Detecting unauthorised and unknown GMO

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Overview of presentation

- *Defining the problem: unauthorised and unknown GMO (UGM)*
  - What are they?
  - Where do they come from?
  - Why are they difficult to detect?
- *What are the solutions proposed and explored within Co-Extra?*
  - Detection
  - Verification
  - Decision support
- *The global dimension:*
  - Trade
  - Regulations
  - Method harmonisation
  - Information exchange

Defining the problem of UGM

- *Unauthorised GMO*
  - = All GMO not authorised for commercial release within relevant jurisdiction
- *May divide into several subgroups*
  - Useful to provide terminology for stakeholders
  - Legal status in a global perspective
  - Information about availability of knowledge (decision support)
- *Legal issue*
  - By definition, presence of UGM is illegal
  - Are some UGM “more illegal” than other?
- *Safety issue*
  - To what extent has a UGM been risk assessed?
  - Is the information available, accessible and reliable?
  - Are some UGM safer than other?

Authorised event
= authorised for commercial release in the relevant jurisdiction

Authorised and 2° approved event

2° approved event
= approved for commercial release in a third party jurisdiction

Transitional event
= pending authorisation within the relevant jurisdiction

Tolerated event
= adventitious presence is tolerated within defined limits within the relevant jurisdiction

Familiar

Non-approved event
= not authorised or approved for commercial release in any jurisdiction

Unknown

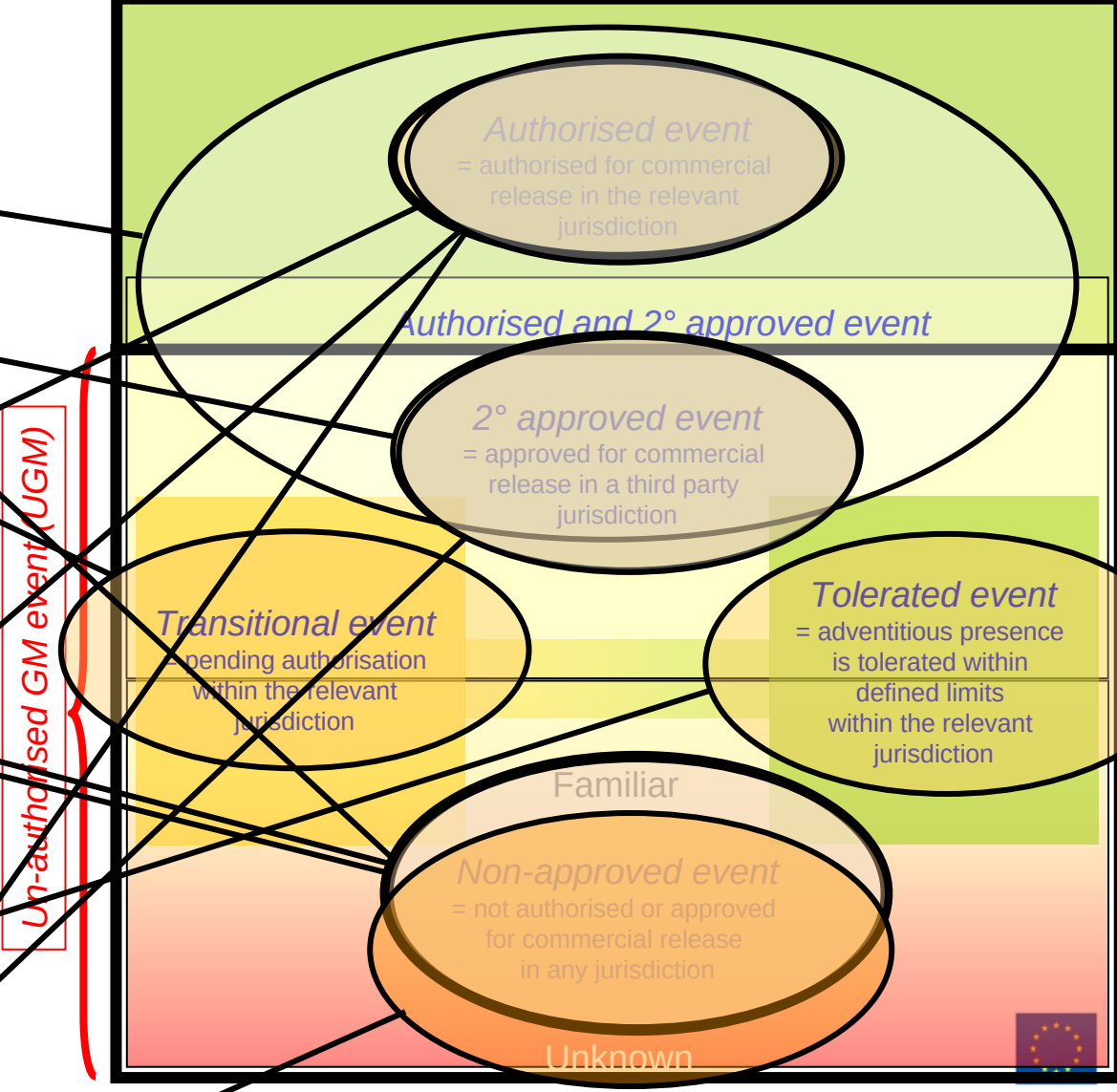
Un-authorised GM event (UGM)

