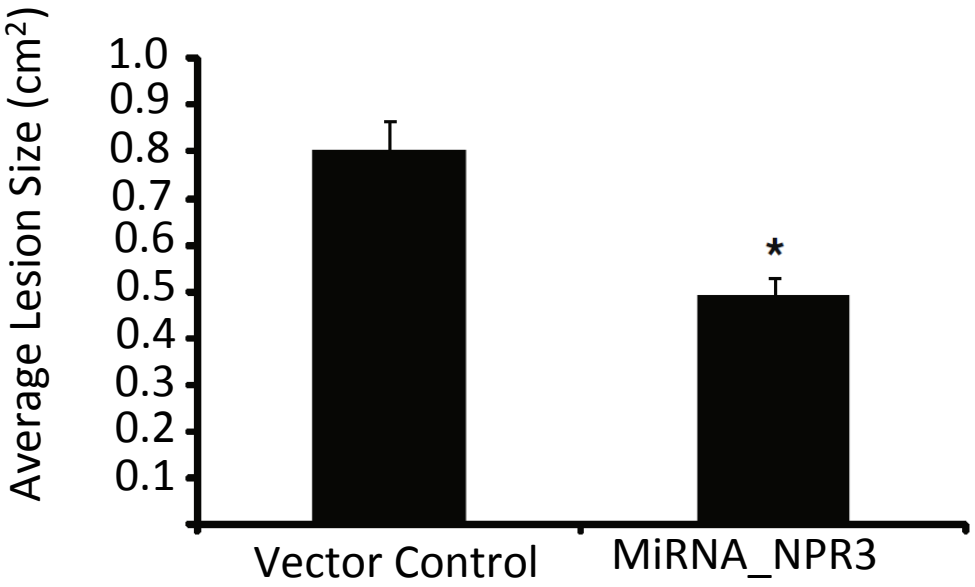
Fister et al., 2016. Plant Methods. Protocol: transient expression system for functional genomics in the tropical tree *Theobroma cacao* L. **12**:19.

TcNPR3 Knockdown via AmiRNA results in enhanced disease resistance







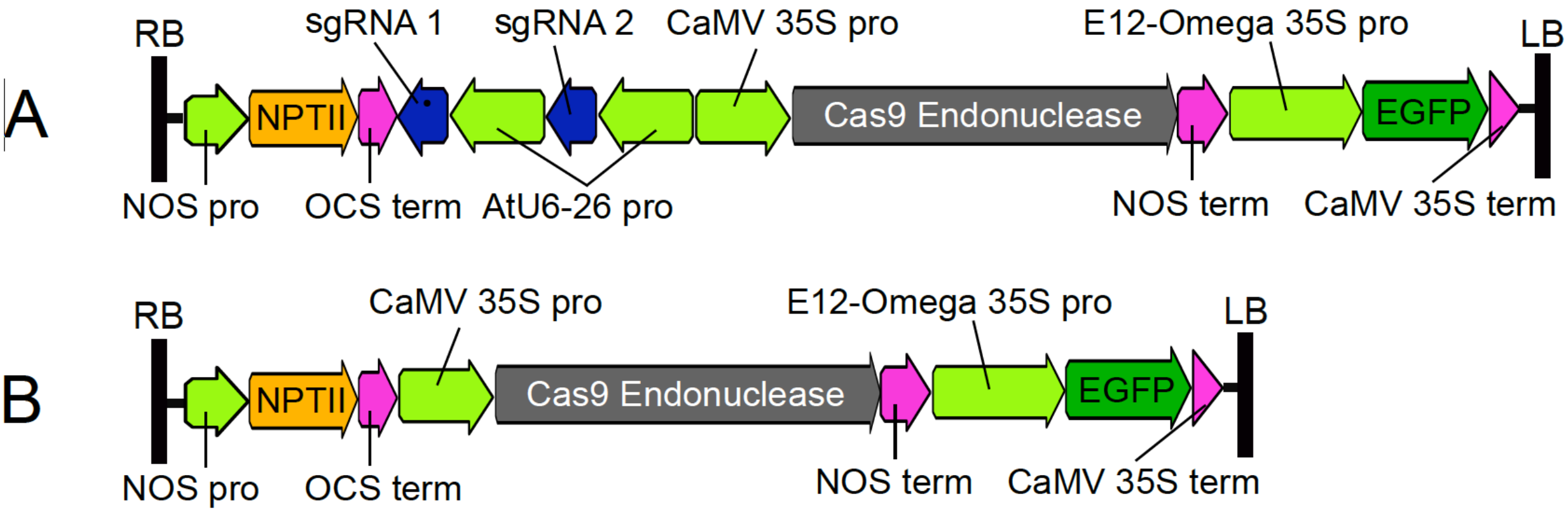


Shi et al., 2013. BMC Plant Biology.

