Progress towards development of WRSP Varieties using biotechnology

SASHA

Sweetpotato Action for Security and Health in Africa

Gorrettie Ssemakula, Marc Ghislain, & Lydia Wamalwa on behalf of the WRSP team

Last year, we had …

- **3 African PhD students** in their last step toward defense and several publications in the pipeline
- **Tested Bt transgenic events** expressing one or a combination of 2 *cry* genes coding for the weevil-toxins: Cry7Aa1, Cry3Ca1, ET33-34:
  - not observed activity against weevils for the 31 transgenic events from Jewel
  - one event 9.5 with low activity against weevils (storage roots harvested from a CFT in Puerto Rico)
  - 64 transgenic events with *cry7Aa1*, *cry3Ca1* genes to test
  - low accumulation of Cry proteins (<0.5 µg / g fresh flesh)
- **New strategy BT & RNAi (GM Corn → GM SP)**
  - develop new *cry* gene constructs: from sweetpotato-like genes to chimeric genes with enhancer 35s promoter, translation enhancers in the 5'UTR, and pro-toxin
  - identify essential weevil genes and test RNAi against weevil larvae
- **Toxicity of undamaged parts of fungal-infected storage roots**
  - Detected ipomeamarone, a toxic phytoalexin, at significant level.

Objectives of year 5 SASHA

SASHA
Sweetpotato Action for Security and Health in Africa

We wanted to:

1. 3 PhD students to graduate and publish
2. Quantify Cry protein accumulation in the remaining 64 transgenic events
3. Finish the *cryET33* plus *cryET34* gene constructs
4. Assess whether higher level of Cry protein accumulation can be expected
5. Transform most competent varieties with new gene constructs
6. Identify essential weevil genes and test RNAi against weevil larvae
7. Confirm ipomeamarone detection at significant levels

African PhD students

Capacity building (Ghent University):

- Individual training on biolistic in Ghent, 2-week visit for molecular technique training at BecA
- PhD students:
  - Abel Sefasi – January 29, 2014
  - Runyararo Rukarwa – August 15, 2014
  - Lydia Wamalwa – thesis submitted, 2014

Publication list

Peer-reviewed journals:

1. **Moses E**; **Solera M**, Kyamanywa S, Mwanga R, Odongo B, Ghislain M, Moar WJ (2010). Toxicity of seven *Bacillus thuringiensis* Cry proteins against *Cylas puncticollis* and *Cylas brunneus* (Coleoptera: Brentidae) using a novel artificial diet. Journal of Economic Entomology 103:1493-1502
2. **Sefasi, A**., Kreuze, J., Ghislain, M., Manrique, S., Kiggundu, A., Ssemakula, G., & Mukasa, S. B. (2012). Induction of somatic embryogenesis in recalcitrant sweetpotato (*Ipomoea batatas* L.) cultivars. African Journal of Biotechnology, 11(94), 16055-16064
3. Ghislain M, Tovar J, Prentice K, Ormachea M, Rivera C, Manrique S, Kreuze J, **Rukarwa R**, **Sefasi A**, Mukasa S, Ssemakula S, **Wamalwa L**, Machuka J (2013). Weevil Resistant Sweetpotato through Biotechnology. Acta Hort. 974:91-98
4. **Sefasi, A**., Ghislain, M., Kiggundu, A., Ssemakula, G. and Mukasa, S. B. (2013). Thidiazuron improves adventitious bud and shoot regeneration in recalcitrant sweetpotato. African Crop Science Journal 21: 85-95
5. **Rukarwa R.J**., Prentice K., Ormachea M., Kreuze J.F. , Tovar J., Mukasa S.B., Ssemakula G., Mwanga R.O.M. and Ghislain M. (2013). Evaluation of bioassays for testing Bt sweetpotato events against sweetpotato weevils. African Crop Science Journal, *21(3), 235-244*
6. Manrique-Trujillo, S.M., Díaz; D., Reaño, R.E., Ghislain, M., and Kreuze, J.F. (2013). Sweetpotato plant regeneration via an improved somatic embryogenesis protocol. Scientia Horticulturae, 161: 95–100
7. **Rukarwa, R. J**., K. Prentice, M. Ormachea, J. F. Kreuze, J. Tovar, S. B. Mukasa, G. Ssemakula, R. O. M. Mwanga, and M. Ghislain (2013). Evaluation of bioassays for testing Bt sweetpotato events against sweetpotato weevils. African Crop Science Journal 21, no. 3: 235-244
8. **Rukarwa R.J**., Mukasa S.B., Odongo B., Ssemakula G and Ghislain M. (2013). Identification of relevant non-target organisms exposed to sweetpotato weevil-resistant Bt sweetpotato in Uganda. 3 Biotech, 1-10
9. **Rukarwa, R. J**., Mukasa, S. B., **Sefasi, A**., Ssemakula, G., Mwanga, R. O. M., & Ghislain, M. (2013). Segregation analysis of *cry7Aa1* gene in F1 progenies of transgenic and non-transgenic sweetpotato crosses. Journal of Plant Breeding and Crop Science, 5(10), 209-213

