Dear or alive.... That is the question

Detection of infectious and non-infectious CGMMV

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CGMMV

- *Cucumber green mottle mosaic tobamovirus* (CGMMV) on cucumber seed is a growing problem
- Very stable, highly infectious virus

- Seed transmitted
- Seed testing
  - DAS-ELISA (coat protein only, antibodies available?)
  - (RT)-PCR or TaqMan
- What does a positive result mean?
  - Is the virus still infectious?

Project Aim

- Develop and validate a test to distinguish between ‘alive’ (infectious) and ’dead’ (non-infectious) *Cucumber green mottle mosaic virus* (CGMMV) on cucumber seeds

- Hypothesis
  - Infectiousness of virus depends on intact viral RNA
  - Less intact RNA means less infectious virus

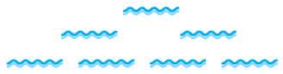
Research set-up

- Method to detect multiple targets necessary
  - Luminex xTAG TSPE assay
    - Generation of multiple (larger) RT-PCR products
    - Linear amplification of each PCR product through TSPE (template spec. primer extension)
    - Fluorescent detection of multiple TSPE products
    - Sum and comparison of fluorescent TSPE signals indicates intactness or absence of virus RNA

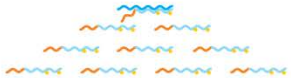
xTAG technology: Workflow summary

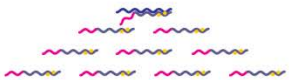
1. Total nucleic acid extraction

2. Multiplex RT-PCR

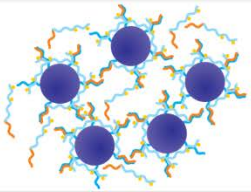


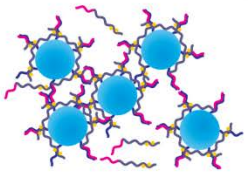
3. Target Specific Primer Extension (TSPE)



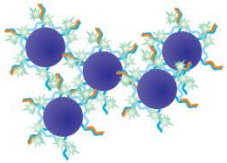


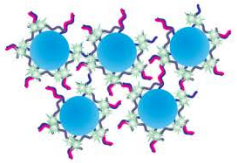
4. Hybridization with MagPlex-TAG beads





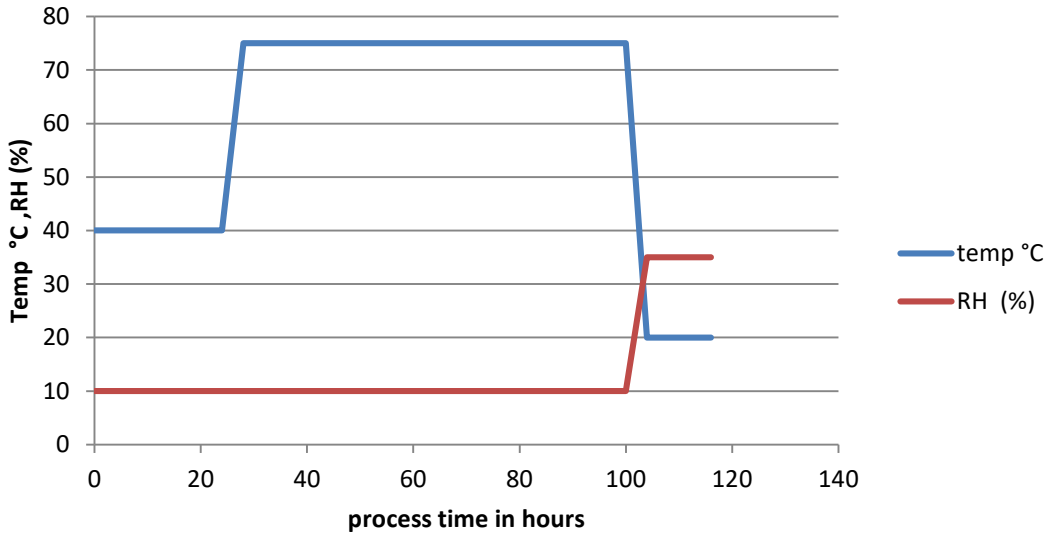
5. Detection and Luminex FlexMAP readout





CGMMV seed transmission

- CGMMV transmitted through seeds *Cucurbitacae*
- Virus is ‘on’ the seed coat
- Disinfection of seed by ‘dry heat treatment’
  - Controlled temperature regime for 120 hours
  - Very critical process: delicate balance between survival of seeds and ‘*death*’ of virus



