Considerations on the development of accurate methods, suitable for the screening, identification and quantification of GMOs

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29 & 30 September 2010

Enlargement/Networking Workshop on Harmonisation of GMO Analysis

European Commission

TAIEX

Technical Assistance Information Exchange Instrument (TAIEX), DG Enlargement

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Technology and Food Science Unit

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Agriculture and Fisheries Policy Area

ILVO

Development of methods for screening, identification and quantification of GMOs

- Introduction - considerations
- Description of “GMO”
- Technical considerations
  - What are the differences at genotypic and phenotypic level
  - Detection versus identification
  - Quantification
    - Reference genes
    - Reference material
  - Analytical difficulty is largely determined by the status of the sample to be analysed
  - Transferability of analytical data throughout the production chain

Development of methods for screening, identification and quantification of GMOs

- General considerations
  - Representativeness of the analytical data for the lot or field
  - Economic feasibility of testing
  - Testing in the context of monitoring or in the context of disputes

- Further information
  - QPCRGMOFOOD
  - SIGMEA
  - COEXTRA
  - ENGL
  - EURL

Introduction - considerations

The overall aim of **plant breeding**

- To develop new plant varieties with improved quality, yield, growing performance

Conventional breeding makes use of existing genetic variation within the gene pool of a species.

The **transgenic** approach and the **new breeding techniques** allow

- To broaden the gene pool from which the breeder can select,
  - by stimulating the development of (natural) variants
- To identify the genotypes of interest more efficiently
- To make use of the cell machinery to make new products

Introduction - considerations

Why GMO detection?

- Legislation
- Consumers choice
- Labeling
- Certainty in trade

Introduction - considerations

Legal context

DIRECTIVE 2001/18/EC on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC

Information on the genetic modification for the purposes of placing on one or several registers modifications in organisms, which can be used for the detection and identification of particular GMO products to facilitate post-marketing control and inspection.

This information should include where appropriate the lodging of samples of the GMO or its genetic material, with the competent authority and details of nucleotide sequences or other type of information which is necessary to identify the GMO product and its progeny, for example the methodology for detecting and identifying the GMO product, including experimental data demonstrating the specificity of the methodology.

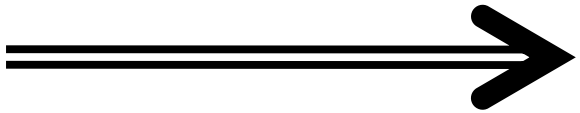
Specific method and reference material

Introduction - considerations

Legal context

REGULATION (EC) No 1829/2003 on genetically modified food and feed:

To facilitate controls on genetically modified food and feed, applicants for authorisation should propose appropriate methods for sampling, identification and detection, and deposit samples of the genetically modified food and feed with the Authority; methods of sampling and detection should be validated, where appropriate, by the Community reference laboratory.



Validated sampling and detection method

Introduction - considerations

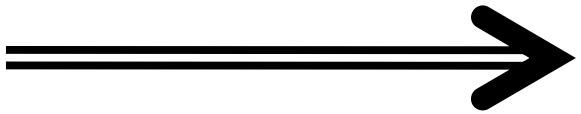
Legal context

REGULATION (EC) No 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of

food and feed products produced from genetically modified organisms and amending Directive

2001/18/EC:

For products intended for direct processing, paragraph 1 shall not apply to traces of authorised GMOs in a proportion no higher than 0,9 % or lower thresholds established under the provisions of Article 30(2), provided that these traces are adventitious or technically unavoidable.'.



Quantitative detection method

Introduction - considerations

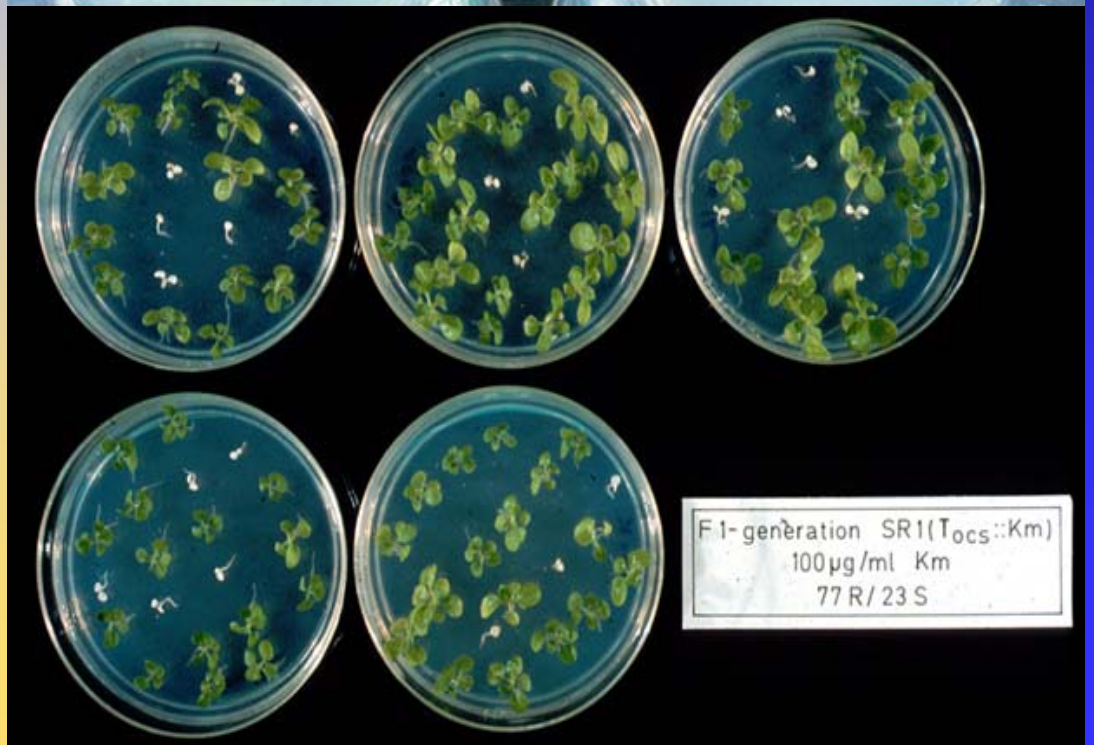
Detection in research context versus detection in function of enforcement

Detection of GMO events in a research context is making use of the same analytical tools as for detection in a monitoring/legal context.

But the experimental setup is different due to the difference in information that is available and the context.

Also the final use of the experimental data is different.