Maize event:
SYN-E3272-5 (Event 3272)
- USA + Canada + Australia
- EU
- Other jurisdictions

Rice event:
BCS-OSØØ3-7 (LL601)
- USA
- EU
- Other jurisdictions

Maize event:
SYN-EV176-9 (176)
- EU
- Other jurisdictions

Cranberry event in academic research laboratory:
- All jurisdictions



Where do UGM come from?

- *"Asynchronous" authorisation and failure to segregate*
  - Usually risk assessed where authorised. Relevance to other jurisdictions?
  - Several cases e.g. of US authorised (deregulated) GMOs found in EU
  - cf. Rapid Alert System for Food and Feed (ec.europa.eu/food/food/rapidalert/)
  - New GMO developing and releasing states – assessments/controls comparable?
  - Illegal use in countries where not authorised of GMOs authorised somewhere else
- *Escapes from field trials and laboratories, etc.*
  - Usually not risk assessed and information limited or unavailable
  - e.g. LL601 and Bt63 rice, Bt10 and E32 maize, pig vaccine
  - Transparency vs. confidentiality (IP, public awareness, trust, etc.)
  - Pollen, bird/rodent, human error, etc.
- *Intended illegal releases*
  - Probably not risk assessed. Purpose may be to cause harm!

Why are UGMs so difficult to detect?

- *Species impurities – multiple species mixed: from which is GM DNA derived?*
- *Principle of all analysis: you need to know what you are looking for*
  - Detection method must be specific and specificity should be validated
  - You need reference material to verify that the method works correctly
  - For most UGM there is neither a specific method nor reference material available
  - Exceptionally, a method and reference material is available (e.g. Bt10 maize)
- *What is introduced in the UGM may be partially known*
  - Methods for GMOs pending authorisation are sometimes available via CRL-GMFF
  - Reference materials are sometimes available from outside of EU
  - Common promoters, terminators, trait genes or cloning vector elements
  - May facilitate detection, but status of evidence may be questionned
- *Completely unknown GMOs*
  - All introduced elements are novel (at least at the DNA sequence level)
  - Need to look for “anything” that is novel in the suspected GMO

