Stories from the lab, stories from the field: advancing crop biotechnology
THE IMPORTANCE OF CASSAVA

ADOPTION AND DEVELOPMENT OF AGBIOTECH

DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

CRISPRing CASSAVA
## THE IMPORTANCE OF CASSAVA

<table>
<thead>
<tr>
<th>CROP</th>
<th>PRODUCTION (Mt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>1’038</td>
</tr>
<tr>
<td>Rice</td>
<td>741</td>
</tr>
<tr>
<td>Wheat</td>
<td>729</td>
</tr>
<tr>
<td>Potato</td>
<td>385</td>
</tr>
<tr>
<td><strong>Cassava</strong></td>
<td><strong>270</strong></td>
</tr>
<tr>
<td>Barley</td>
<td>144</td>
</tr>
<tr>
<td>Sorghum</td>
<td>104</td>
</tr>
</tbody>
</table>

FAOSTAT, 2014
Cassava, a world crop
• Originates from South America (Mato grosso)
• Cassava ranks in the top 5 staple crops
• Daily food for more than 600 million – 1 billion people
• In Sub-Saharan African regions, up to 60% of the daily calories intake
• Industrial crop for starch & biofuel production (Nigeria, China, India, Thailand, Indonesia)
THE IMPORTANCE OF CASSAVA
Cassava starch

Most traded starch globally

Value ≈ 2 billion $

Cassava starch Exports (2011-2015)

Largest exporter: Thailand
Largest producer: Nigeria

Source: COMTRADE

Source: CIAT
THE IMPORTANCE OF CASSAVA
New market opportunity: WAXY

- In most cassava varieties, the amylose content varies from **17% to 25%**

- **Waxy starch (low amylose)** is of high interest to the food industry because of its **lower starch retrogradation** (loss of water after the cooked product cools down)

- Cassava starch paste has proved to be **50% clearer** and **twice as resistant** to freezing as waxy maize.

- Cassava starch is also **more soluble** and **absorbs more water** with limited water losses after refrigeration and freezing
High quality affordable beer for lower income consumers

70% of the extract supplied by cassava and the remaining coming from barley malt

Clear lager look-alike: 6.5% v/v alcohol

10% excise vs. 40% for imported lager

Allowing pricing at 25 MT (0.33 $) vs. 35 MT (0.46 $) for mainstream economy beer

Impala lager launched in Mozambique by SAB Miller

Adapted from G. Van Houten (SAB)
**THE IMPORTANCE OF CASSAVA**

**Cassava Yield**

- Cassava yields up to **90 t ha\(^{-1}\)** (Columbia and India, experimental conditions)
- Under experimental conditions, **50-60 t ha\(^{-1}\) in East Africa** (Fermont et al., 2009)

<table>
<thead>
<tr>
<th>Continent</th>
<th>Average Yield (t/Ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asia</td>
<td>24.1</td>
</tr>
<tr>
<td>South America</td>
<td>15.5</td>
</tr>
<tr>
<td>Africa</td>
<td>9.2</td>
</tr>
</tbody>
</table>

(data from FAO, 2014)
THE IMPORTANCE OF CASSAVA
Cassava Yield

World cassava yield (1960 – 2014)
THE IMPORTANCE OF CASSAVA
Cassava Agrosystems
THE IMPORTANCE OF CASSAVA
Cassava Agrosystems

**Agrosystems**
Agronomical practices
Fertilizers

...  

**Abiotic stresses**
Salt stress
Drought stress
Cold stress

...  

**Biotic stresses**
Pests (whitefly, mealybug, mite, etc.)
Cassava Mosaic Disease
Cassava Brown Streak Disease
Cassava Bacterial Blight

...  

**Post-harvest**
Post-harvest Physiological Deterioration
Secondary deterioration
MAJOR CONSTRAINTS TO CASSAVA PRODUCTION

SOUTH AMERICA
- Bacterial blight
- Drought
- Post-harvest
- Pests

AFRICA
- Viral diseases
- Post-harvest
- Drought
- Pests

INDIA
- Viral diseases
- Pests
- Post-harvest

SE ASIA
- Post-harvest
- Pests
THE IMPORTANCE OF CASSAVA
Conventional breeding

- Cassava is highly **heterozygous** (selection of dominant traits, difficulty to identify parental lines with good breeding value)

- Cassava has a relatively **long life cycle** (assessment of several traits is long and difficult)

- **Flowering** is genotype- and environment-dependent

- **Initial breeding efforts** were towards the development of high yielding varieties (70’s and 80’s)
THE IMPORTANCE OF CASSAVA
Cassava Biotechnology