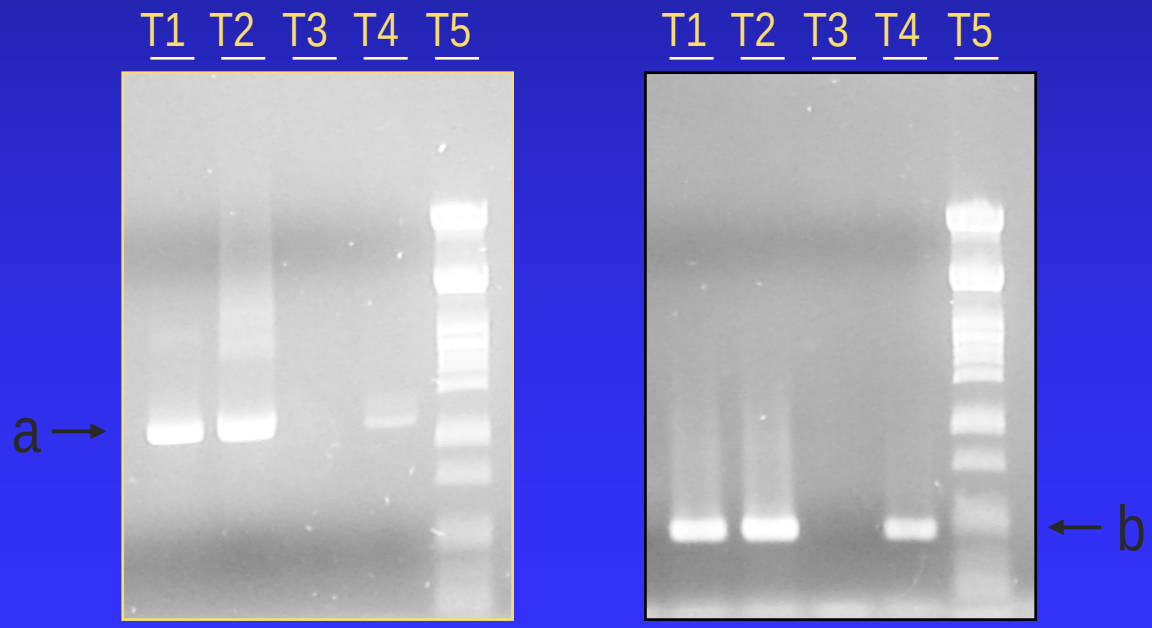
: **1983**



The first transgenic plants

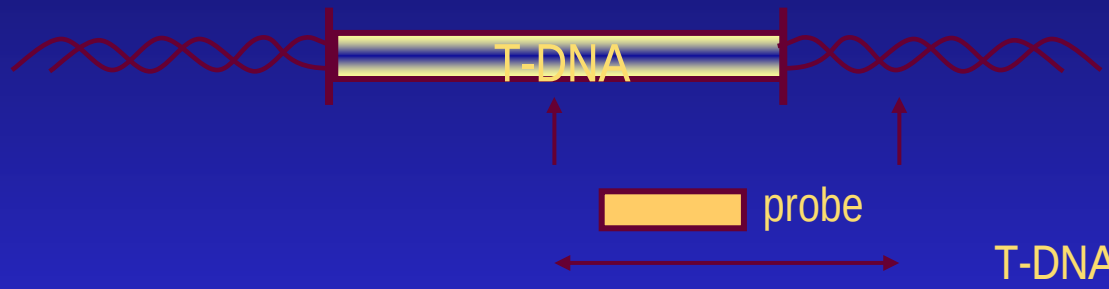
transcriptional fusions between the nos promotor and the coding sequence of the neomycinphosfotransferase gene or the chloramphenicolacetylase gene (cat) derived from prokaryotic antibiotic resistance markers, resulting in kanamicin and chloramphenicol resistance respectively

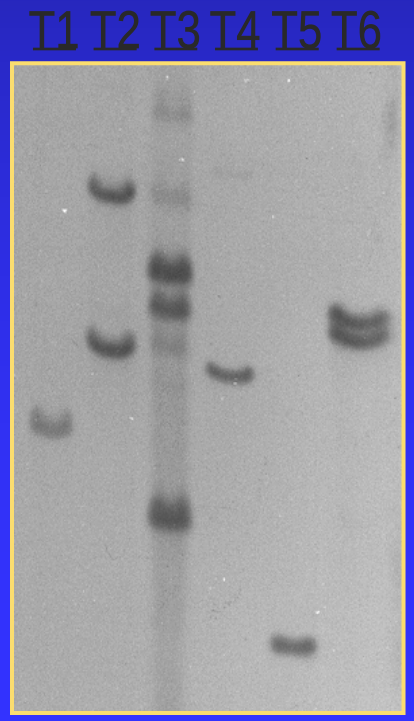
Screening of transformed plants by PCR



Identification of transgenic plants by Southern analysis

Simple transgene insert





The T-DNA plant junctions are different in every transformant; the number of fragments indicates the number of T-DNA copies

Introduction - considerations

GMO

≠

Non-GMO

GMO differs from the convential plant by the presence of a foreign DNA-fragment

The detection of the GMO is based on this small difference

Introduction - considerations

- What is the **quality character** to be detected
  - Absence of a transgenic trait or GMO
  - Presence of a specific transgenic trait or GMO
- GMO is a **collective term**, and as such it is not an analyte that can be detected in an analytical experiment
- What is the difference between **a GMO and a non-GMO**
  - The presence of a DNA fragment that is inserted in the plant genome(s) by using a DNA tranformation process

Introduction - considerations

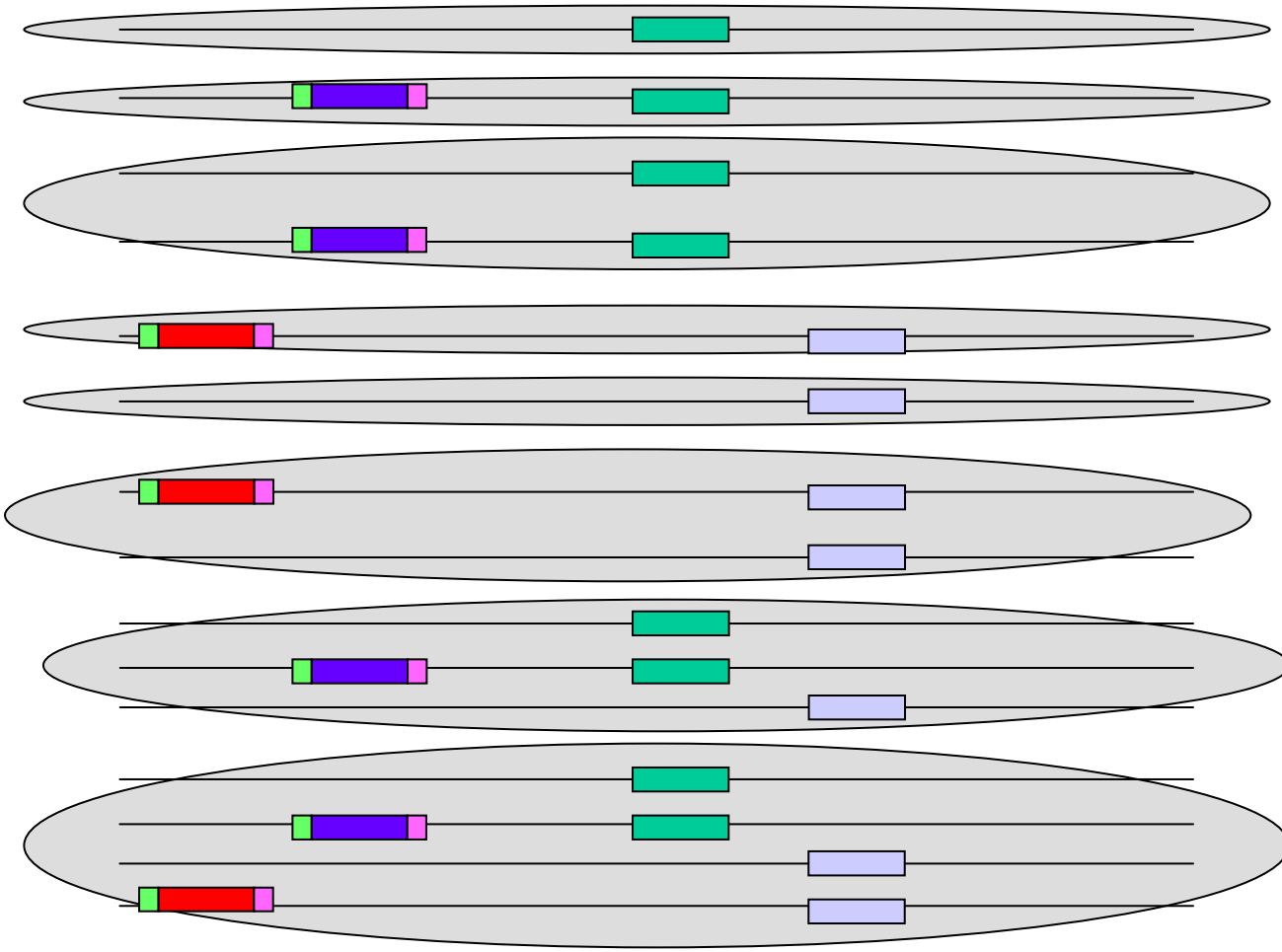
- How can the presence of such a fragment be demonstrated
  - Demonstrating the presence of the **inserted DNA** or the **expressed products** from it
- How to distinguish GMO that contains the same DNA fragment and or expresses the same phenotype
  - Demonstrating the presence (of one) of the for each event unique **DNA junction fragment(s)**

Introduction - considerations

- In order to implement the legislation on labeling **detection methods for GMOs** are necessary
  - How to distinguish a GMO from a non-GMO?
  - How to distinguish different GMOs?
  - How to quantify the GMO content per ingredient in a product?
- Where to get the necessary information for developing the detection method and strategy?
- At the moment in the EU a new event will only be authorised if a validated method and reference material is available. Info see EURL

Introduction - considerations

Does a product contain a GMO? If yes which GMO events are present.



Maize wt

Maize GMO

Maize wt + Maize GMO

Soya GMO

Soya wt

Soya GMO + Soya wt

Maize wt + Maize GMO+ Soja wt

Maize wt + Maize GMO +
Soya GMO + Soya wt

Description of “GMO”

Biotechnology

- Modern biotechnology refers to a number of techniques that involve the intentional manipulation of genes, cells and living tissue in a predictable and controlled manner to generate changes in the genetic make-up of an organism.
- recombinant DNA techniques (rDNA or genetic engineering).
  - 1953 discovery of the structure of deoxyribonucleic acid DNA and the way genetic information is passed from generation to generation.
  - to produce desired changes in an organism through the direct manipulation of its genes in a controlled and less time-consuming fashion in comparison to traditional biotechnology techniques.