Publication list (pending)

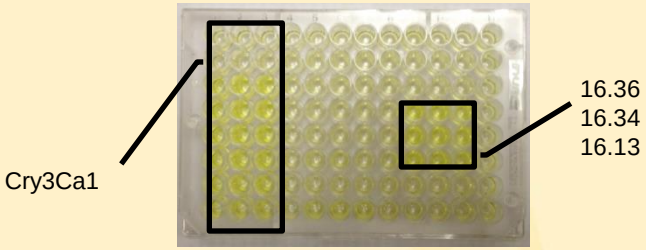
Peer-reviewed journals:

1. Hernández-Martíneza, P., Vera-Velasco, N.M., Martínez-Solís, M., Ghislain, M., Ferré, J., & Escriche B. (2014). Shared binding sites for the *Bacillus thuringiensis* proteins Cry3Bb, Cry3Ca and Cry7Aa in the African sweetpotato pest *Cylas puncticollis* (Brentidae). **Manuscript submitted.**
2. **Wamalwa. L. N**., Cheseto, X., Ouna, E., Kaplan, F., Maniania, N. K., Machuka, J., Torto, B. & Ghislain M. (2014). Ipomeamarone accumulation at potentially toxic levels in apparently healthy parts of sweetpotato (Ipomoea batatas L. Lam) storage roots infected by *Rhizopus stolonifer*. **Manuscript submitted**.
3. **Wamalwa, L. N**.; Tovar, J. C.; Prentice, K., Indieka, A. S.; Machuka, J.; Kreuze, J. & Ghislain M. (2014). Identification of Sub-Saharan African sweetpotato cultivars suitable for efficient genetic transformation. **Manuscript in preparation**.
4. Labarta, R. A., Kiiza, B., Hareau, G. G., Ndirigwe, J., Nasona, G. B., Twiyeze, M., Mwanga, R. O. M., Kreuze, J. F. & Ghislain, M. (2014). Potential economic impacts of transforming genetically a food security crop. The case of weevil and virus resistant sweetpotato in the African Lakes zone. **Manuscript in preparation**.

Cry protein accumulation

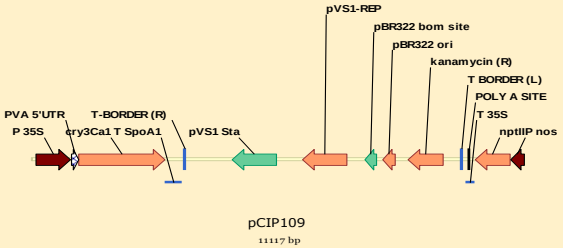
(in events with SP-like *cry* genes)

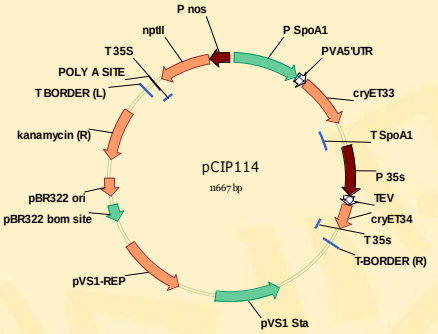
- All 64 transgenic events from ABL plus 15 from BecA produced storage roots in the greenhouse
- Total soluble proteins were extracted from 60 and tested by DAS-ELISA
- **Few events have been detected with 0.5 to 1.4 ug Cry3Ca1 / g fresh flesh**, assuming additional Cry7Aa1 protein, these events are worth now testing activity against weevils (LC50 is 0.4 ug / g artificial diet)



New *cry* gene constructs

- Previous **sweetpotato-like *cry* genes** were based on the use of the sweetpotato β-amylase and sporamin genes (storage root expression and wound induced) where coding sequence was replaced by codon-optimized sequence of the cry7Aa1, cry3Ca1 toxin, and the ET33-34 as a fused protein.
- **New *cry* genes** are based on the use of enhancer 35s promoter, PVA and TEV translation enhancers in 5'UTR, pro-toxin for *cry7Aa1* and *cry3Ca1*, and separate genes for *cryET33* and *cryET34*:
  - **pCIP109** bears [d35s-PVA 5' UTR ; cry3Ca1 pro-toxin codon optimized – 3'UTR sporamin]
  - **pCIP112** bears [d35s-PVA 5' UTR ; cry7Aa1 pro-toxin codon optimized – 3'UTR β-amylase] and [d35s-PVA 5' UTR ; cry3Ca1 pro-toxin codon optimized – 3'UTR sporamin]
  - **pCIP114** bears [PspoA1-PVA 5' UTR ; cryET33 pro-toxin codon optimized – 3'UTR sporamin] and [d35s-TEV 5' UTR : cryET34 pro-toxin codon optimized – 3'UTR 35s]
  - **pCIP115** is same as pCIP114 but PspoA1 is replaced by d35s promoter.