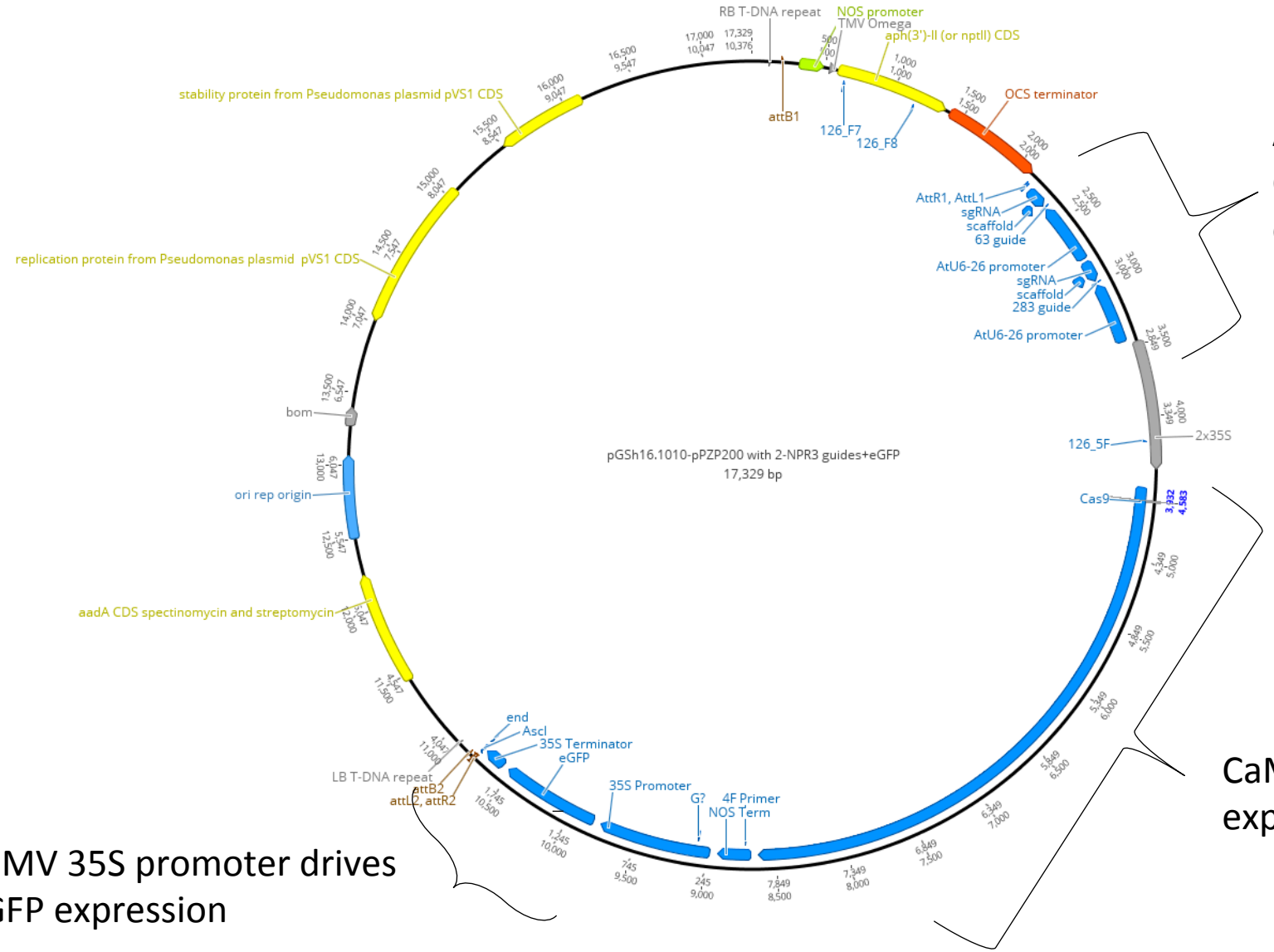
But… this is a transgenic approach

- 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.