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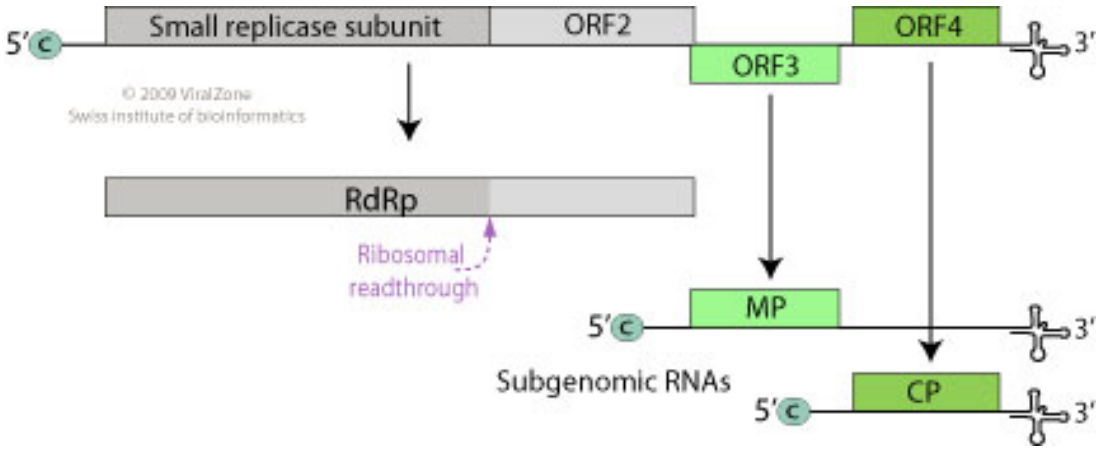
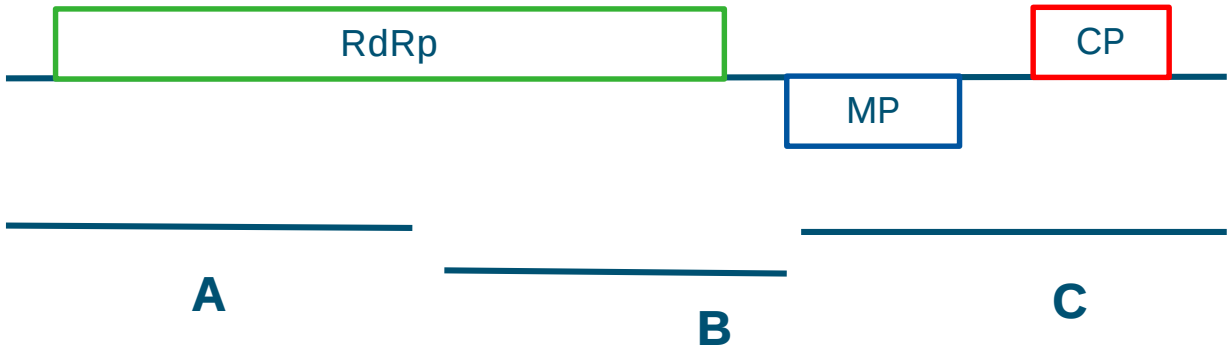
CGMMV genome

- *Cucumber green mottle mosaic virus*
- Tobamovirus
  - ss RNA (+-sense) ± 6500 nts
  - 4 ORFs: RdRp (+readthrough), MP and Coat Protein



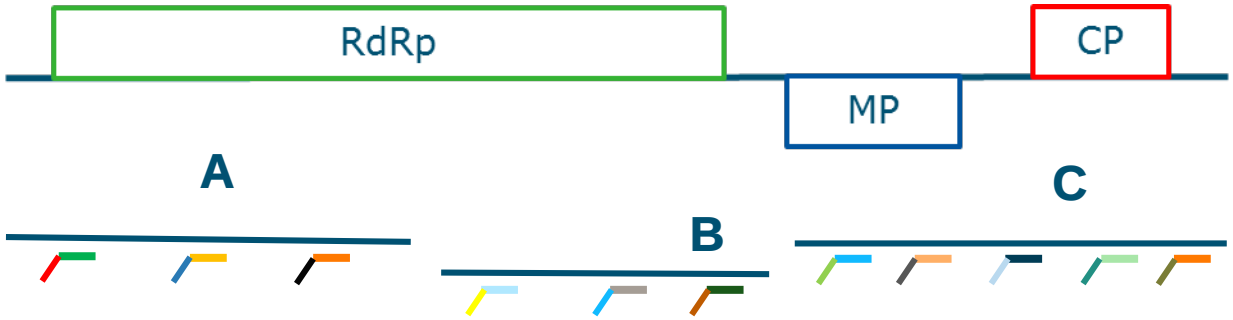
Research Set-up CGMMV

- Design of multiple RT-PCR primer sets to cover the CGMMV genome
  - Preferably ‘large’ RT-PCR products (1,5 – 2 Kb)
  - A, B and C cover ± 80% of total genome