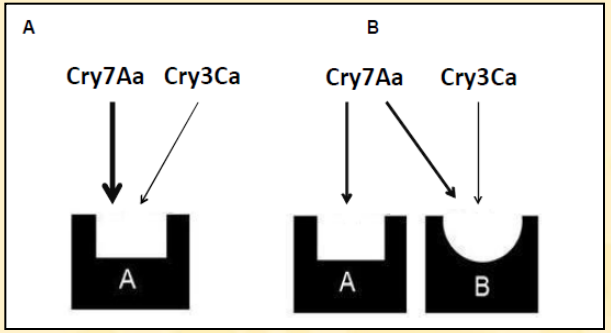


Expression of new *cry* genes

- Transient expression in leaves of *Nicotiana benthamania* (for 35s only):
  - Both *cry7Aa1* and *cry3Ca1* genes are functional
  - *cry3Ca1* gene accumulates much more Cry protein

pCIP	p35s:cry7Aa1	p35s:cry3Ca1
	ug/gr	
112 S1 -Nb	0.162	2.918
112 S2 -Nb	0.094	0.426
112 S3 -Nb	0.117	3.434

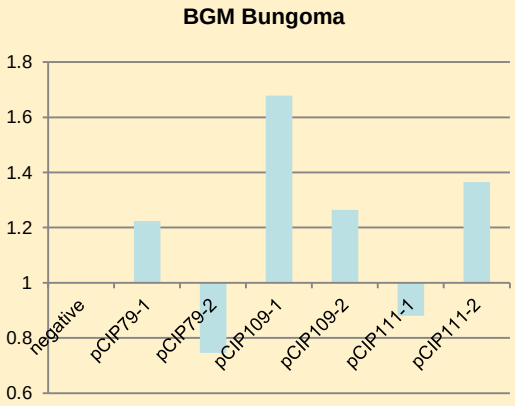
- Partial competition of Cry7Aa1 and Cry3Ca1 for the same binding site of weevil midgut between (University of Valencia, Spain)

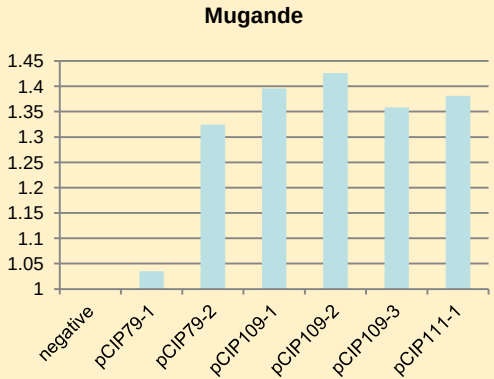


- Focus on ***cry3Ca1***

Expression of new *cry* genes

- Does the new *cry3Ca1* gene express more protein than the previous version?
  - **Transient expression in storage root slices**:
    - Agro-infiltration with vacuum could not detect Cry proteins with DAS-ELISA
    - Biolistic to introduce 3 plasmids encoding cry3CA1 into cells:
      - Target area is small and unpredictable
      - Expression was observed but differences were not reliable





Biolistic devise available at Kawanda in Uganda and Ghent University Belgium

Genetic transformation

- **At ABL:**
  - Imby and Jonathan
  - pCIP109, 112, 114
  - Somatic embryogenesis
- **At BecA / KU:**
  - Ukerewe by organogenesis
  - Kakamega 7 by somatic embryogenesis
  - pCIP109, 112, 114, 115
- **At DDPSC:**
  - PIPI531122
  - pCIP109, 114 (and mix of the 2)

Variety	At	cry3Ca1	cry7Aa1 + cry3Ca1
Imby	ABL	11	14
Jonathan	ABL	5	15
Ukerewe	BecA	0	2

Of the 28 events sent from ABL, 11 survived (Imby) and are under multiplication, new shipment is on its way with 37 events