Description of “GMO”

Modern biotechnology refers to a number of techniques that involve the intentional manipulation of genes, cells and living tissue in a predictable and controlled manner to generate changes in the genetic make-up of an organism.

What is a GMO – transgenic event (EU2001/18)

- Art 2 Definition Art 2(2) "genetically modified organism (GMO)" means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination;
  - Within the terms of this definition:
  - (a) genetic modification occurs at least through the use of the techniques listed in Annex I A, Part 1;
  - (b) the techniques listed in Annex I A, Part 2, are not considered to result in genetic modification;
- Art 3 Exemptions
  - 1. This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.
  - 2. This Directive shall not apply to the carriage of genetically modified organisms by rail, road, inland waterway, sea or air.

Description of “GMO”

ANNEX I A TECHNIQUES REFERRED TO IN ARTICLE 2(2)

- PART 1
- Techniques of genetic modification referred to in Article 2(2)(a) are *inter alia*:
- (1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;
- (2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;
- (3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Description of “GMO”

ANNEX I A TECHNIQUES REFERRED TO IN ARTICLE 2(2)

- PART 2
- Techniques referred to in Article 2(2)(b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/methods other than those excluded by Annex IB:
- (1) in vitro fertilisation,
- (2) natural processes such as: conjugation, transduction, transformation,
- (3) polyploidy induction.

Description of “GMO”

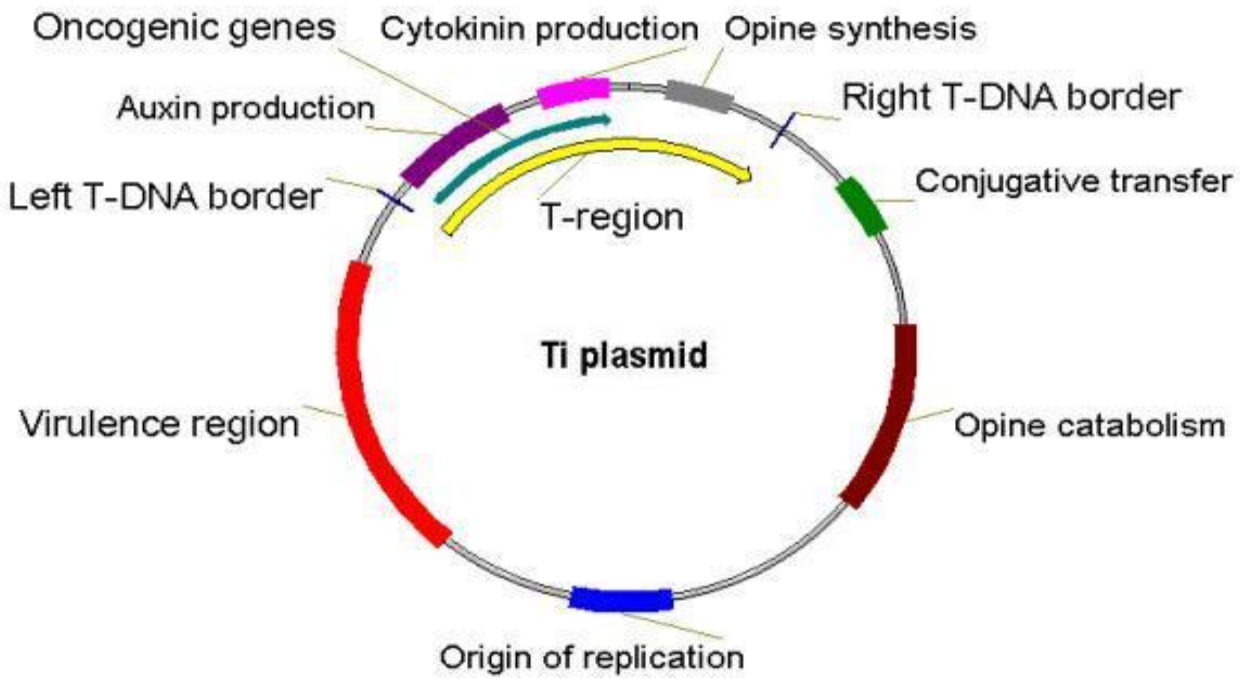
ANNEX I B TECHNIQUES REFERRED TO IN ARTICLE 3

- Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:
- (1) mutagenesis,
- (2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.

Description of “GMO”

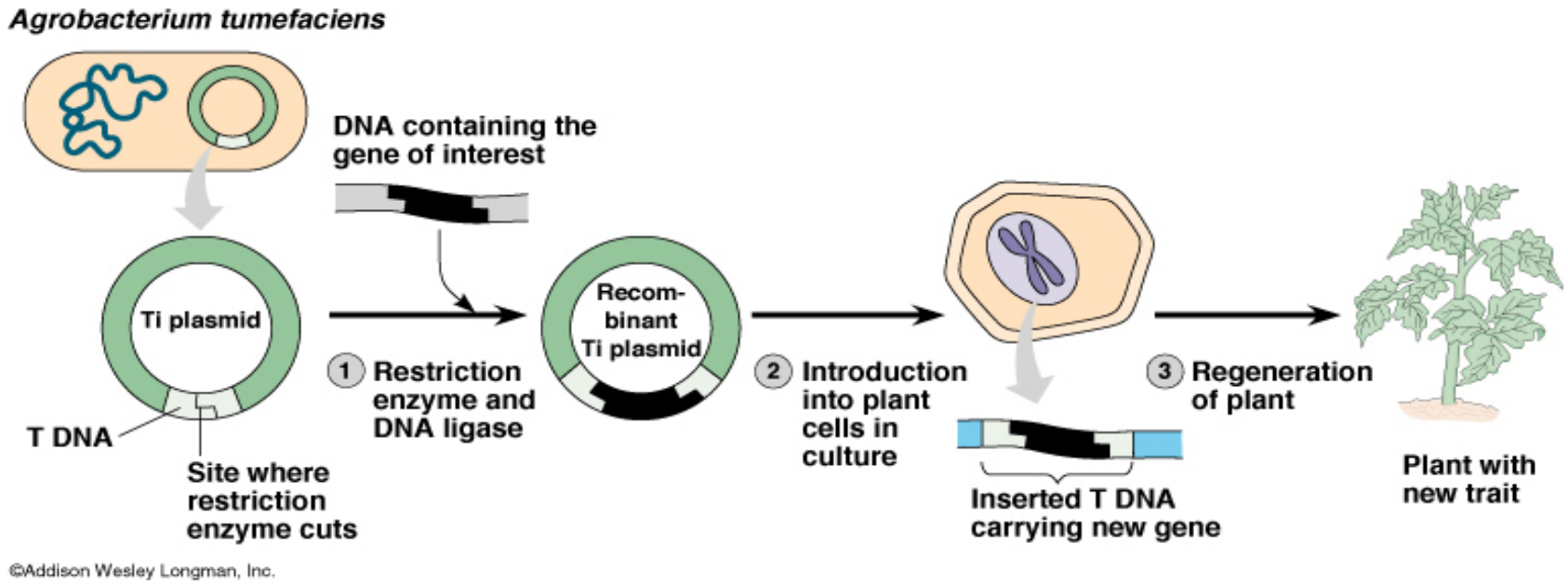
Agrobacterium-mediated transformation

The genome of *Agrobacterium tumefaciens* C58 comprises



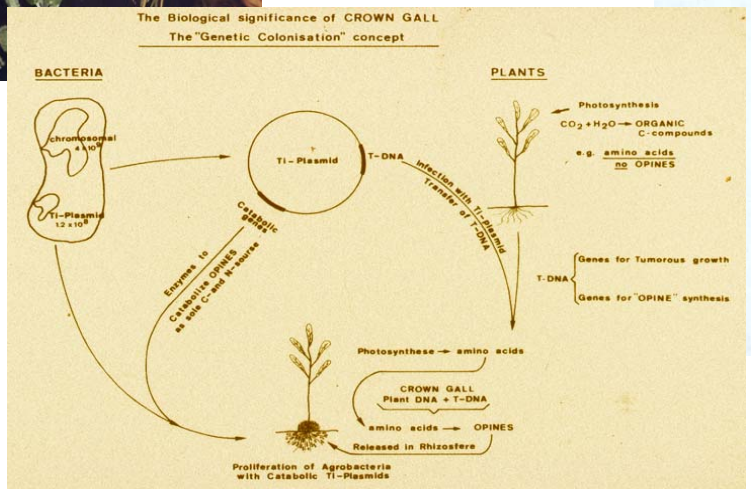
Description of “GMO”