Solutions proposed and explored - detection

Qualitative differential analysis

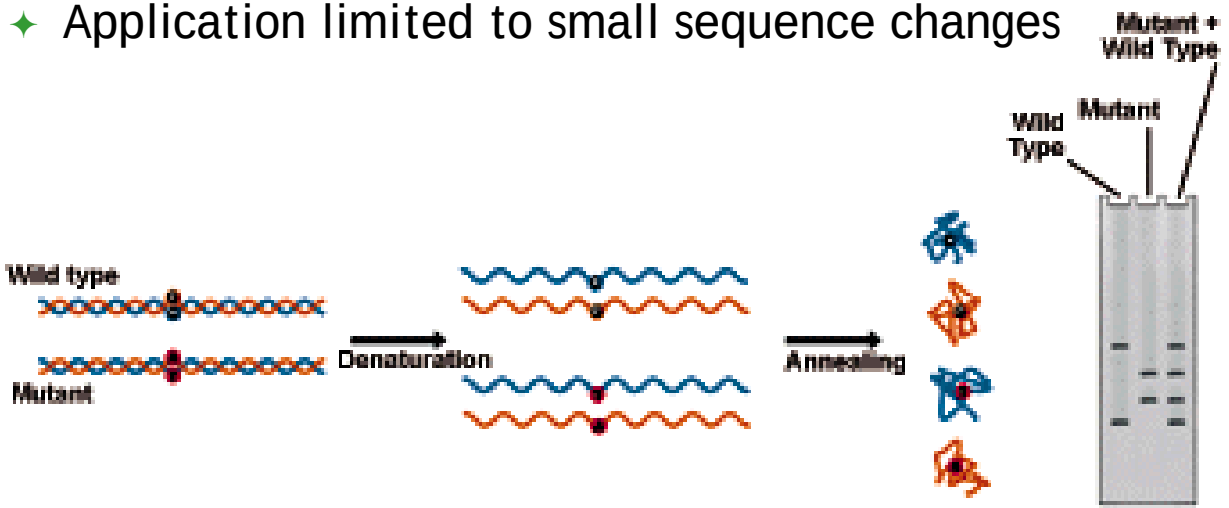
- Mutation/substitution screening
  - Observations of small nucleotide changes may indicate divergent origin
- The “matrix” approach
  - Targeted amplification followed by identification of amplified targets
  - Need for broad spectrum of screening modules
- Anchor PCR fingerprinting approach
  - Each GMO produces a specific anchor PCR fingerprint. Fragments can be sequenced
- High density microarray approaches
  - Direct hybridisation of genomic DNA to “profiling” microarrays
- Transcript sequencing and subtraction analysis
  - High throughput sequencing: GM subtracted from non-GM transcriptome

Quantitative differential analysis

- Quantitative differential PCR

Mutation/substitution screening

- Comparison of DNA strands between reference and possible UGM
  - Divergent origins often associated with substitutions / sequence changes
  - Substitutions / sequence changes alter migration in electrophoresis
  - Applicable to commonly used elements, e.g. P35S, 3'-nos, EPSPS
    - Can tabulate data from reference materials for comparison
  - Application limited to small sequence changes



The “matrix” approach

First proposed for GMOchips project in FP5. Explored in multiple variants

- *Screening for potentially introduced elements*
  - Promoters, terminators, trait genes, vector & potentially fusion elements
  - Simplex, oligoplex or multiplex detection method
  - Allelic diversity may result in false negatives
- A priori *tabulated relationship between GMOs and the elements screened for*
  - This is often referred to as the “matrix” (in a mathematical sense, not to be confused with the material subject to analysis, e.g. a foodstuff = product)
  - The element is defined by its detection method, not by its popular name
    - For example, there are several CaMV P35S varieties, not all detected by same method
- A posteriori *comparing the results of the screening with the “matrix”*
  - Result is a list of GMOs that may be present in the product
  - Perfect matches vs partial matches vs incompatible results, relative to “matrix”
  - Detection of elements not found in any authorised GMO → presence of UGM
    - Provided non-GM source can be excluded

The “matrix” approach

Analytical module/ GMO event	Screen A	Screen B	Screen C	Screen D	Screen E	Screen F	Screen G	Screen H
GMO_One	+	+	+	-	-	+	-	-
GMO_Two	+	+	-	-	-	+	-	-
GMO_Three	+	+	-	+	-	-	+	-
GMO_Four	+	-	-	+	-	-	+	-
GMO_Five	-	+	+	-	-	-	-	+
GMO_Six	-	-	-	-	+	-	-	+
GMO_Seven	+	+	-	-	+	-	-	-
GMO_Eight	-	-	+	-	-	+	-	-
GMO_Nine	-	-	-	+	-	-	-	-
GMO_Ten	-	-	-	-	-	-	+	-
Sample_1	+	+	+	+	-	+	-	+
Sample_2	-	+	+	-	-	+	-	+
Sample_3	-	+	-	-	+	-	+	-