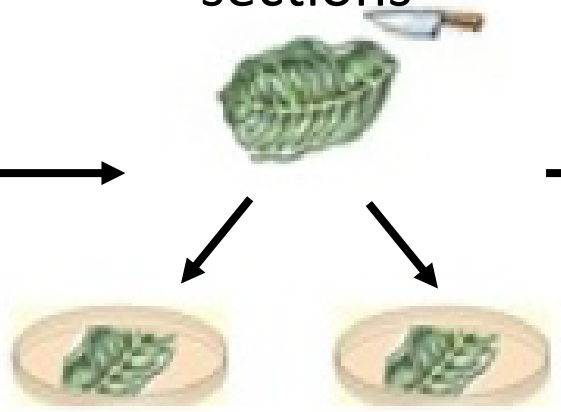
CaMV 35S drives Cas9 expression

CaMV 35S promoter drives EGFP expression

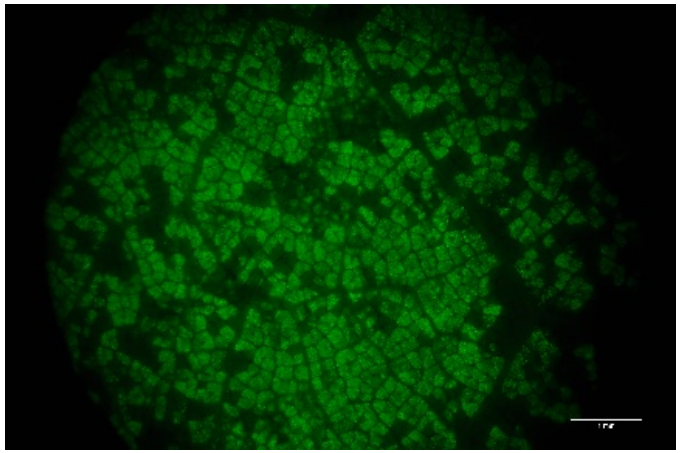
Transformation of Cacao Leaves with CRISPR-Cas9 Vector

Collect Leaves

Cut into sections



Vacuum Agroinfiltrate

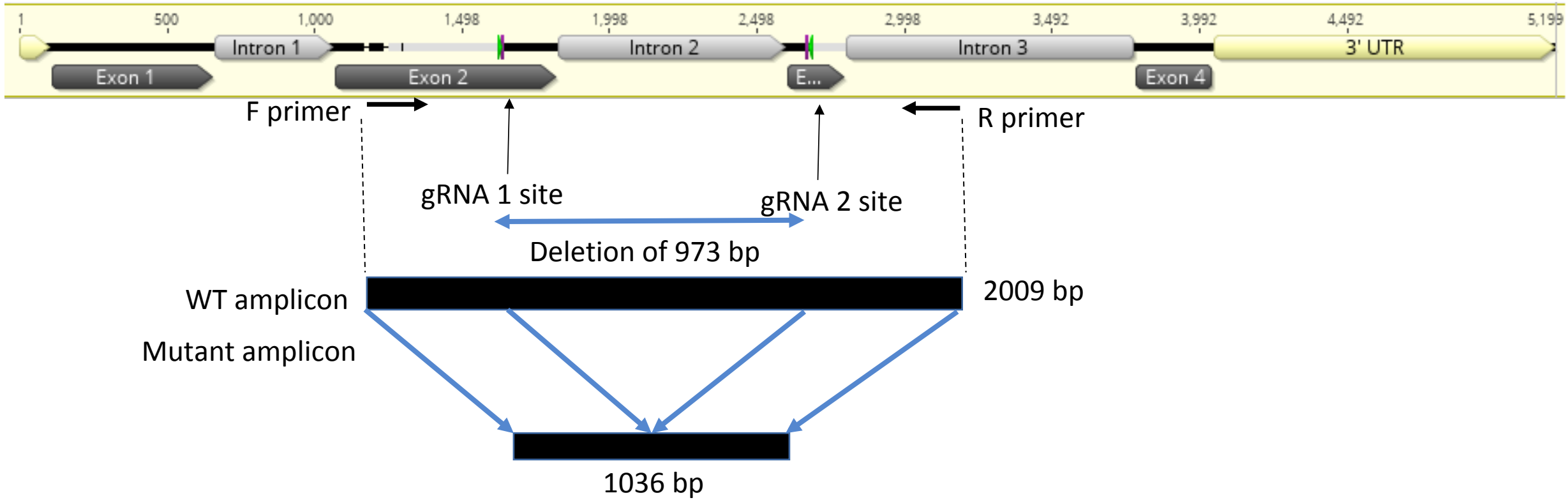


Screen for successful transformation

Extract DNA for Detection of Mutations

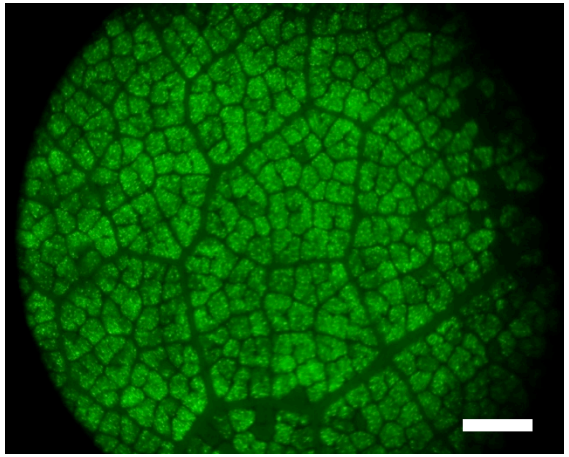
If CAS9 cleaves NPR3 at both targeted sites, we expect a 973 bp deletion and gene inactivation