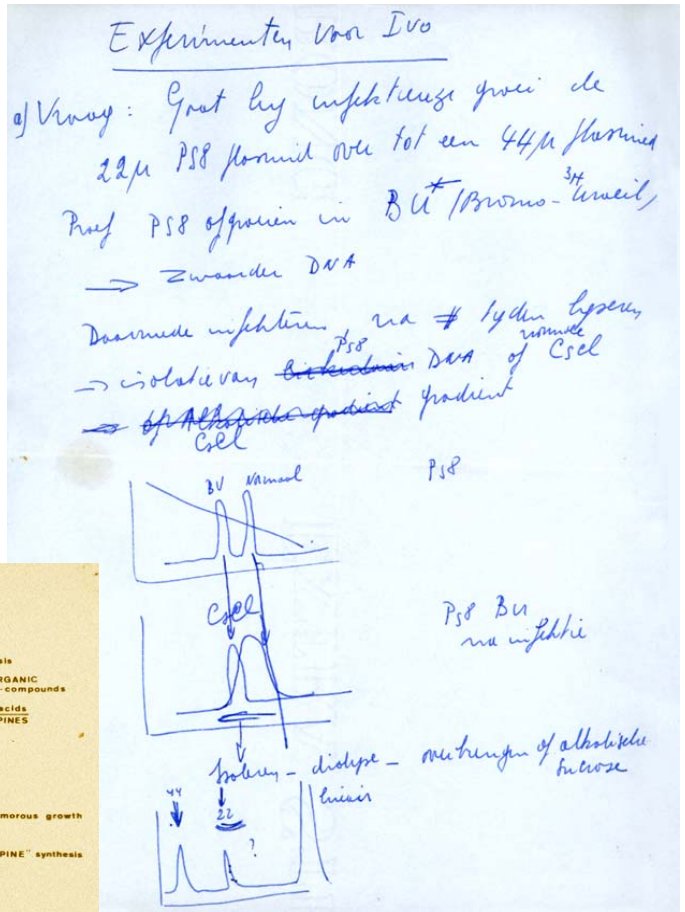
- Agrobacterium mediated Gene transfer
- Recombinant DNA technologie
- Transformatie van planten

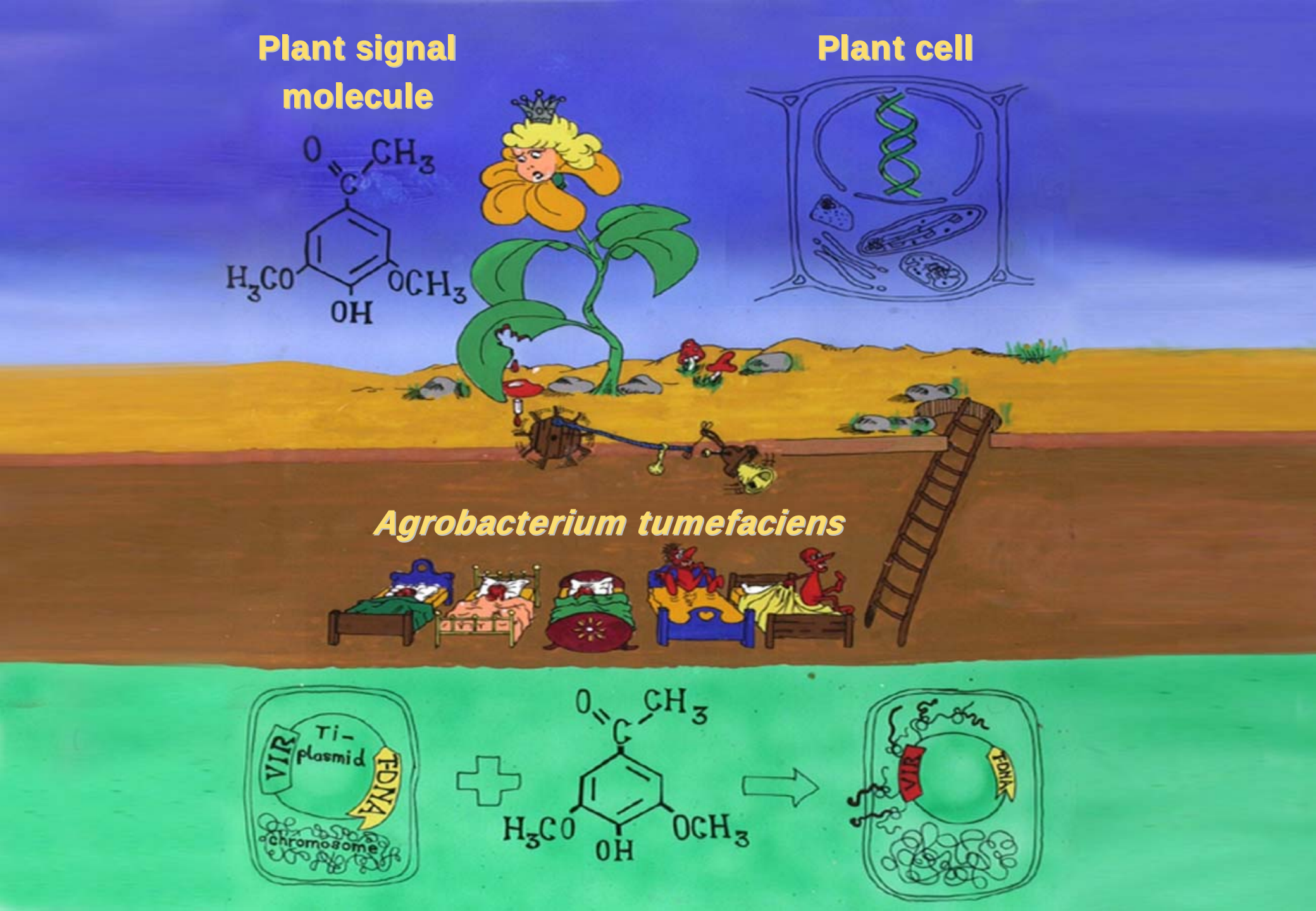


Description of “GMO”

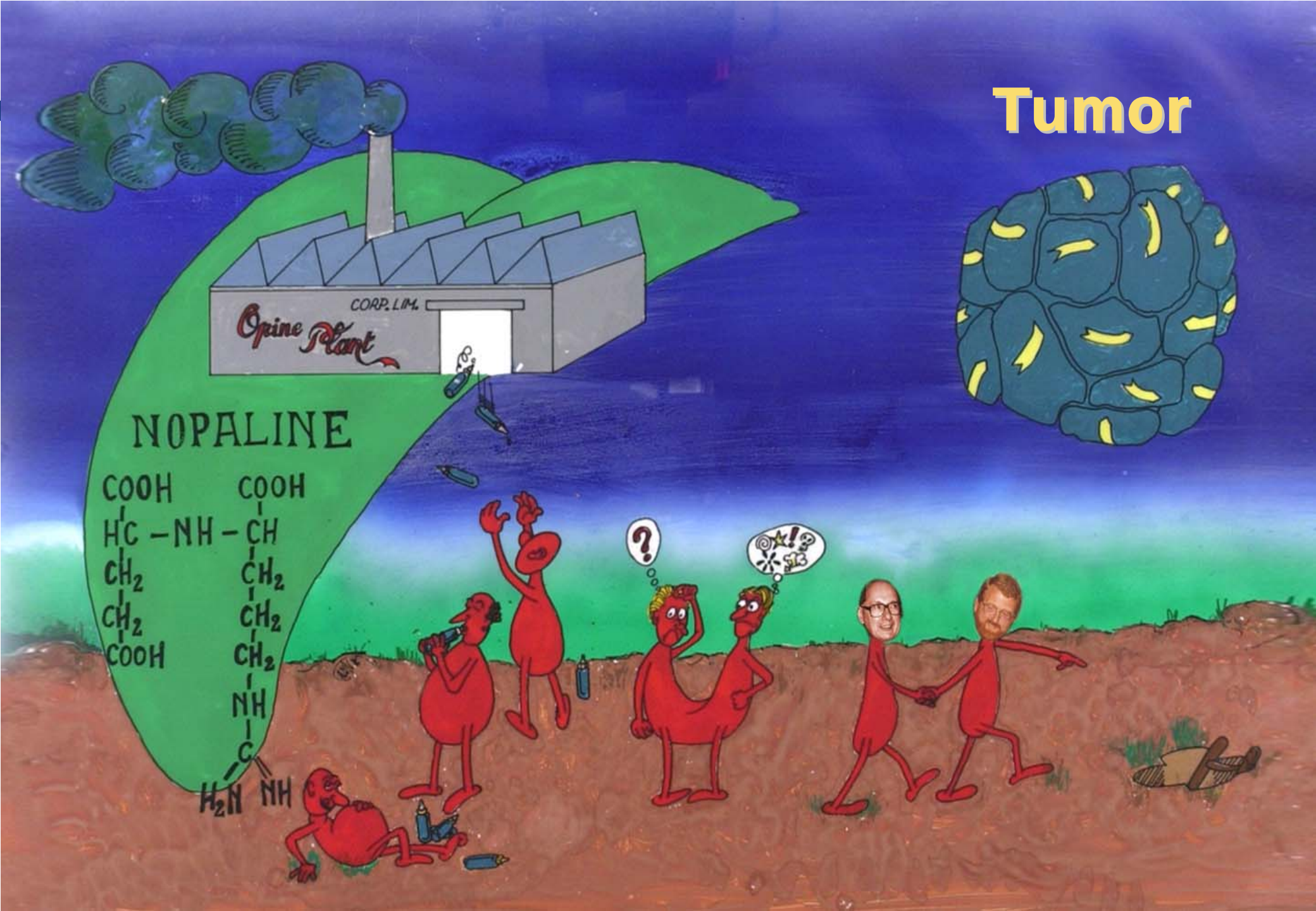
Agrobacterium tumefaciens mediated gene transfer