To do – Bt sweetpotato

- Graduation of Lydia Wamalwa, finish pending publications
- Finalize quantification of Cry3Ca1 protein accumulation from 64 + 15 events bearing the **SP-like *cry* genes** and then test best expressers for activity against the weevils by bio-assays at BecA
- Characterize putative transgenic events with the **new cry3Aa1 gene** and produce at least 30 transgenic events with the **new *cryET33* + *cryET34* genes** and produce storage roots from all confirmed transgenic events with new *cry* genes for screening by DAS-ELISA and best ones for activity against weevils
- Then …

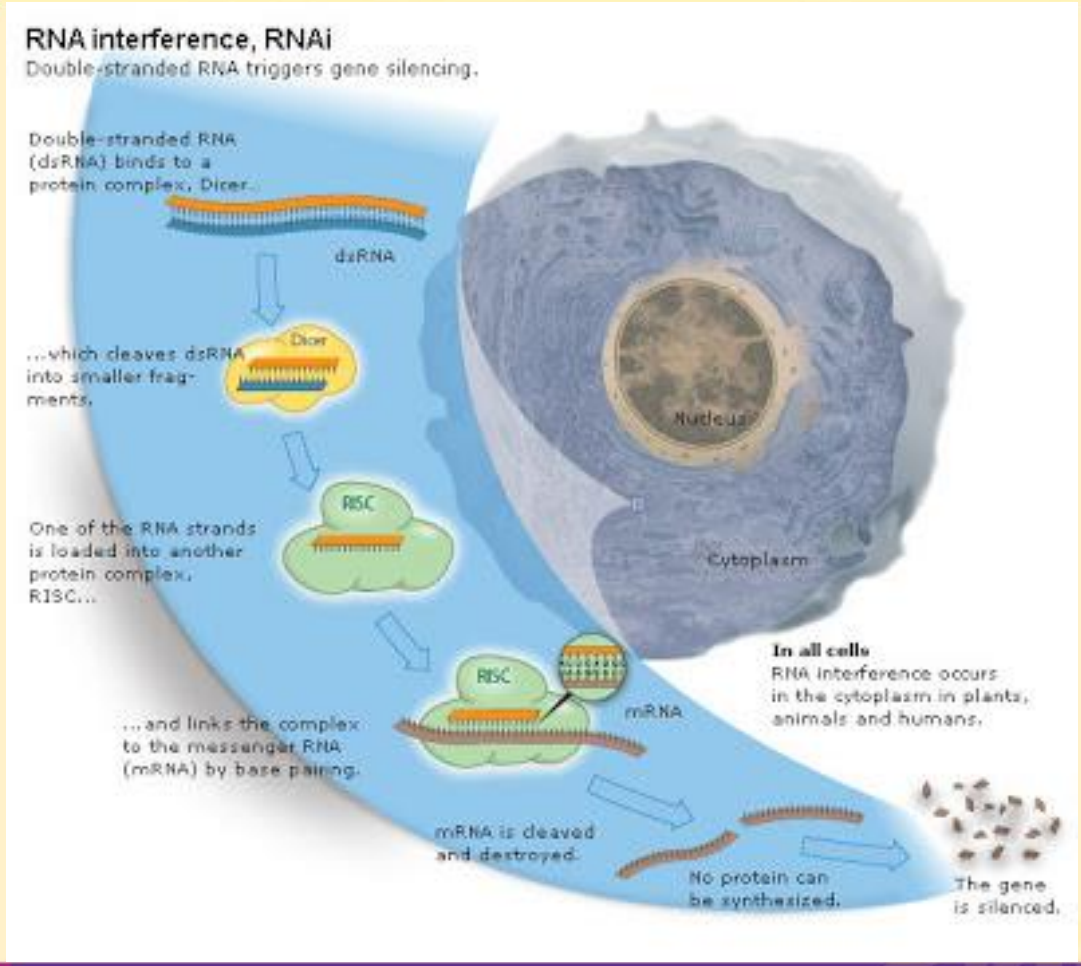
RNAi strategy

- Rationale: We may never reach enough Cry protein in the storage root for full control of the weevils
- Our strategy: Funding from SASHA, GCE9, and University of Ghent
    - Profs. Guy Smagghe and Lieve Gheysen, Katterinne Prentice, PhD student, Ghent University, BE
    - Ana Bailey and Chuck Niblett, Venganza ltd, USA
    - J. Kreuze, M. Ghislain CIP, and G. Ssemakula NaCRRI, SSA
- Similar to Bt (Cry3Bb1)+ RNAi (*Snf7*-RNAi) corn for resistance against western corn rootworm (*Diabrotica virgifera*)

Figure 2: F1 plants expressing a V-ATPase A dsRNA are protected from WCR feeding damage. The plant on left is a non-transgenic control with average root damage, whereas the plant on the right shows the average root protection seen when the transgene is expressed

Baum, James A., et al. "Control of coleopteran insect pests through RNA interference." ***Nature biotechnology*** **25.11 (2007): 1322-1326**

How does it work?