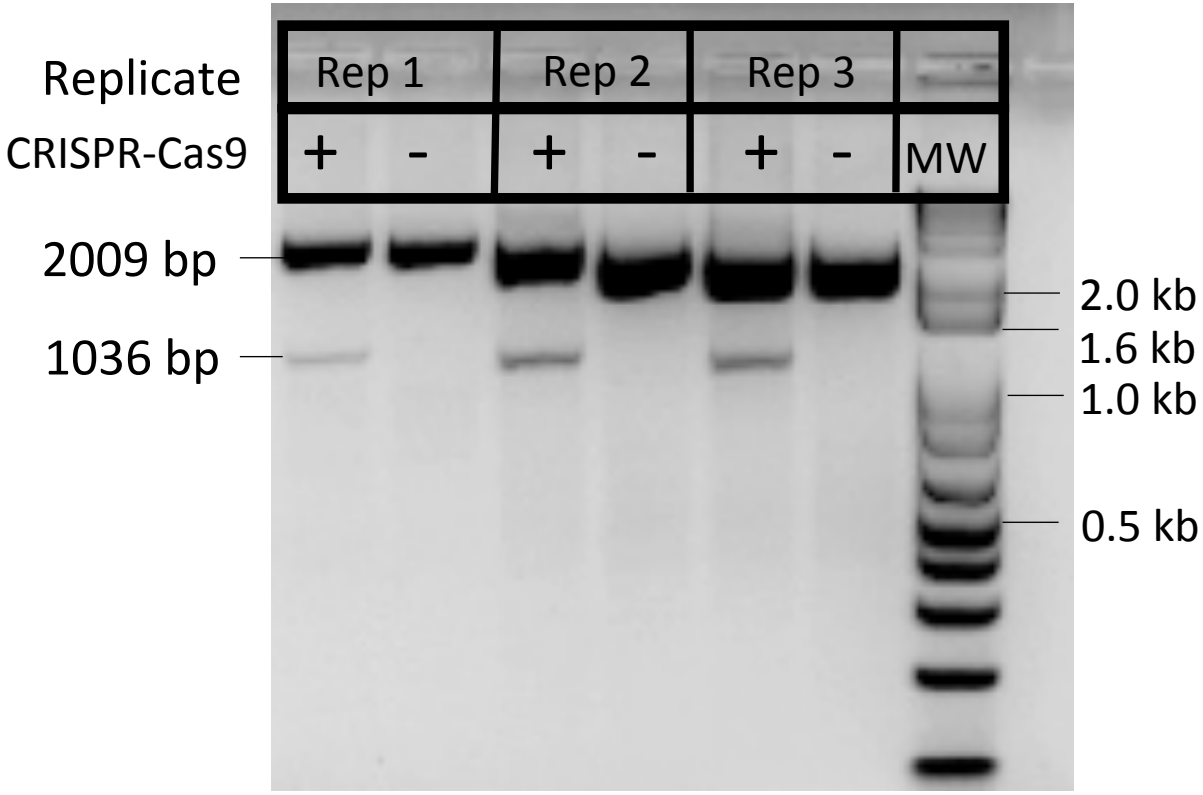
NPR3 Gene Model



Detection of edited cacao genomic DNA

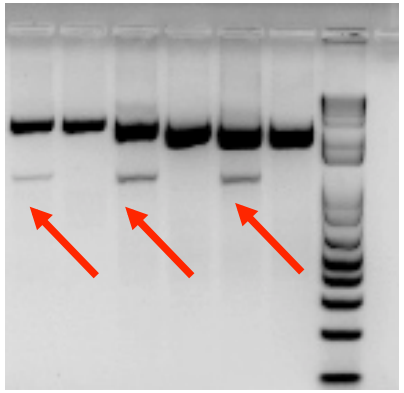
Very high transformation success rate

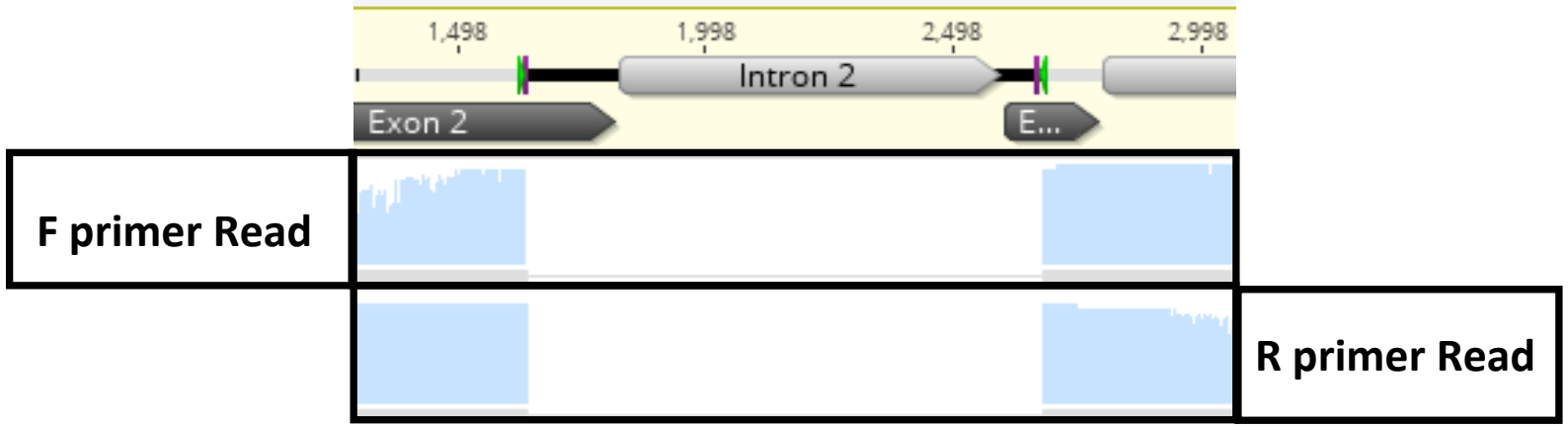




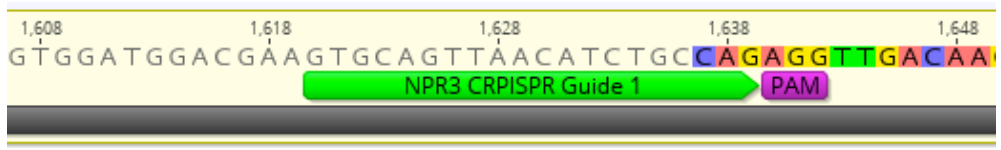
Upper and lower bands were purified and cloned.

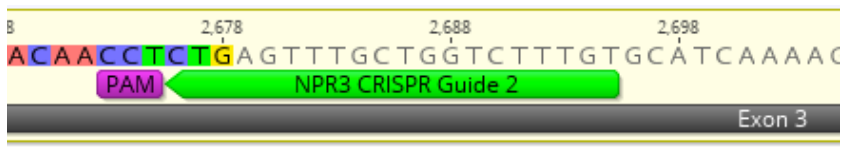
Precise 973 bp Deletion in NPR Gene Was Detected



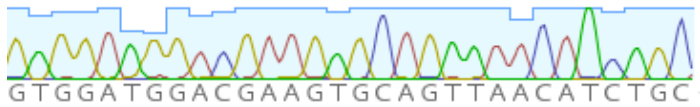


32% of total DNA was cut (molar basis)