Sample_1	Sample_2	Sample_3
Perfect	Part_missing	Mostly_missing
Perfect	Part_missing	Mostly_missing
Part_missing	Mostly_missing	Mostly_missing
Part_missing	Negative	Mostly_missing
Perfect	Perfect	Mostly_missing
Part_missing	Part_missing	Partly_missing
Part_missing	Part_missing	Partly_missing
Perfect	Perfect	Negative
Perfect	Negative	Negative
Negative	Negative	Perfect
A,B,C,D,F,H/none		
	B,C,F,H/none	
		G/B,E

Strong indicator of UGM

Figure adapted from: A. Holst-Jensen, Biotechnology Advances (in press), doi:10.1016/j.biotechadv.2009.05.025

The “matrix” approach

The principle is already implemented in many laboratories

Contributions in Co-Extra project:

- Targets explored and development of modules for new targets:
  - Common promoters, terminators, vector elements incl. selection marker genes, trait genes and event specific fusion motifs
- Developments and assessment of amplification and detection
  - Multiplexing (oligoplexing), mostly combining oligoplex amplification
  - PCR and non-PCR based amplification techniques
  - Capillary gel electrophoresis and array hybridisation for detection
- Data analysis
  - Primer specificity software (UniquePrimer)
  - Software for application with particular assays
  - Decision Support System module (database) designed for MA application

The “matrix” approach

Related publications from Co-Extra project:

- Heide et al. (**2008**). Detection of eight maize events by qualitative, multiplex PCR and fluorescence capillary electrophoresis. *Eur. Food Res. Technol.* 227 (2): 527-535; doi: 10.1007/s00217-007-0751-4
- Heide et al. (**2008**). Determination of eight genetically modified maize events by quantitative, multiplex PCR and fluorescence capillary gel electrophoresis. *Eur. Food Res. Technol.* 227 (4): 1125-1137; doi: 10.1007/s00217-008-0828-8.
- Morisset et al. (**2008**). NAIMA: target amplification strategy allowing quantitative on-chip detection of GMOs. *Nucleic Acids Res.* 36: e118; doi: 10.1093/nar/gkn524
- Chaouachi et al. (**2008**). A high-throughput multiplex method adapted for GMO Detection. *J. Agric. Food Chem***.** 56: 11596-11606; doi: 10.1021/jf801482r
- Prins et al. (**2008**). Optimised padlock probe ligation and microarray detection of multiple (non-authorised) GMOs in a single reaction. *BMC Genomics* 9:584; doi: 10.1186/1471-2164/9/584
- Leimanis et al. (**2008**). Validation of the performance of a GMO multiplex screening assay based on microarray detection. *Eur. Food Res. Technol.* 227 (6): 1621-1632; doi: 10.1007/s00217-008-0886-y
- Hamels et al. (**2009**). A PCR-microarray method for the screening of genetically modified organisms. *Eur. Food Res. Technol.* 228: 531-541**;** doi: 10.1007/s00217-008-0960-5
- Nakken, S., Aussedat, O., Kristoffersen, A.B., Holst-Jensen, A. & Tengs, T. (**2009**). UniquePrimer - a web utility for design of specific PCR primers and probe. *Annals of Microbiology* 59(2): 1-3