Double stranded RNA:

- RNA viruses
- Hairpin from nuclear (trans)genes
- Synthetic dsRNA

Key features:

- 21-25 nucl. perfect match
- No protein formed
- Autocatalytic

Transcriptome analysis

(*Cylas puncticollis* and *C. brunneus*)

RNAseq (GSL at NCSU) on total RNA extracted from larvae (UG)

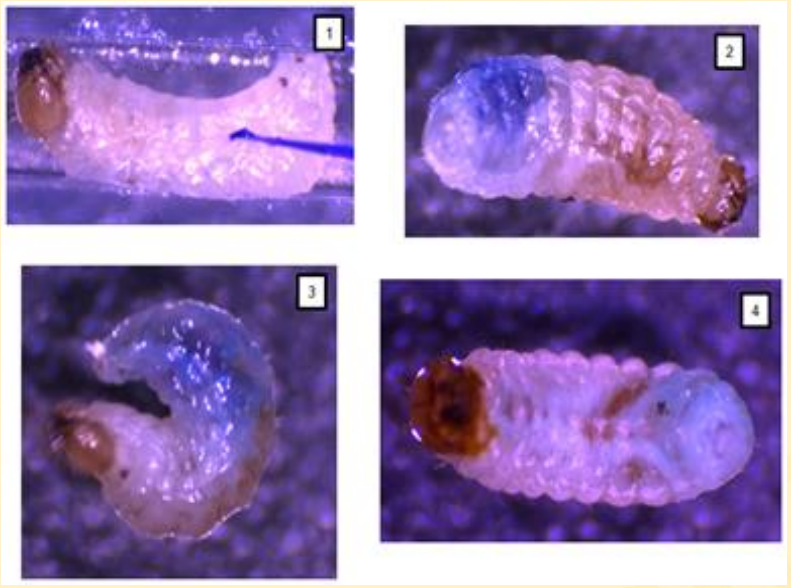
Findings:

- Presence of genes required for dsRNA uptake and RNAi-based gene silencing
- Sequences of 24 essential genes and target sequences identified for both species

dsRNA	Gene
G1	Vha68-2 F1 ATP synthase β subunit
G2	V/A-type ATP synthase catalytic subunit A
G3	Synaptobrevin, isoform A
G4	Pfk phosphofrutokinase
G5	adenylate kinase-2
G6	Focal adhesion kinase isoform D
G7	gamma-coatomer protein, isoform C
G8	delta-coatomer protein, isoform A
G9	alpha-coatomer protein, isoform D
G10	TBP-associated factor 1, isoform D
G11	lethal (2) NC136, isoform B
G12	Proteasome 20 kD subunit
G13	DNA pol interact tpr cont. prot. 47 Kd
G14	alpha-Adaptin, isoform A
G15	Mad1
G16	Ubiquitin conjugating enzyme E2
G17	RNA pol beta subunit
G18	RNA helicase
G19	ribosomal protein S13e
G20	DNA polymerase alpha 50 kD
G21	vATPase A
G22	vATPase D
G23	RPL19
G24	Snf7

Nano-injection with dsRNA

1. Weevil colonies established at Ghent University
2. Automated-nano-injection apparatus FemtoJet (Eppendorf)



(1) Injection with blue dye. **(2)** after 0-1 min. **(3)** 1 h **(4)** 1 day.

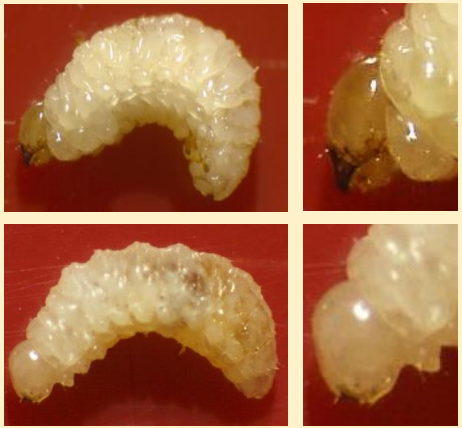
Transcription suppression

Nano-injection of dsRNA for lacasse2 gene (involved in cuticle tanning) in 2nd instar larvae of *C. puncticollis* (362 bp at 0.2 µg/mg BW)