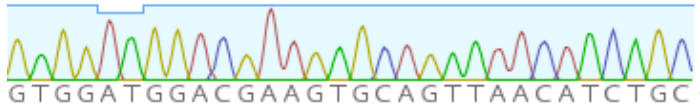




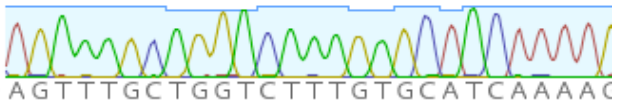
Leaf 1

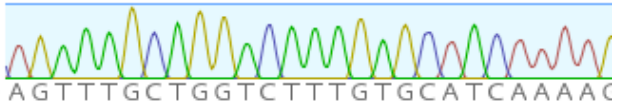


Leaf 2

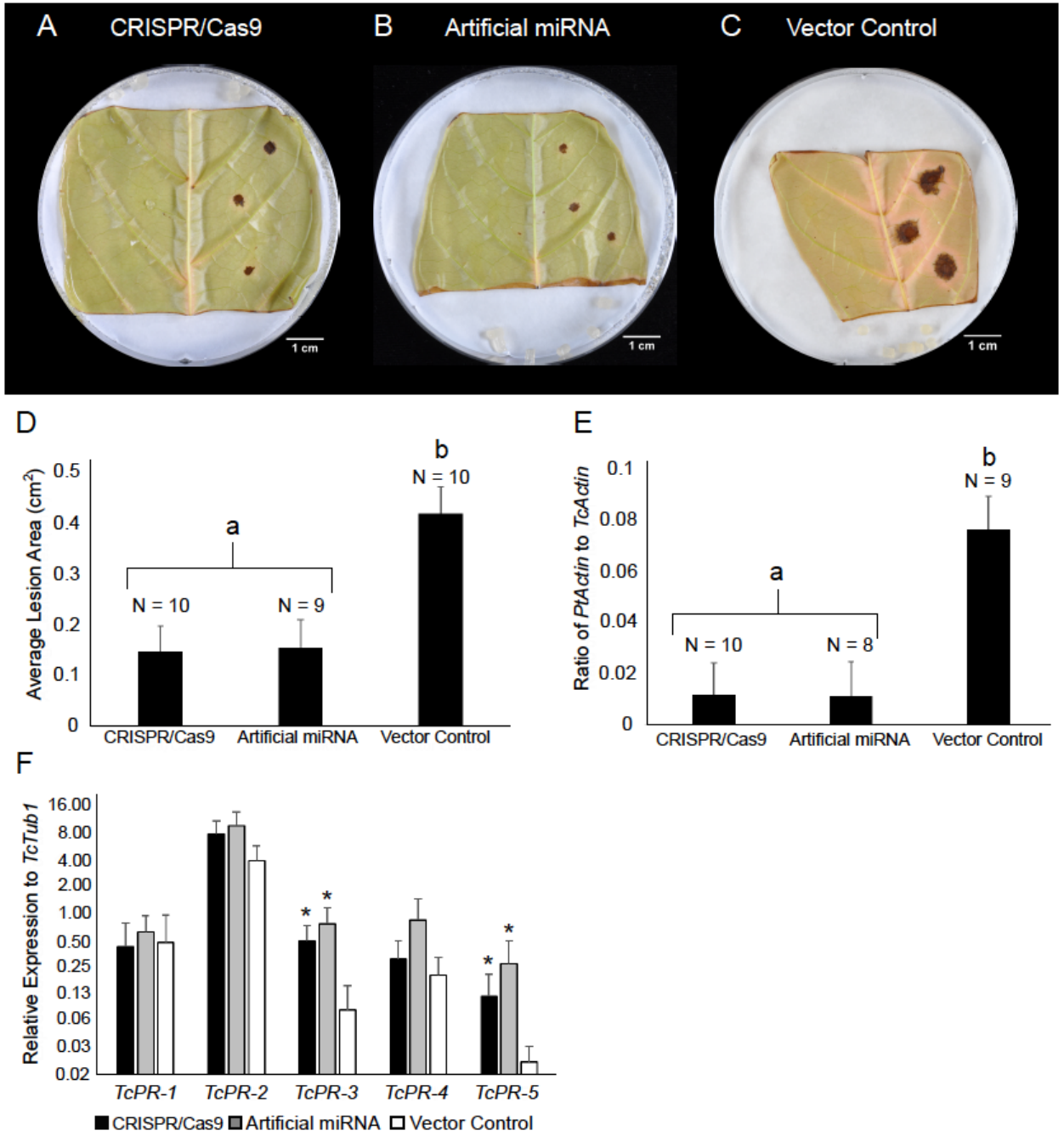


973 bp deletion



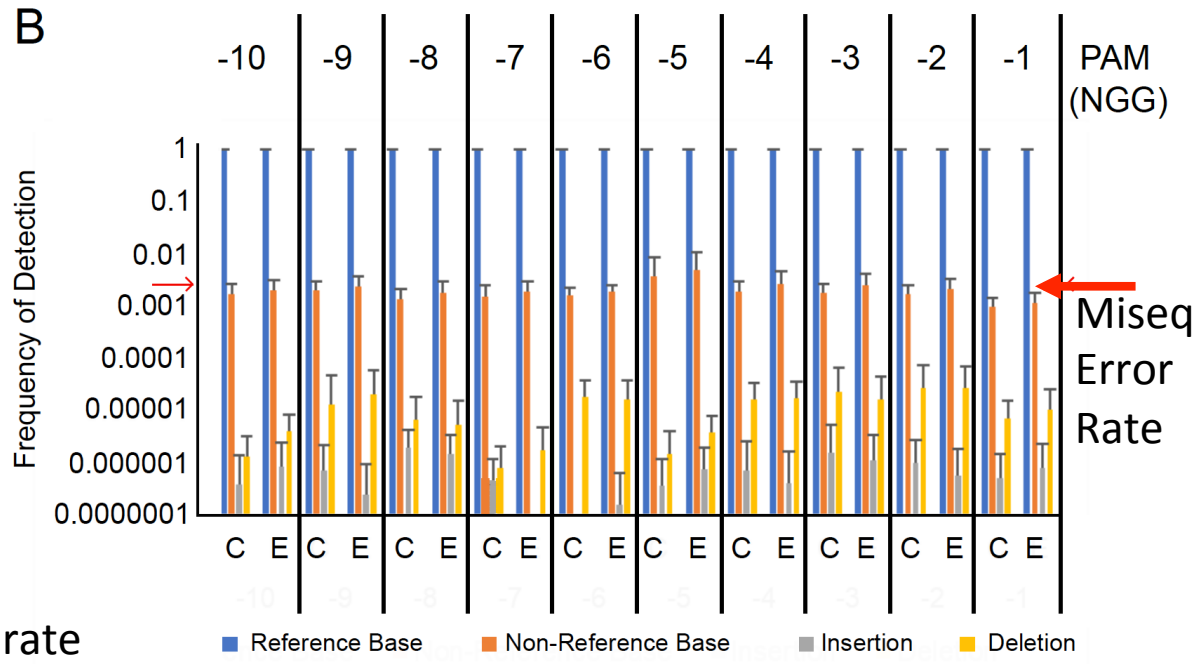