Research Set-up CGMMV

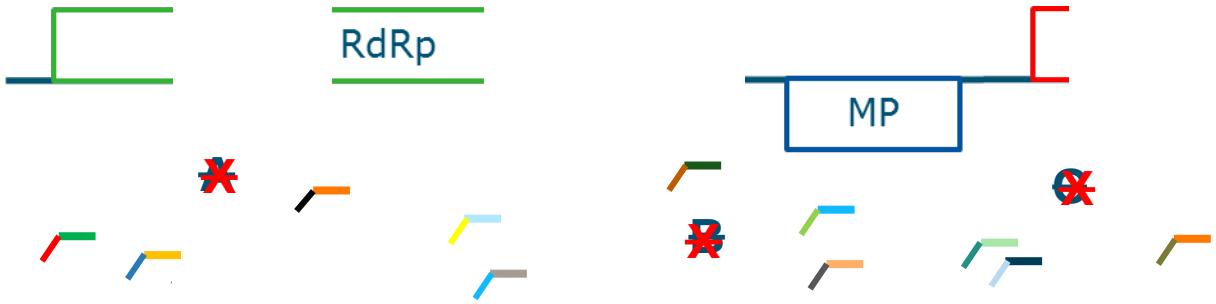
- Design of TSPE primers



- A total of 11 TSPE signals
  - A = 3 signals
  - B = 3 signals
  - C = 5 signals

Research Set-up CGMMV

- Breakdown of CGMMV RNA



- No RT-PCR products formed
- No TSPE primers will bind
- No TSPE signals will be generated

TSPE primer selection

- Initial testing of TSPE sets on individual RT-PCR products of CGMMV K3 isolate (on Nb and Cucumber)
  - Combined TSPE sets

template	TSPE 273	TSPE 707	TSPE 1208	TSPE 2268	TSPE 2484	TSPE 2780
Nb A	31.832	225	20.584	319	251	234
Cu A	30.692	253	11.778	350	236	237
Nb B	378	326	533	69.701	934	3.152
Cu B	433	326	427	64.517	611	2.024

- Differences in signal strength between TSPE primers but no cross-reactivity

Detection of CGMMV on seeds

- Isolate ZZB-545 on cucumber seeds

template	TSPE 273	TSPE 707	TSPE 1208	Back-ground
ZZB545	15.583	7.578	7.865	207

template	TSPE 2268	TSPE 2484	TSPE 2780	Back-ground
ZZB545	19.477	1.769	13.854	433

template	4382	4578	4938	5357	5988	Back-ground
ZZB545	4.296	605	2.420	8.721	7.391	660

CGMMV detection after dry heat treatment

- Dry heat treatment of ZZB-545 seed batch (Bert Woudt, Syngenta)
  - TSPE primers for PCR-fragment A

template	TSPE 273	TSPE 707	TSPE 1208	Back-ground
Healthy seed	302	255	270	666
No treat	22.740	13.191	14.845	486
Heat	370	296	345	425
K3	22.257	363	22.971	926

CGMMV detection after dry heat treatment

- Dry heat treatment of ZZB-545 seed batch (Bert Woudt, Syngenta)
  - TSPE primers for PCR-fragment B

template	TSPE 2268	TSPE 2484	TSPE 2780	Back-ground
Healthy seed	261	238	265	370
No treat	33.086	6.243	46.587	377
Heat	761	231	289	408
K3	84.626	1.949	9.974	442