MATFORSK

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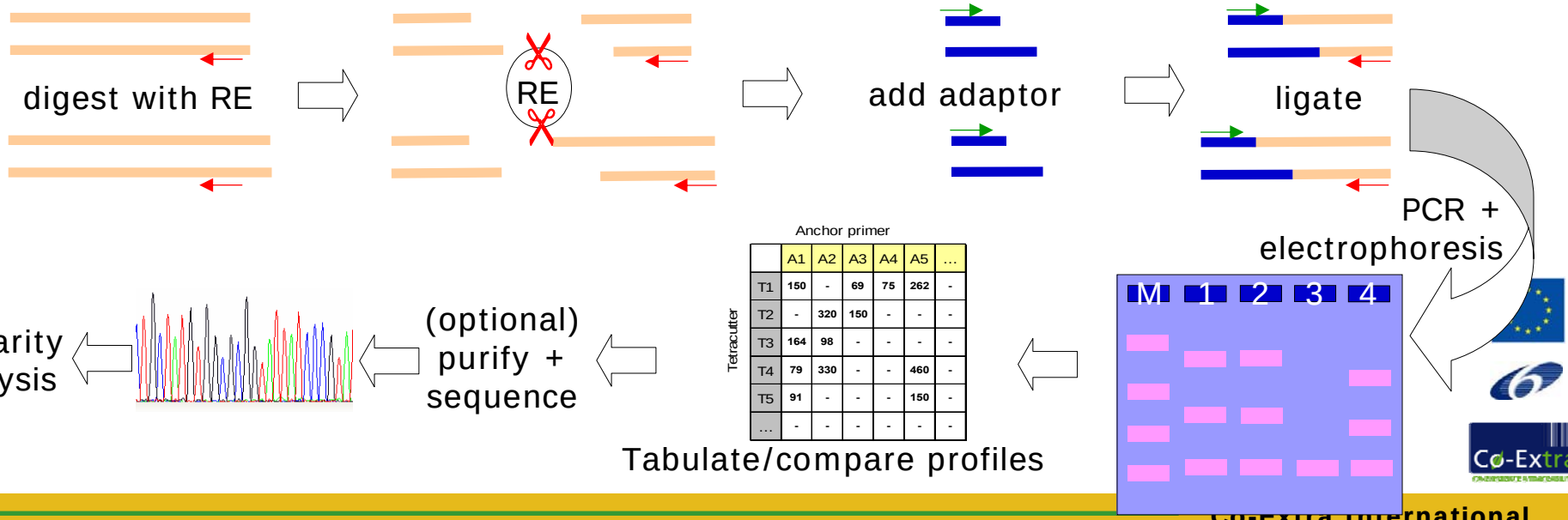
eppendorf Array Technologies

Veterinærinstituttet National Veterinary Institute

Anchor PCR fingerprinting approach

- *Anchor PCR – Semitargeted PCR, captures fragment adjacent to anchor*
  - DNA fragmented with restriction enzyme (RE), adaptor ligated to fragment
  - PCR with anchor primer ← and adaptor primer →
  - Result = fingerprint profile specific for GMO + RE + adaptor + anchor
  - Fingerprint profiles can be tabulated (size per fragment per profile)
  - Suspected UGM subject to anchor PCR profiling. Profile matched against known



Anchor PCR continued

A Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	150	-	69	75	262	-
T2	-	320	150	-	-	-
T3	164	98	-	-	-	-
T4	79	330	-	-	460	-
T5	91	-	-	-	150	-
…	-	-	-	-	-	-

B Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	-	-	-	-	-	-
T2	-	-	-	-	-	-
T3	-	-	-	-	-	-
T4	-	-	-	-	-	-
T5	-	-	-	-	-	-
…	-	-	-	-	-	-

C Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	150 76	-	69	75 69	262 366	-
T2	-	320 169	150	-	-	-
T3	164 321	98 199	-	-	-	-
T4	79 360	330 360	-	-	460 220	-
T5	91	-	-	-	150 220	-
…	-	-	-	-	-	-

D Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	350	-	69	-	-	-
T2	-	250	150	-	-	-
T3	240	110	-	-	-	-
T4	130	360	-	-	-	-
T5	230	-	-	-	-	-
…	-	-	-	-	-	-

E Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	-	320	69	67	310	-
T2	-	-	150	-	-	-
T3	59	190	-	-	-	-
T4	-	-	-	250	-	-
T5	-	420	-	-	-	-
…	-	-	-	-	-	-

F Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	-	96	-	-	120	-
T2	-	150	-	-	-	-
T3	-	230	-	96	-	-
T4	-	260	-	-	300	-
T5	-	-	-	-	190	-
…	-	-	-	-	-	-

X Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	150	-	69	75	262	-
T2	-	320	150	-	-	-
T3	164	98	-	-	-	-
T4	79	330	-	-	460	-
T5	91	-	-	-	150	-
…	-	-	-	-	-	-

Y Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	150 350	-	69	75	262	-
T2	-	320 250	150	-	-	-
T3	164 240	98 110	-	-	-	-
T4	79 130	330 360	-	-	460	-
T5	91 230	-	-	-	150	-
…	-	-	-	-	-	-