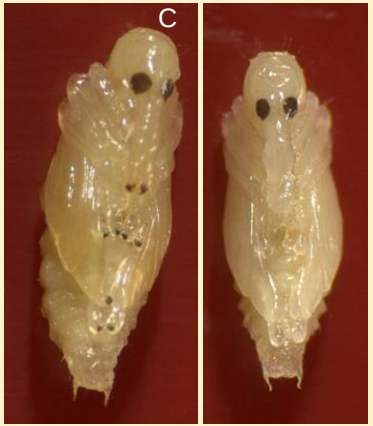
Persistence of the TS effect (>2 weeks after injection, no recovery)

Larvae - control (dsGFP)

Inhibition of *Laccase2* in *C. puncticollis*



Larvae (dsLac2)



Pupa- control (dsGFP)

Pupa (dsLac2)

Adult- control (dsGFP)

Adult (dsLac2)

Adult (dsLac2)

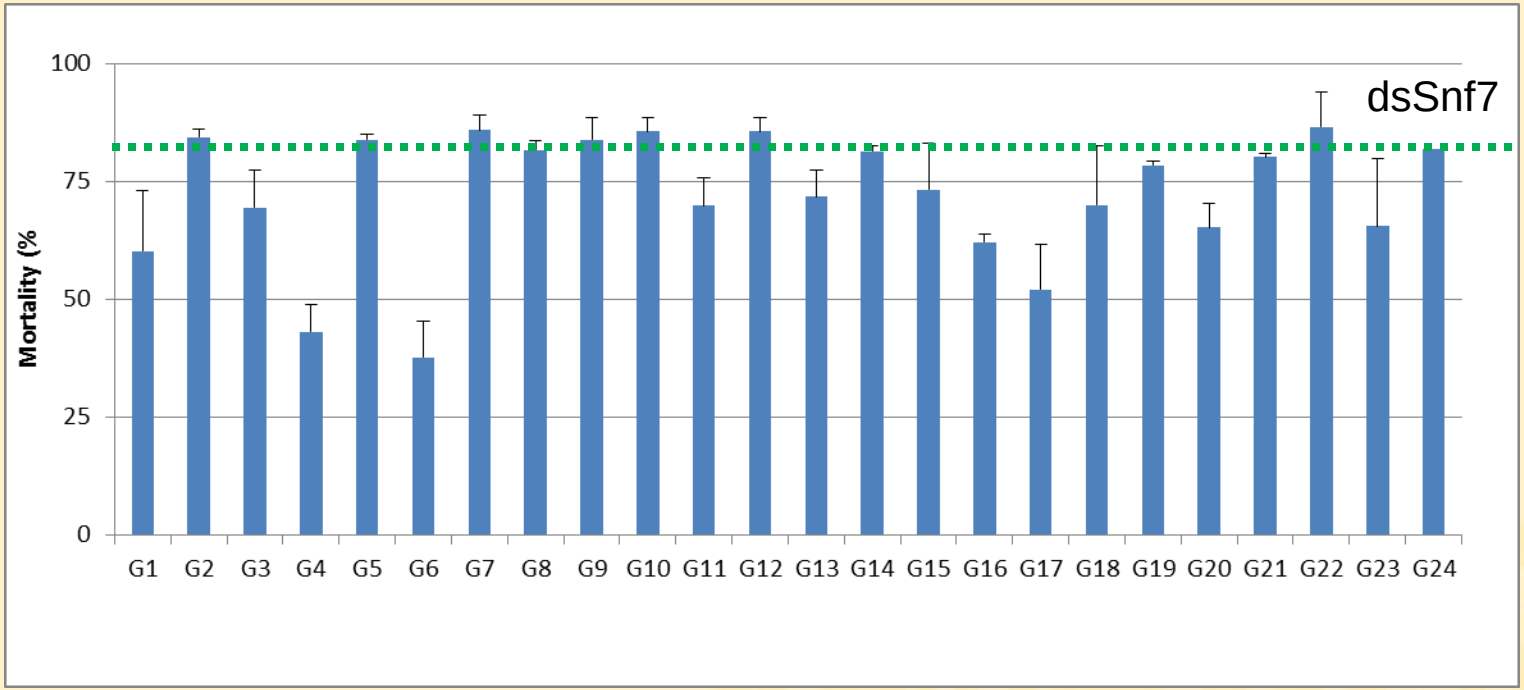
Day 3 **Day 5** **Day 10** **Day 13**

TS of essential genes in *C.p.*

dsRNA for 24 target genes in 2nd instar larvae of *C. puncticollis* (400 bp average, 500 ng/mg BW)

6-8 dsRNA as good as *Snf7* (resistance to WCR)