Results on CGMMV infected plant material

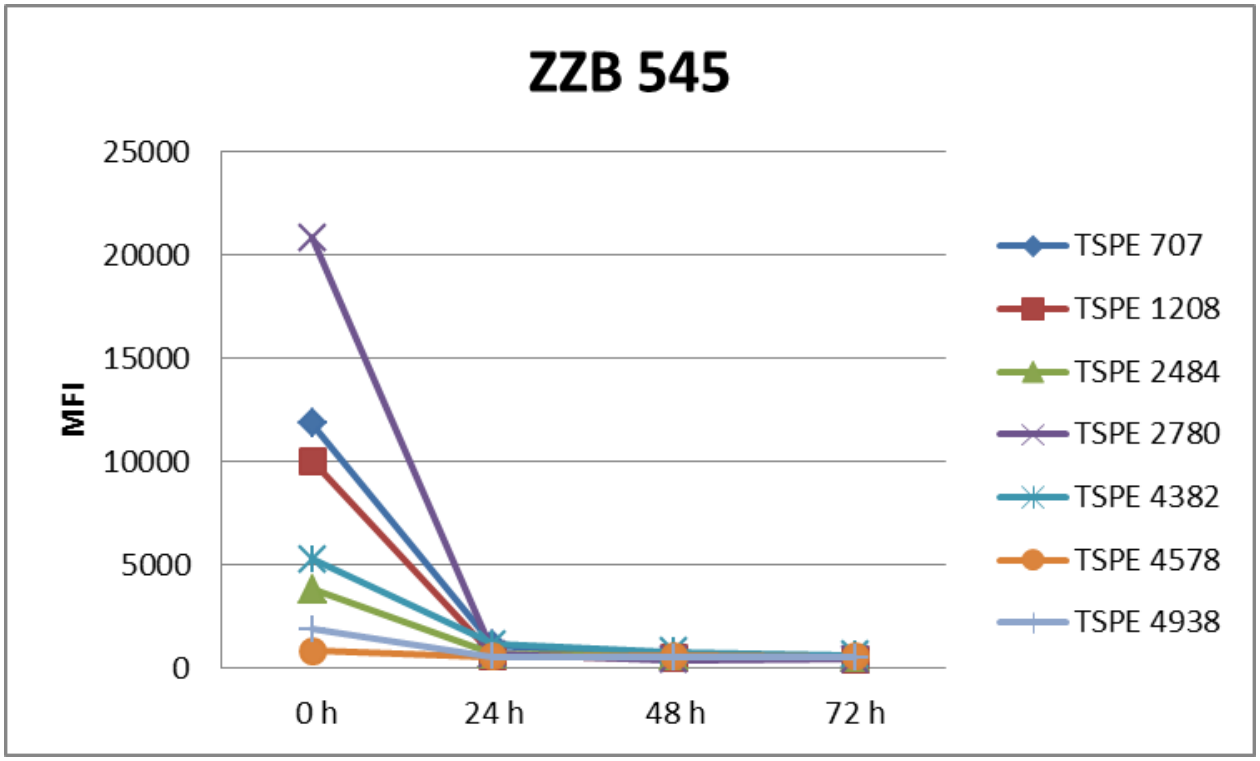
- xTAG TSPE amplicon detection
  - Successful amplicon detection with 11 TSPE-primers
- Detection of CGMMV isolates (infected leaves)
  - >15 different CGMMV isolates detected
  - Replications always consistently positive
- Detection of CGMMV isolates (on seed batches)
  - All 4 available seed batches clearly positive
  - Replications always consistently positive
  - Destruction of amplicons abolishes TSPE signals

CGMMV seed treatment

- Time course experiment on CGMMV heat treated seeds
  - Three seed batches Naktuinbouw (ZZB545, ZZB636 and ZZB637)
  - Heat treatment performed by Syngenta Seeds (Bert Woudt)
  - Samples taken at 0, 24, 48 and 72 hours
    - RNA extraction on crushed seeds (4 x 100 seeds)
    - DAS-ELISA tests all positive
  - Luminex xTAG assay
    - 4 replicates of 100 seeds on each time point

Luminex assay on heat treated seeds

- Average of Luminex signals (MFIs) of four replicates
  - ZZB 545 as an example



Assay validation

- Assay scope
  - the detection of intact RNA of CGMMV (*Cucumber green mottle mosaic virus)* in cucumber seeds using a Luminex xTAG assay
- Performance characteristics (EPPO PM 7-98)
  - Trueness
  - Analytical sensitivity and specificity
  - Selectivity
  - Repeatability
  - Reproducibility

Assay validation

- Trueness
  - Conformation by DAS-ELISA, RT-PCR and sequence analyses of amplicons
- Sensitivity
  - 3 replicates of dilutions series of RNA (seeds and leaves)
  - Consistent detection of 100x dilutions
- Specificity
  - 4 seed lots + 9 known CGMMV isolates all positive
  - KGMMV (related tobamovirus) = negative
  - CMV (unrelated cucumber virus) = negative

Assay validation

- Selectivity
  - 5 different seed lots of unknown origin and assumed different cultivars were all positive with similar negative values
  - Test performance not influenced by cultivar
- Repeatability and Reproducibility
  - 4 replicates, 2 extraction methods, 3 seed batches
  - Samples (0, 24, 48 and 72 hrs) of heat treatments
  - 8 repetitions of each timepoint on each seed batch
  - No differences between repetitions