Z Anchor primer

Tetracutter	A1	A2	A3	A4	A5	…
T1	230	-	69	130	-	-
T2	-	40	150	-	360	-
T3	350	150	-	-	-	-
T4	150	320	-	-	-	-
T5	300	-	-	-	-	-
…	-	-	-	-	-	-

High density microarrays

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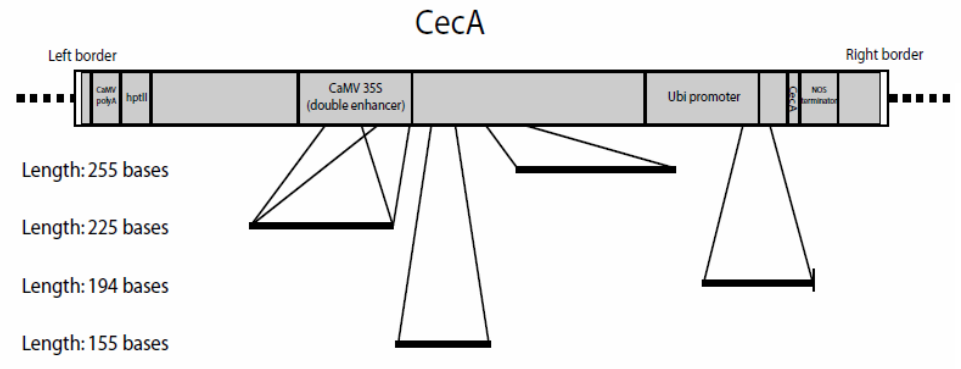
- *Very high number of probes, each motif absent in non-GM genome*
  - Targeting of probes depend on purpose
    - Selected among all motifs not found in non-GM genome
    - Selected among all motifs that could have GM origin (e.g. vectors)
- *Labeling and hybridisation of DNA to microarray*
  - Total genomic DNA or whole genome amplified DNA (non-selective amplification)
  - Separate arrays for non-GM and suspected UGM, or two-colour system
- *Bioinformatics analysis to identify positives*
  - Probes with higher signal in suspected UGM sample are likely positives
  - Windows tiled through overlapping probe motifs to identify true positives
  - Positive windows can be exploited for (anchor-)PCR and sequencing



Tengs, T., Kristoffersen, A.B., Berdal, K.G., Thorstensen, T., Butenko, M.A., Nesvold, H. & Holst-Jensen, A. (**2007**) Microarray-based method for detection of unknown genetic modifications. ***BMC Biotechnology*** 7: 91; doi: 10.1186/1472-6750-7-91

Co-Extra

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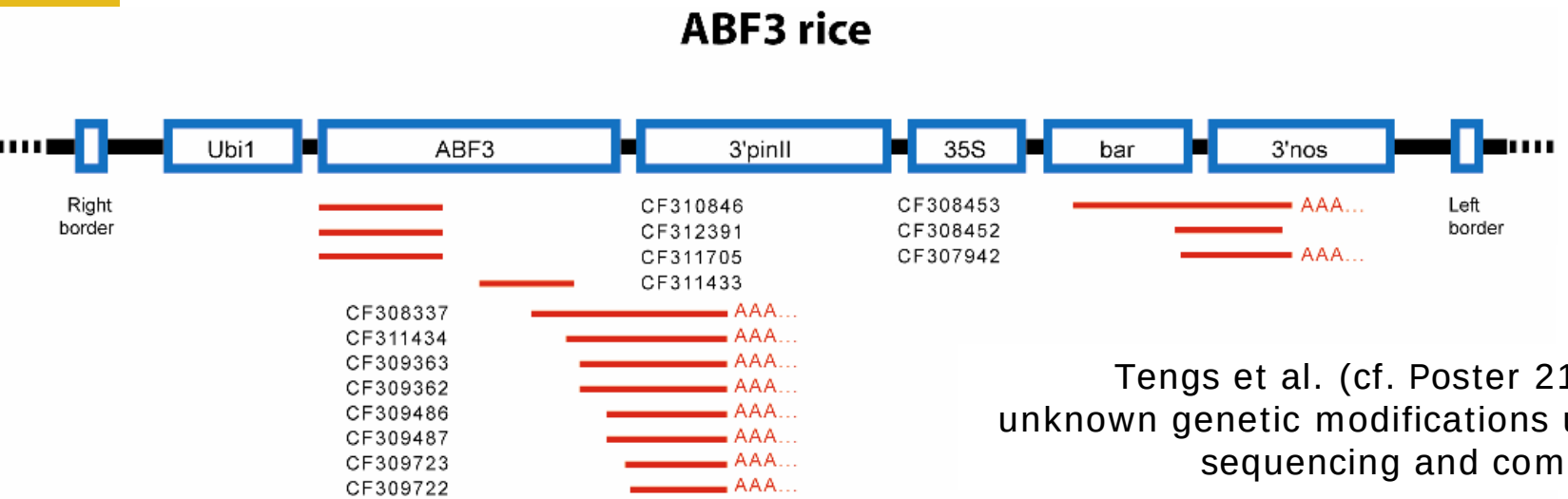
Transcriptome sequencing