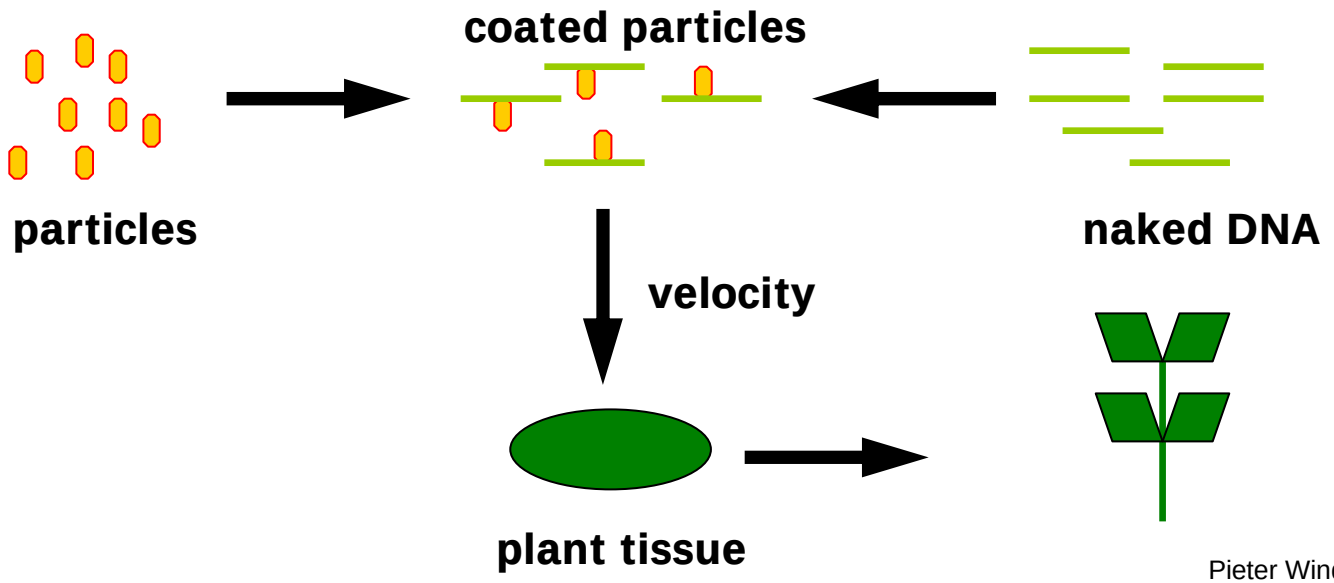

Plant signal molecule
Plant cell
Agrobacterium tumefaciens
VIR
Ti-plasmid
T-DNA
chromosome
VIR
T-DNA


Transfer and integration
of T-DNA


Tumor
CORP. LIM.
Opine Plant
NOPALINE
COOH
COOH
H'C – NH – CH
CH2
CH2
CH2
CH2
CH2
COOH
CH2
NH
C
H2N
NH

Description of “GMO”