- Possibility to engineer traits not present in the cassava germplasm and in wild-relatives
- Rapid introgression of improved traits in farmer- & industry-preferred cultivars
- Combination of multiple improved traits
  
- Genetic transformation is a tedious process restricted to a few cultivars
- Cassava-specific trait with no available data from model plant species
- Public opposition to GM in countries where cassava is grown
Figure 1 Factors in the adoption and development of agbiotech in sub-Saharan Africa.
Agrobacterium-mediated transformation of friable embryogenic calli and regeneration of transgenic cassava
Effective transfer of tropical crop biotech to African R&D institutions

TRAINEE IN EUROPE

BecA (Nairobi, Kenya)

Farmer-preferred cultivars
Serere, Ebwanatereka, Kibandameno

Hands-on WORSHOPS in TZ

MARI (Dar es Salaam, Tanzania)

Proof-of-concept model cultivar (60444)

Hands-on WORSHOPS in SA

Witwatersrand University
(Jo’burg, SA)

Model (60444) and industry-preferred (T200) cultivars

BNARI (Accra, Ghana)

Farmer-preferred cultivar (ADI 001)
ADOPTION AND DEVELOPMENT OF AGBIOTECH IN SUB-SAHARAN AFRICA

CTCRI (Trivandrum, India) Tech Transfer

BNARI (Accra, Ghana) Tech Transfer, virus resistance

FUC (Fortaleza, Brazil) Tech transfer, drought resistance

University of Zimbabwe Tech transfer

BecA, IITA (Nairobi, Kenya) Tech transfer, drought, field trials

IAG (Hanoi, Vietnam) Tech transfer

LIPI (Bogor, Indonesia) Tech transfer, post-harvest

MARI (Tanzania) Tech transfer

Witwatersrand (SA) Tech transfer, virus resistance

U. Los Andes (Columbia) Tech Transfer, bacterial blight

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DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

CRISPRing CASSAVA
MAJOR CONSTRAINTS TO CASSAVA PRODUCTION

- **SOUTH AMERICA**: Bacterial blight, Drought, Post-harvest, Pests
- **AFRICA**: Viral diseases, Post-harvest, Drought, Pests
- **INDIA**: Viral diseases, Pests, Post-harvest
- **SE ASIA**: Post-harvest, Pests
**Cassava Mosaic Disease**

- Whitefly-transmitted
- Infection front moving at 30-100km/year
- Severe symptoms
- Infections at early developmental stage

Africa-wide losses due to CMD

24% of total production

India losses due to CMD

40% in Tamil Nadu

**Cassava Brown Streak Disease**

- Genome sequence
  - Released in 2009, 2 viral species
- Highly infectious on CMD-resistant germplasm deployed in the 80-90’s
- Mild leaf symptoms, dark brown-black necrotic rot of the tuberous roots

Losses in east Africa estimated at 100 million USD

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Alicai et al., 2007

Kiboko (Kenya) field trial

Alicai et al., 2007

A

B
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

Geminivirus genome organization

Geminivirus diversity

Patil & Fauquet, 2009, MPP
Targeting coding sequences (AC1)

Binary vector for cassava transformation

DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava

Correlation between hairpin-derived small RNAs and virus resistance

Vanderschuren et al., 2009, Plant Molecular Biology
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

Development of high-throughput inoculation methods to screen for virus resistance

AGROCLONE TANDEM REPEAT DNA A

AGROCLONE TANDEM REPEAT DNA B

Lentz et al., 2018, Plant Methods
Field trial at Alupe KARI field station (Kenya) in collaboration with Masinde Muliro University of Science and Technology (MMUST)
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

Collaboration with Prof. Hassan Were (MMUST, Kenya)
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

Before the hailstorm

After the hailstorm

Hailstorm

CMD Symptom Scores

Planting 2

Average
Symptom Score

Weeks After Planting

5 10 15 20 25 30

60444 (WT)
pC1300
duAC1-2
duAC1-101
duAC1-152
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Diversity of viral populations in the field

VIRUS GENOME SEQUENCING

DEPTH

ACCURACY

HiSeq2000

100nt paired reads

~600bp contigs

BLAST against database of Sanger sequenced virus clones to identify dominant ‘strain’

Clone virus

Sanger Sequence

Manual Assembly
CIrcular DNA Enrichment SEQuencing (CIDER-SEQ)

DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Diversity of viral populations in the field

Mehta et al., Nucleic Acids Research, under review
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Diversity of viral populations in the field

Circular DNA Enrichment Sequencing (CIDER-SEQ)

Software development

High quality sequences from geminiviruses in plant tissues

Mehta et al., Nucleic Acids Research, under review
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Diversity of viral populations in the field