14 days after injection

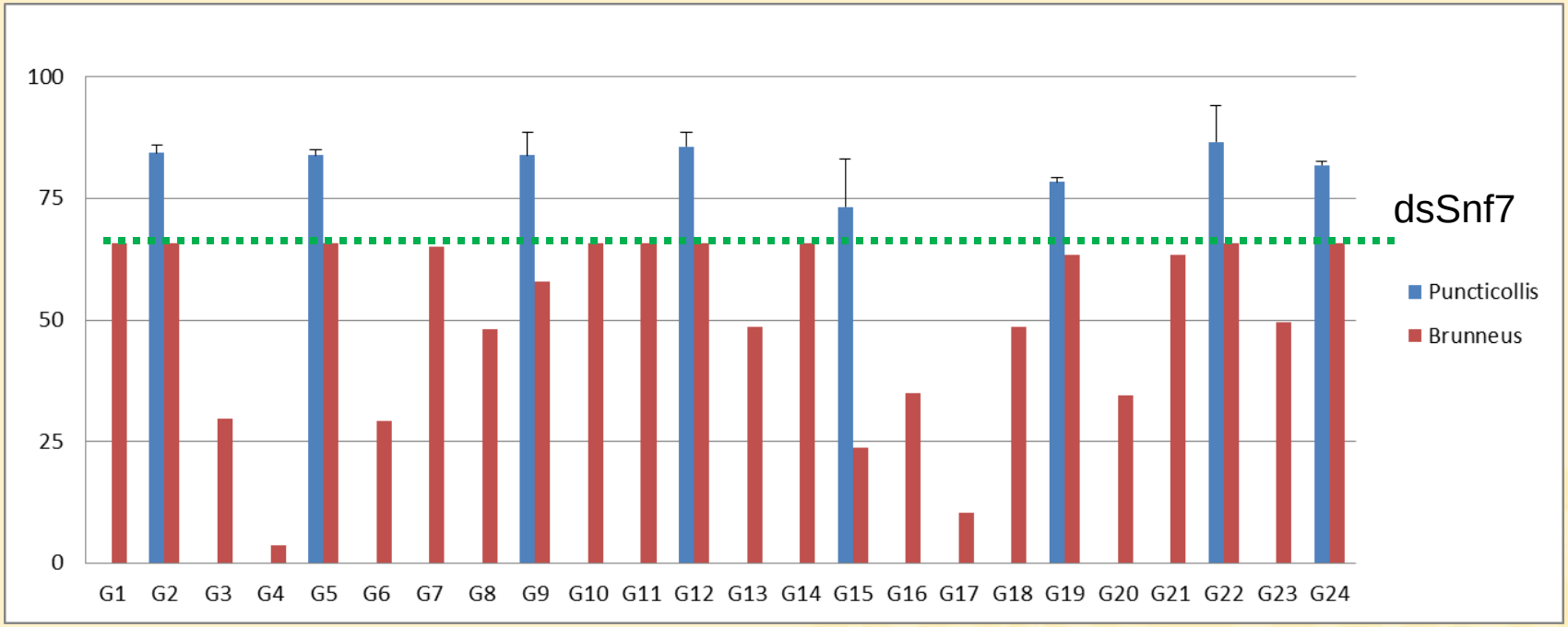


TS of essential genes in *C.b.*

dsRNA for 24 target genes in 2nd instar larvae of *C. brunneus* (same dsRNA and conditions)

Slower but same best dsRNA for *C. puncticollis*

14 days after injection



Feeding with dsRNA

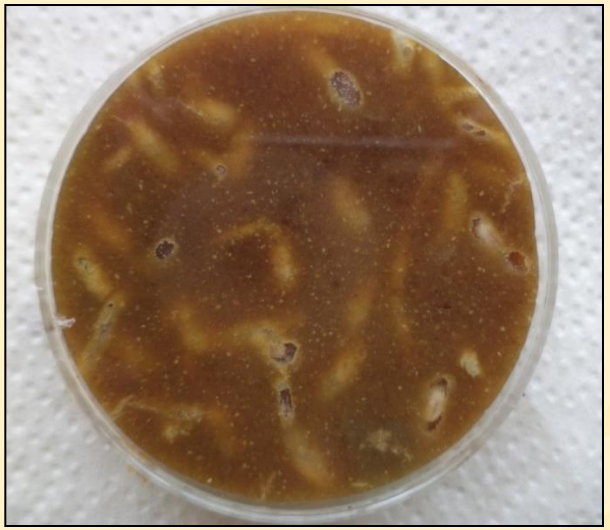
1. **Soaking:** Larvae were soaked for 24 h and afterwards they were transferred to root slices
   - Real-time quantitative PCR on *Lac2* gene to monitor TS

Larvae with blue body after 24 h, indicating the ingestion of the blue dye solution