NPR3 Editing INCREASES Disease Resistance and Increases Expression of Specific PR Genes



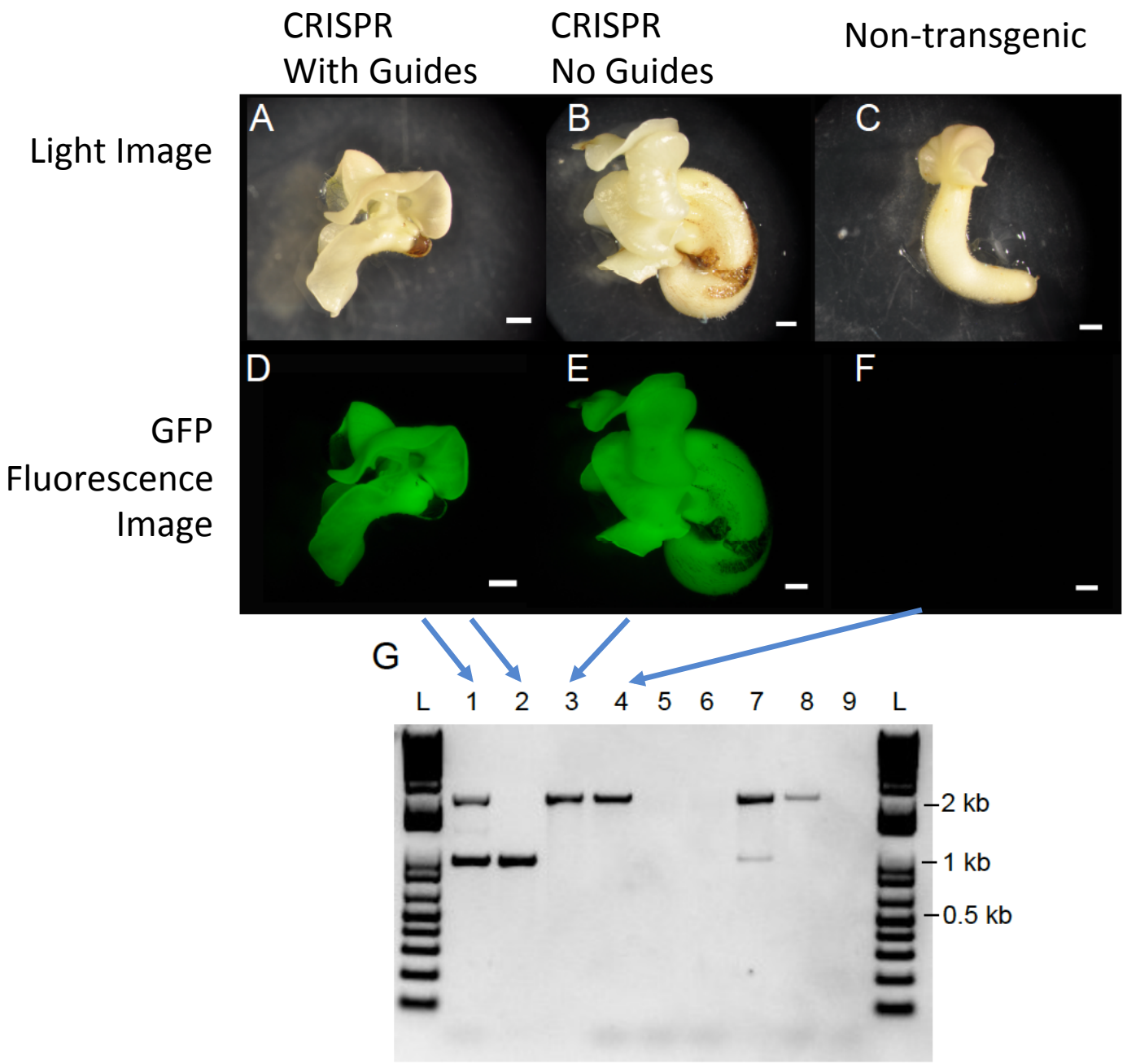
Off Target Mutations NOT Detected at 5 Most Closely Related Sites in Cacao Genome