Identity to ACMV-NOg genomic sequence

<table>
<thead>
<tr>
<th>Transgenic Lines</th>
<th>Control Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsAC1 152-2, n=137</td>
<td>60444 WT-2, n=153</td>
</tr>
<tr>
<td>dsAC1 152-1, n=77</td>
<td>60444 WT-1, n=61</td>
</tr>
<tr>
<td>dsAC 101-2, n=79</td>
<td>Empty Vector, n=62</td>
</tr>
</tbody>
</table>

%identity to transgene-derived dsRNA
DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA
Diversity of viral populations in the field

Phylogenetic analysis of virus genomes detected in control and transgenic lines

Wild Type & Empty Vector  
\( n = 278 \)

dsAC1 transgenics  
\( n = 375 \)

Mehta et al., Nucleic Acids Research, under review
THE IMPORTANCE OF CASSAVA

ADOPTION AND DEVELOPMENT OF AGBIOTECH

DEVELOPING AND TESTING VIRUS RESISTANT CASSAVA

CRISPRing CASSAVA
CRISPRing CASSAVA

- Flowering 6 to 16 months after planting
- **Irregular** and **non-uniform** flowering pattern (difficult crossing)

CASSAVA GROWTH CYCLE

Cassava grows from stakes cut from the plant's stems. After 3 months, some of its fibrous roots begin to swell with starch relocated from the leaves. Most of the root starch forms after the sixth month, when the plant also achieves maximum canopy size.
CRISPRing CASSAVA

- Expression of Arabidopsis FT protein triggers early flowering in cassava (starting at 2 – 3 months after multiplication)

- Fertile flowers are induced by FT under greenhouse conditions

McGarry and Kragler, 2013, TiPS

FT-expressing cassava

Bull et al., 2017, Plants
CRISPRing CASSAVA

- Granule-bound starch synthase (GBSS) and protein targeting to starch (PTST) can be targeted to generate waxy starch

- Single copy genes in cassava
## CRISPRing CASSAVA

### A. GBSSsgRNA4 target

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>gbss-TB</th>
<th>gbss-TW</th>
<th>gbss-TAB</th>
<th>gbss-TAH</th>
<th>gbss-TAU</th>
<th>gbss-DAO</th>
<th>gbss-TAQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Reads</td>
<td>97.6</td>
<td>48.6</td>
<td>44.1</td>
<td>44.1</td>
<td>43.5</td>
<td>45.1</td>
<td>46.0</td>
<td>96.3</td>
</tr>
<tr>
<td>Total # Reads</td>
<td>2934</td>
<td>2742</td>
<td>2570</td>
<td>2565</td>
<td>3021</td>
<td>2786</td>
<td>2860</td>
<td>3112</td>
</tr>
</tbody>
</table>

### B. PTSTsgRNA2 target

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ptst-TE</th>
<th>ptst-TAC</th>
<th>ptst-TAI</th>
<th>ptst-TAK</th>
<th>ptst-DAO</th>
<th>ptst-DAO</th>
<th>ptst-TAQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Reads</td>
<td>91.1</td>
<td>46.3</td>
<td>92.8</td>
<td>92.5</td>
<td>82.8</td>
<td>56.2</td>
<td>48.2</td>
<td>59.5</td>
</tr>
<tr>
<td>Total # Reads</td>
<td>1942</td>
<td>1428</td>
<td>1053</td>
<td>794</td>
<td>1190</td>
<td>1594</td>
<td>1828</td>
<td>1615</td>
</tr>
</tbody>
</table>

### C. Diagram showing frameshift in WT and various genotypes

- **WT**: Normal sequence without frameshift.
- **gbss-TB**: Decrease in % reads with frameshift.
- **gbss-TW**: Similar to WT.
- **gbss-TAB**: Minor frameshift.
- **gbss-TAH**: Significant frameshift.
- **gbss-TAU**: Minor frameshift.
- **gbss-DAO**: Frameshift.
- **gbss-TAQ**: Frameshift.

### D. Diagram showing amino acid positions and frameshift

- **WT**: Normal sequence.
- **ptst-TE**: Frameshift in amino acids.
- **ptst-TAC**: Frameshift.
- **ptst-TAI**: Frameshift.
- **ptst-TAK**: Frameshift.
- **ptst-DAO**: Frameshift.
- **ptst-TAQ**: Frameshift.
- **ptst-TBC**: Frameshift.