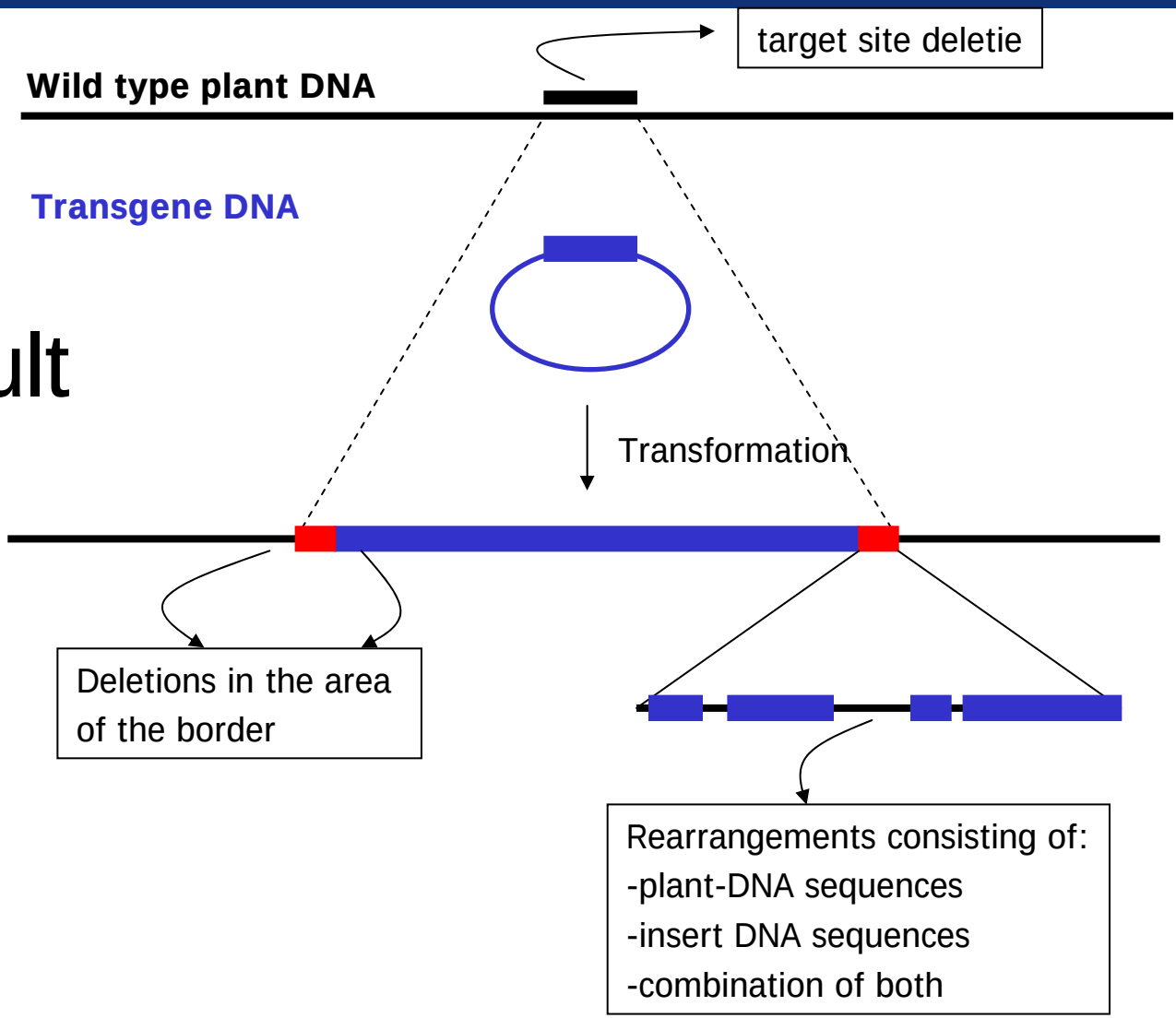
- Direct gene transfer



Pieter Windels, 2004

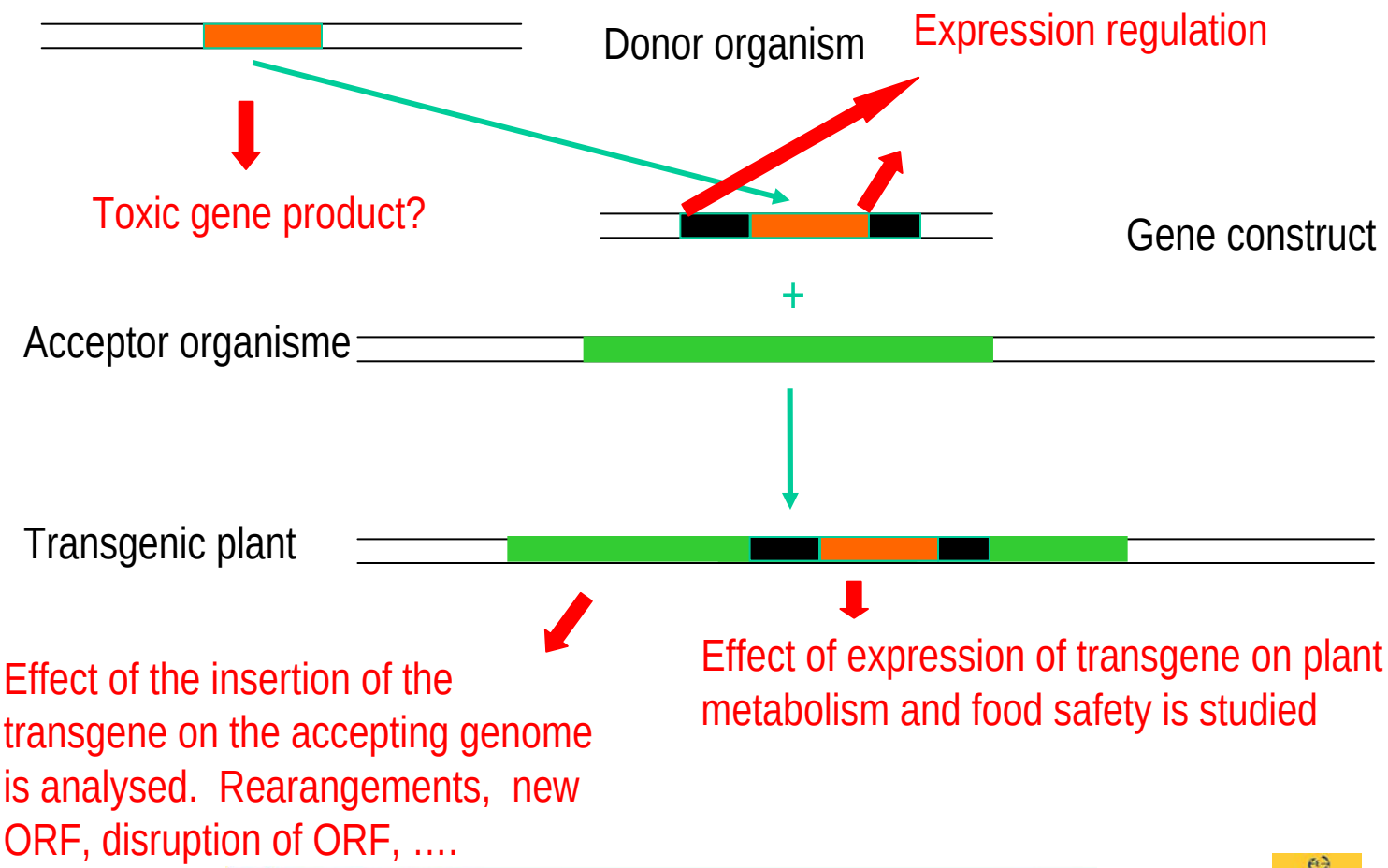
Description of “GMO”

The result



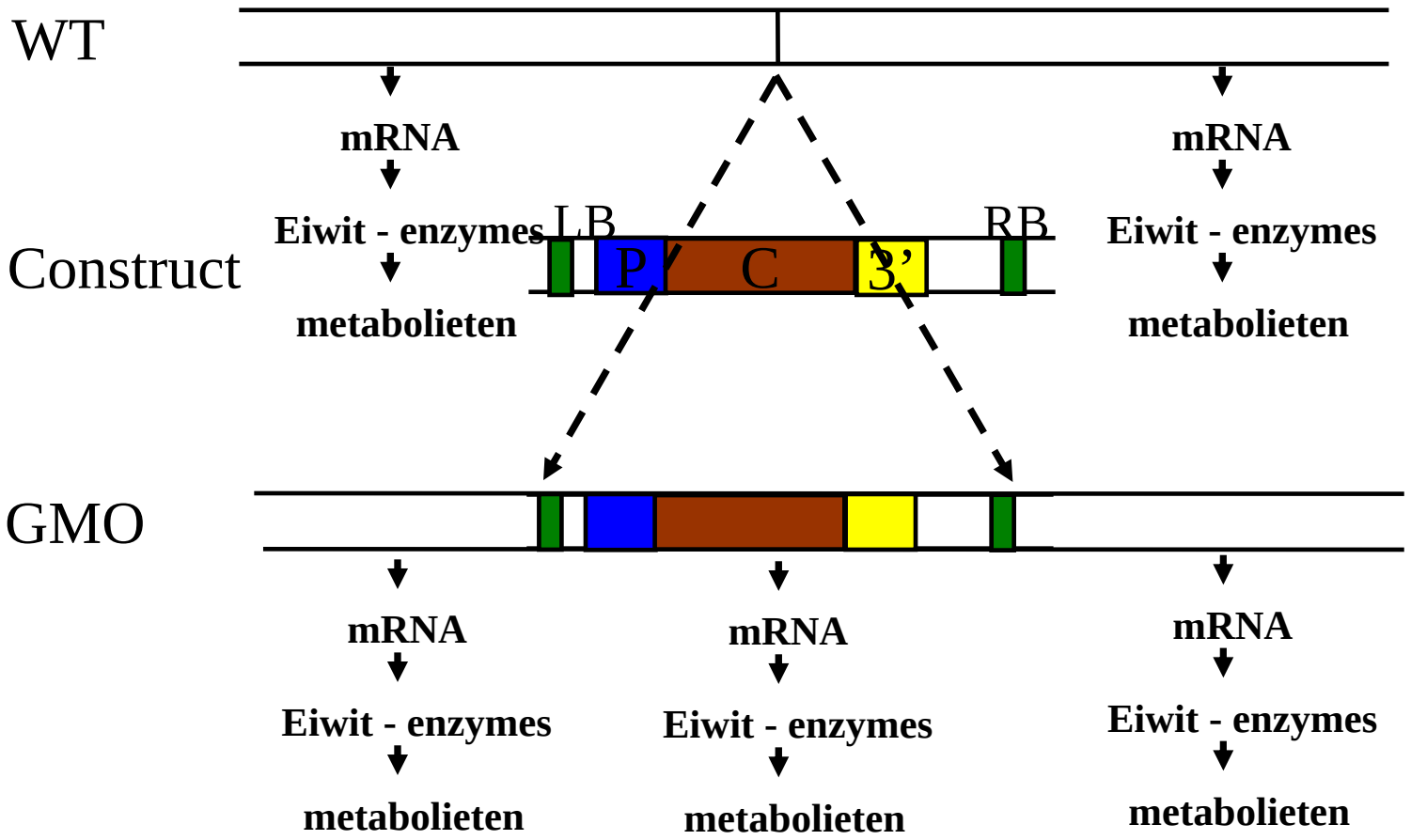
Description of “GMO”

In the risk evaluation of GMOs the effect of the inserted genes as well as the changes in the accepting genome are evaluated