ID	Sequence	Mismatches (#)
sgRNA 1	GTGCAGTTAACATCTGCCAGAGG	
sgRNA 1 Offtarget 1	GTGCCATTAATATCTGCCACAGG	3
sgRNA 1 Offtarget 2	GTGCAGTAAATGGCTGCCAGAGG	4
sgRNA 1 Offtarget 3	GTGCAGTTGACATCTCCCTGAGG	3
sgRNA 1 Offtarget 4	GAGCAGTTAAAAGCTCCCAGAGG	4
sgRNA 2	ACAAAGACCAGCAAACTCAGAGG	
sgRNA 2 Offtarget 1	TAAAAGACAAGCAAACACAGAGG	4
sgRNA 2 Offtarget 2	AAATAGACAAGCAAACTAAGAGG	4
sgRNA 2 Offtarget 3	ACAAATACCAGCAAATTCAAAGG	3
sgRNA 2 Offtarget 4	ACAAAGACAAGCAAACAGAGAGG	3
sgRNA 2 Offtarget 5	ACTAGAAGCACCAAACTTTGAGG	7



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?

Acknowledgements:

Special thanks to:

Penn State - Yinong Yang

Bastian Minkenberg

UC Berkeley - Brian Staskawicz

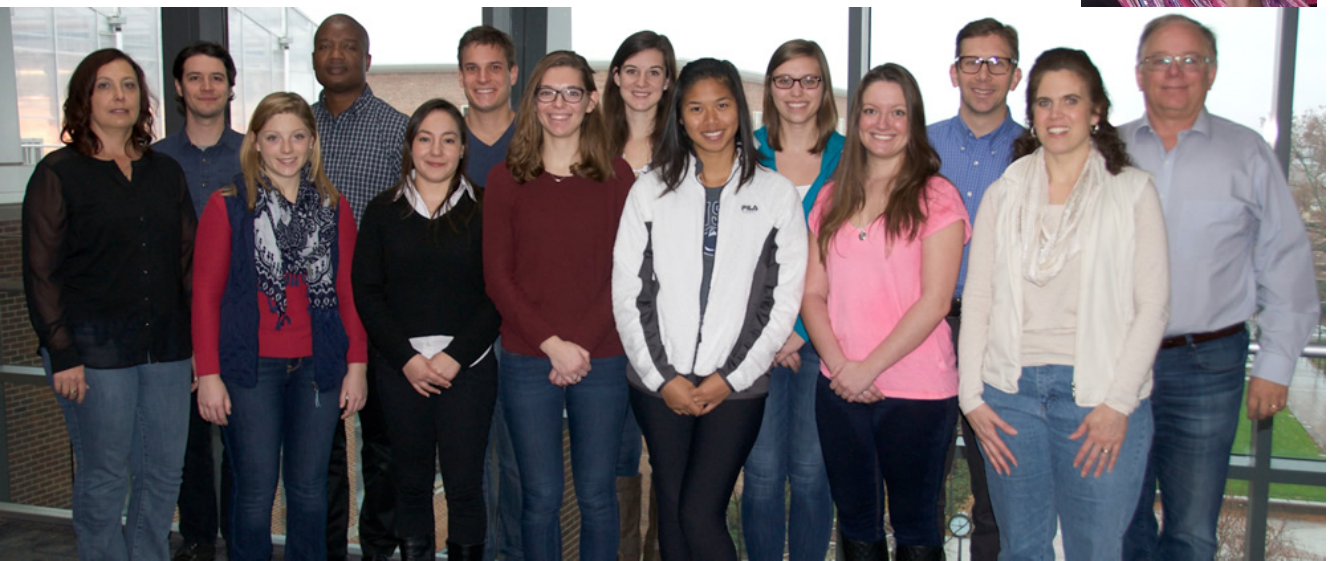
Daniela Thomazella

Douglas Dahlbeck

Andrew Fister

Lena Sheaffer

The Guiltinan-Maximova Lab



PennState
College of Agricultural Sciences

Program in the Molecular Biology of Cacao

USDA

NSF

This work is supported by the USDA National Institute of Food and Agriculture Hatch project 1003147 and NSF Plant Genome Research Award 1546863.