---

Bull et al., 2018, Science Advances
**CRISPRing CASSAVA**

**A.**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>gbss-TAH</th>
<th>gbss-TAB</th>
<th>gbss-DAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="https://example.com/image1.png" alt="Image" /></td>
<td><img src="https://example.com/image2.png" alt="Image" /></td>
<td><img src="https://example.com/image3.png" alt="Image" /></td>
<td><img src="https://example.com/image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**B.**

<table>
<thead>
<tr>
<th></th>
<th>Root 1</th>
<th>Root 2</th>
<th>Root 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td><img src="https://example.com/image5.png" alt="Image" /></td>
<td><img src="https://example.com/image6.png" alt="Image" /></td>
<td><img src="https://example.com/image7.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>gbss-TAH</strong></td>
<td><img src="https://example.com/image8.png" alt="Image" /></td>
<td><img src="https://example.com/image9.png" alt="Image" /></td>
<td><img src="https://example.com/image10.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>ptst-TAC</strong></td>
<td><img src="https://example.com/image11.png" alt="Image" /></td>
<td><img src="https://example.com/image12.png" alt="Image" /></td>
<td><img src="https://example.com/image13.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**C.**

![Graph showing % amylose](https://example.com/graph.png)

**D.**

![Densitometry analysis](https://example.com/densitometry.png)

**Iodine** staining of amylose (blue color)
CRISPRing CASSAVA

A.  

B.  

C.  

D.  

<table>
<thead>
<tr>
<th>GBSSsgRNA4 target</th>
<th>% Reads</th>
<th>#Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>gbss-TAH S1-1</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>gbss-TAH S1-2</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>gbss-TAO S1-1</td>
<td>42.8</td>
<td>35</td>
</tr>
<tr>
<td>gbss-TAO S1-2</td>
<td>53.3</td>
<td>45</td>
</tr>
</tbody>
</table>

Bull et al., 2018, Science Advances
Schematic representation of the designed NPBT and conventional breeding for trait improvement. (A) Pathway for breeding of recessive traits using a wild relative or mutagenized plant as parent material. Multiple crosses required to introgress a homozygous mutant into a farmer-preferred genotype. Several years required to generate improved variety. (B) NPBT described here for accelerated flowering and segregation of genome-edited lines. Agrobacterium-mediated stable transformation provides transgenic lines with different genome edited populations. Several mutant lines are screened (phenotype and genotype) and a single segregation from selfing can occur ex situ in a glasshouse environment yielding T-DNA-free progeny with the homozygous mutation. Transformation to field-testing can be achieved in approximately 2.5 years.
### ACKNOWLEDGEMENTS

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Hassan Were  

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**University of Greenwich**  
Maruthi Gowda  

**INRA**  
Jean-Luc Gallois  

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PLANT GENETICS LAB

http://www.gembloux.ulg.ac.be/plant-genetics/
Vitamin B6 deficiency has been hypothesized to contribute to nodding syndrome.

<table>
<thead>
<tr>
<th></th>
<th>Cassava consumption</th>
<th>Vitamin B6 deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>64%</td>
</tr>
<tr>
<td>Nodding cases</td>
<td>100%</td>
<td>73%</td>
</tr>
</tbody>
</table>

Fotz et al. 2013

Could the parasite behind onchocerciasis, better known as river blindness, explain the odd "nodding" seizures in a growing number of African children? Figure 2. Epidemic curve of nodding syndrome cases in Kitgum District, Uganda, by year of onset. Modified from Fotz et al. (6).
NUTRITIONAL IMPORTANCE OF VITAMIN B6

Vanderschuren et al., 2013, Frontiers in Plant Science

Physiological functions
- prevention of cardiovascular disease
- red blood cell formation
- relief of depression
- antitumor agent
- hormone function
- immune system function

Biochemical functions
- nucleic acid biosynthesis
- lipid metabolism
- glucose metabolism
- amino acid metabolism
- hemoglobin biosynthesis
- neurotransmitter biosynthesis
- potent antioxidant
Transgenic PDX1-PDX2 cassava accumulate high levels of B6 in leaves and roots (up to 9-fold & 16-fold over wild-type levels, respectively)

Collaborator: Fitzpatrick Lab

Li et al., 2015, Nature Biotechnology
Hypothesis of human intestinal vitamin B6 metabolism

Albersen et al., 2013, PloSONE
After boiling, transgenic PDX1-PDX2 leaves and roots retain high levels of B6 (up to 22-fold & 9-fold over wild-type levels, respectively).

Based on Caco-2 cells assay, increased vitamin B6 levels in transgenic cassava results in increased amount of bioavailable vitamin B6.

500 g of cooked cassava roots or 50 g of cooked cassava leaves per day provide recommended daily intake of vitamin B6.

Monica Albersen (U Utrecht)

Li et al., 2015, Nature Biotechnology
ACKNOWLEDGEMENTS

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