Conclusions

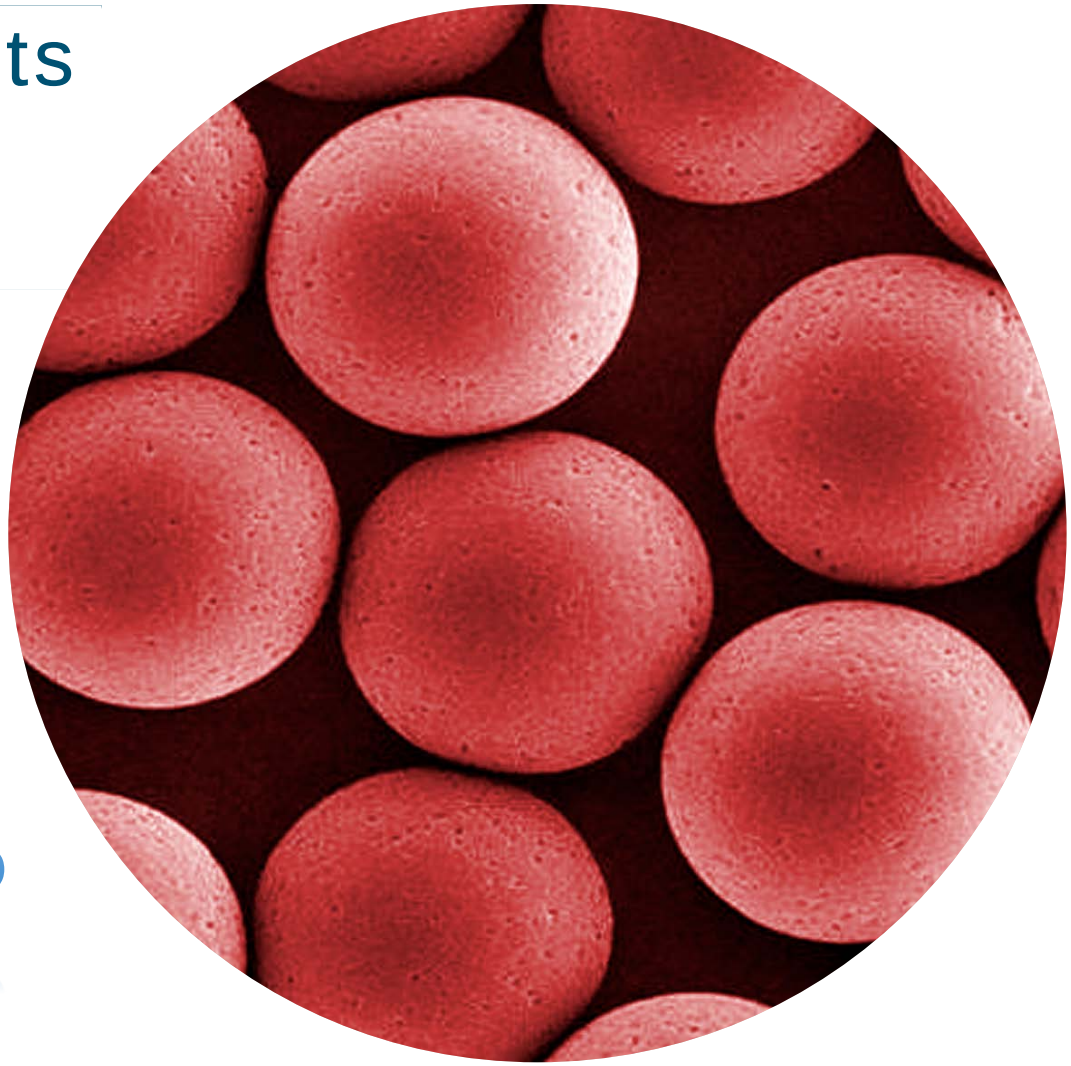
- Multiplex CGMMV Luminex assay with 11 data-points successfully developed
- Reliable detection of multiple isolates
  - On plant material and different seed batches
- All heat-treated seed lots become negative in Luminex assay but remain positive in ELISA
- Assay was successfully validated

- Confirmation of Luminex results through bio-assay in still pending
  - Is the virus really ‘dead’?

Acknowledgements

Bert Woudt
Syngenta Seeds

Harrie Koenraadt
Naktuinbouw



QUESTIONS?

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