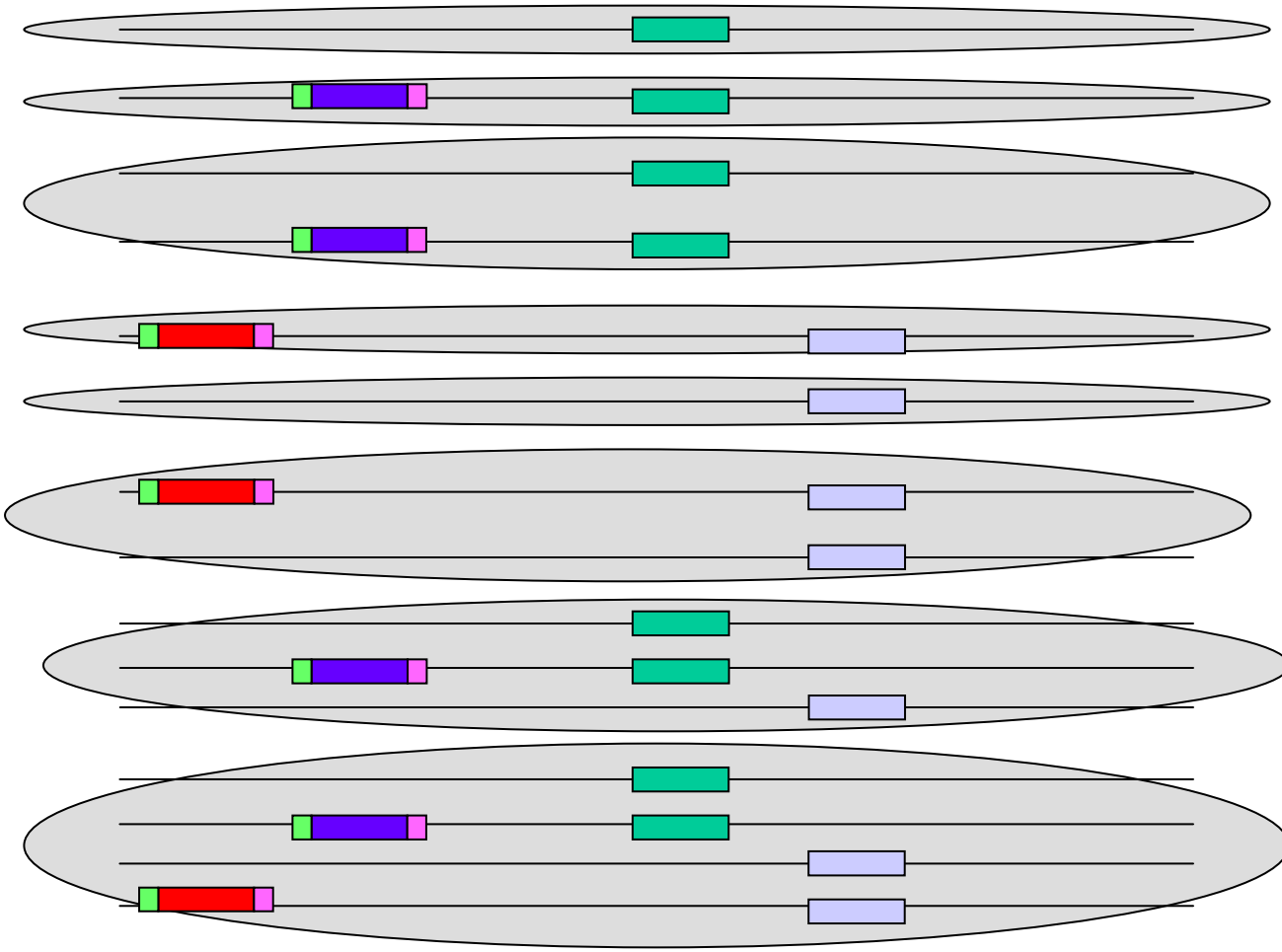
Define target to be quantified

What are the differences at genotypic and phenotypic level?



Define target to be quantified

Does a product contain a GMO? If yes which GMO events are present.



Maize wt

Maize GMO

Maize wt + Maize GMO

Soya GMO

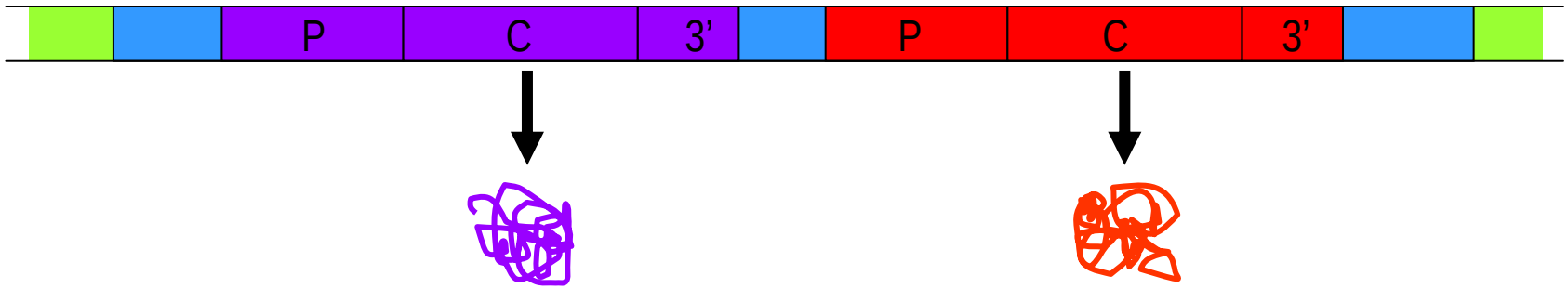
Soya wt

Soya GMO + Soya wt

Maize wt + Maize GMO+ Soja wt

Maize wt + Maize GMO +
Soya GMO + Soya wt

Define target to be quantified



Detection methods

Nucleic Acid-Based Detection

Only possible when the genomic DNA is stably changed or modified

- amplification-based methods
  - polymerase chain reaction (PCR)*
  - fingerprinting/fragment profiling methods
  - iso-thermal amplification
- sequencing (cDNA, gDNA)
- hybridisation-based methods
  - Southern blot
  - Micro-array (low*- and high-density)

Detection methods

Protein-Based Detection

Only possible if a new protein is present in the plant and the primary product

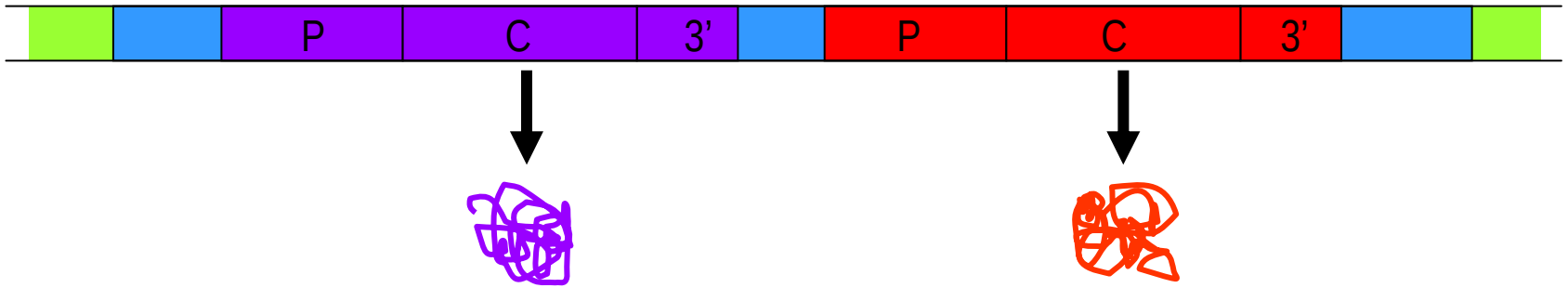
- sequencing (proteins, peptides)
- 1D- and 2D protein gel electrophoresis
- immuno-based methods (qualitative / quantitative), …

Metabolite analysis

Only possible if a new metabolite is present in the plant and the primary product

- Gas chromatography, in combination with MS
- High performance liquid chromatography (HPLC). in combination with MS
- DART, NIRS, …