2. **Artificial diet** (just started)

To do – RNAi strategy

- Improve soaking assay with lipofectamine, larvae evaluated for longer than 10 days and sampled every 3 days for qPCR.
- Artificial diet: implies subcloning the gene sequence in vector for producing higher quantities of dsRNA



Depending results from ingestion (dsRNA degraded before reaching cells, or mortality observed even after only 10 days) we will solicit a phase II GCE9 grant (**November 3rd, 2014 at 1:00 PM (Pacific Time)**

Health concerns from weevil damages

- When weevils (which contribute 28-100% production losses in sweetpotatoes) damage sweetpotatoes, poor farmers cut off the infested part for human consumption: the infected part is fed to farm animals
- Secondary infection by microbes arises, which in turn causes the plant to elicit production of phytoalexins with different toxicities.
- In sweetpotatoes, furanoterpenoids such as ipomeamarone (ipm), dehydro-ipomeamarone (dehydro-ipm), 4-ipomeanol and 1,4-ipomeadiol are known to cause hepatoxicity, pneumonia, lung edema, and cattle deaths (acute toxicity reported in rats fed with infested SP from **250 mg/kg**)

Hence, would a weevil-resistant sweetpotato reduce some health concerns in addition to food security?

Detection of ipomeamarone

Partnership with ICIPE (B. Torto, N. Maniania), University of Florida and Kaplan Schiller Research, and Kenyatta University.

- Sweetpotatoes were purchased from markets in Nairobi [Kemb, Naspot, Nyawo and Bungoma] while weevil-infested samples from KARI Marigat
- We isolated and identified the fungus *Rhizopus stolonifer* from weevil-infested storage roots
- **Furanoterpenoids** were extracted using established procedures such as GC-MS and HPLC
- Both **ipomeamarone** (*ipm) and* **dehydro-*ipm*** were identified

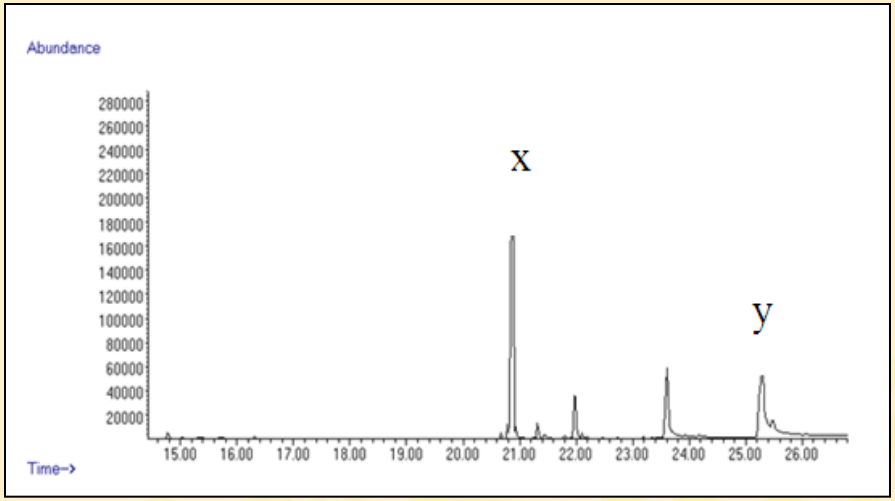


Figure 2: Representative GC-MS total ion chromatogram of purified furanoterpenoids, *ipm* and dehydro-*ipm* indicated as x and y, respectively

Preliminary results

- The main furanoterpenoid produced was *ipm* and its precursor, *dehydro-ipm*.
- High *ipm* levels, up to 4,500 mg/kg in sweetpotato samples were elicited by presence of *R. stolonifer* which far exceed toxicity levels in experimental mice with 250 mg/kg
- The peak of *ipm* was at 4 cm from inoculation site into the healthier-looking region

To do - health concern

- Increase number of samples to confirm preliminary results
- Then publish the study
- Develop a larger study to address:
  - which furanoterpenoids are present in microbe infected sweetpotatoes in farmers' fields
  - furanoterpenoid levels in sweetpotatoes from farmers' fields;
  - varietal response to fungal infection resulting in *ipm* accumulation;
  - degradation of *ipm* after processing infested roots and accumulation in metabolism in farm animal and human organisms and
  - management of the problem by the rural poor.


200-dead-cows-mystery-sol_n_815864.html

200 Dead Cows Mystery Solved In Wisconsin: Toxic Moldy Potatoes To Blame

The Huffington Post | Travis Walter Donovan

First Posted: 01/29/11 05:30 PM ET | Updated: 05/25/11 06:30 PM ET

Thank you Asante sana