- *Isolate mRNA from suspected UGM, convert to cDNA with reverse transcriptase*
- *Option 1: Subtract against cDNA library from non-GM*
- *Perform high-throughput DNA sequencing on the suspect cDNA library*
- *Option 2: Subtract against DNA sequence database with non-GM sequences*
- *Apply bioinformatics to identify potential GMO-derived sequence motifs*
- *Exploit identified motifs to verify by (anchor-)PCR and sequencing*



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Tengs et al. (cf. Poster 21). Characterization of unknown genetic modifications using high throughput sequencing and computational subtraction

Quantitative differential PCR

- *Quantify at least two targets – hypothetically equal quantities*
  - Significant difference in quantity means that hypothesis is falsified
- *Representative example: screening element and multiple GMO events*
  - For screening element *S*: $[S] = Q_S$
  - For all authorised GMOs (*A*, *B*, …) containing *S*: $[A + B + \ldots] = Q_{Auth} = Q_A + Q_B + \ldots$
  - Taking into consideration all measurement uncertainty factors
  - Hypothesis: $\mu = Q_S - Q_{Auth} = 0$

Cankar, K., Chauvensy-Ancel, V., Fortabat, M.N., Gruden, K., Kobilinsky, A., Zel, J. & Bertheau, Y. (**2008**). Detection of nonauthorized genetically modified organisms using differential quantitative polymerase chain reaction: application to 35S in maize. ***Anal. Biochem.*** 376 (2): 189-199; doi:10.1016/j.ab.2008.02.013

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Co-Extra

Decision support

- *A modular decision support system is developed within the Co-Extra project*
  - Separate module on UGM
  - User is asked to input available information on the product
    - Origin, species, any available test results, whether or not under IP, etc.
  - System classifies the product as high, medium or low risk of UGM
  - Also separate module for assessment of method fitness-for-purpose
  - Also separate module for application with the “matrix” approach (the database module)

- For more information on the Decision Support System (DSS) see presentations by Marko Bohanec, Kristina Gruden and Esther Kok in session 5, Thursday morning from 11:00 to 12:00

Jožef Stefan Institute, Ljubljana, Slovenia

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Co-Extra

The global dimension

UGM is a global and increasing concern: Europe, China, Japan, USA…

Trade vs. safety

- *Unauthorised GMOs may affect trade and availability of products*
  - e.g. LL601 rice, low level presence of asynchronously authorised GMOs, etc.
- *Safety is rarely documented / confirmed for UGM, therefore undesirable*

Regulations

- *Zero tolerance? What would this mean (LOD/LOQ)? At what cost? How document?*
  - Different consequences for different "legal" classes, cf. proposed classification?
- *When do methods have to be available? Can they be shared globally?*

Method harmonisation

- *Same methods and sensitivity implemented by the stakeholders?*
- *Can the method applied discriminate UGM from authorised?*
- *Are methods applied that will detect and verify presence of UGM?*

Information exchange

- *Traceability facilitates origin identification and retraction when needed*
- *Developments, field trials, reference materials, detection methods, etc.*
  - Transparency facilitates monitoring and identification, and may reduce risks!

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