Detection methods

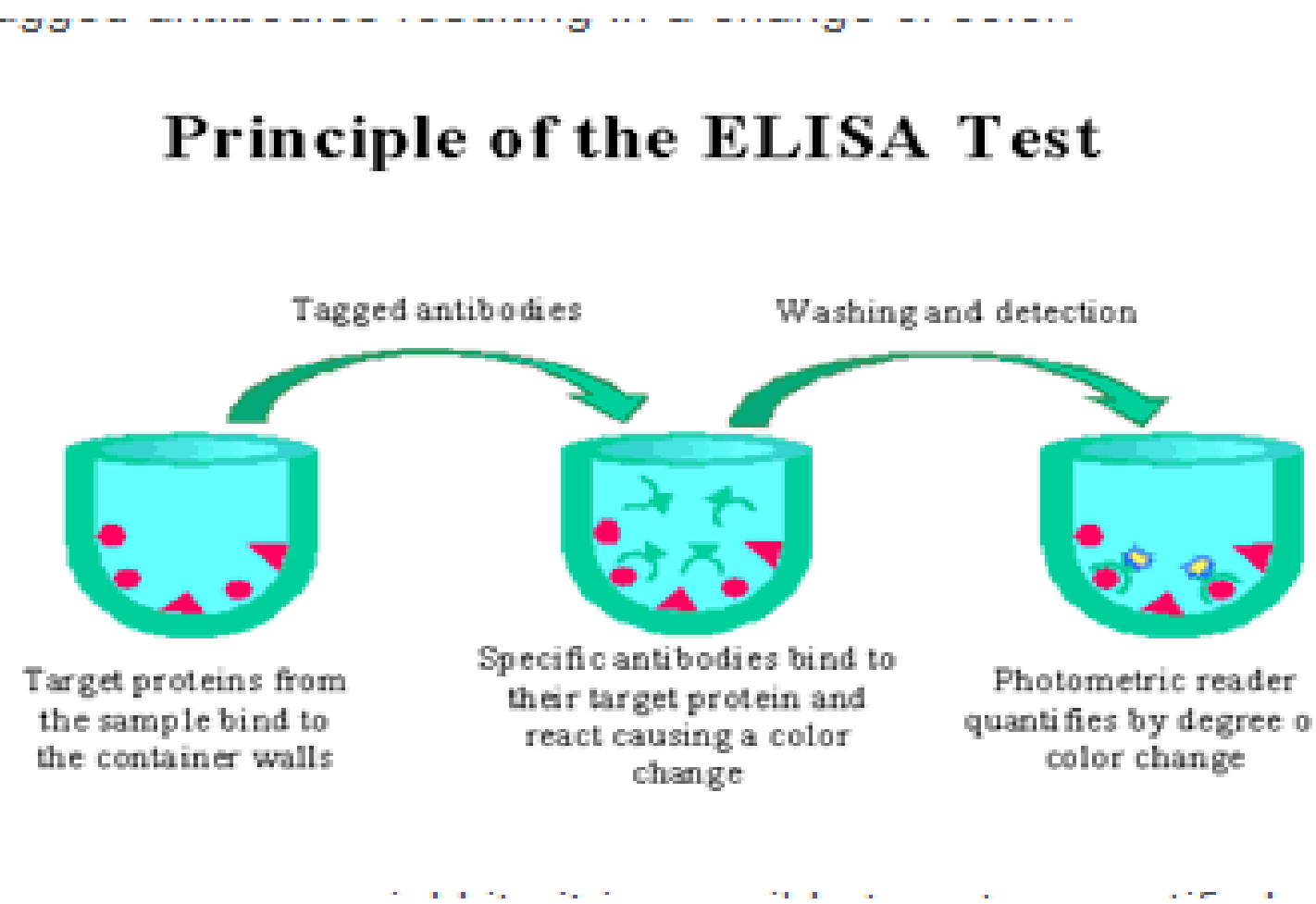


- Screening for phenotype
- Detection of genetic elements and derived products: Screening for the presence of GMOs
- Detection of fusion's between genetic elements
- Detection of fusion region between plant DNA and inserted DNA: Screening for the presence of a particular GMO

Phenotypic screening

Technically possible, but only possible on living plants, time consuming and labor intensive. NOT for FOOD

Detection methods

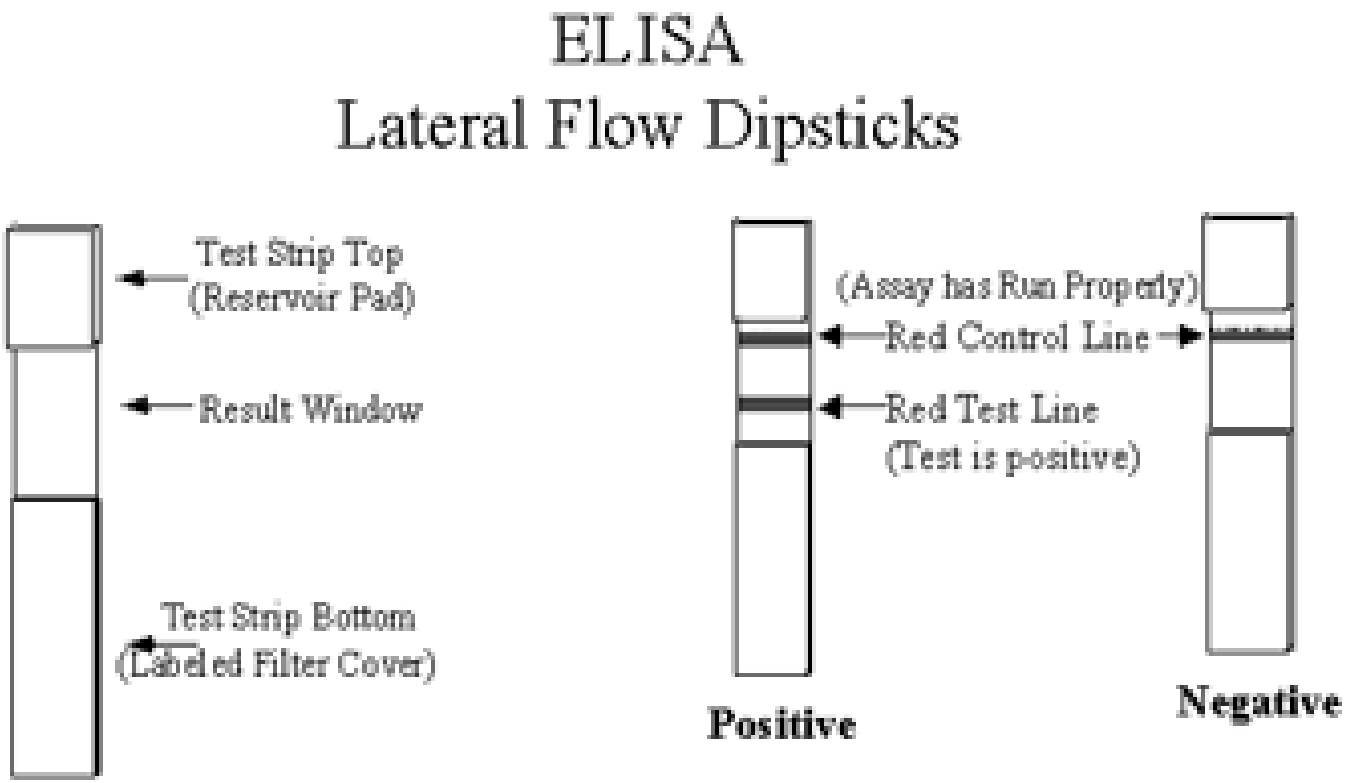


Only possible if the matrix to be analysed contain proteins and if antibodies are available.

Epitopes might change during processing of the raw product, which might effect the quantification.

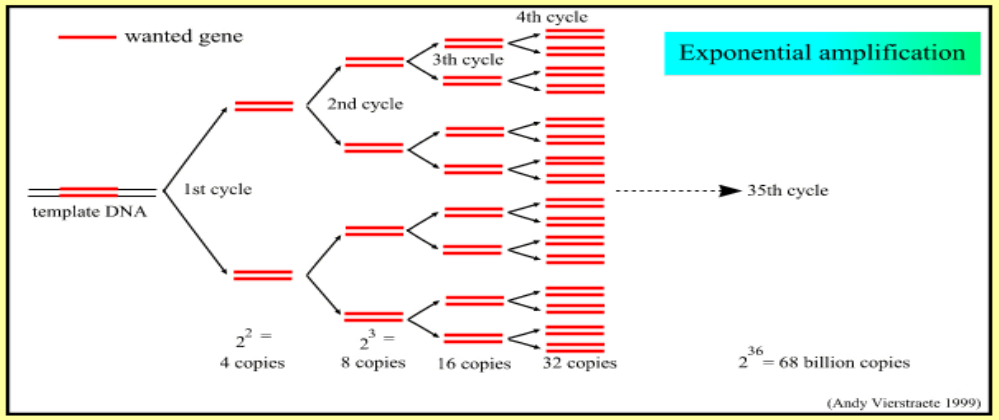
If the trait is not expressed detection is not possible

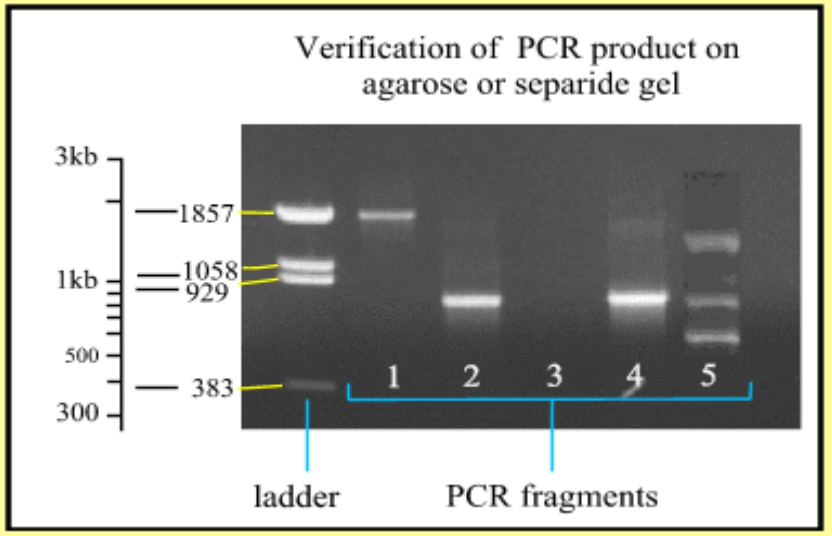
Detection methods

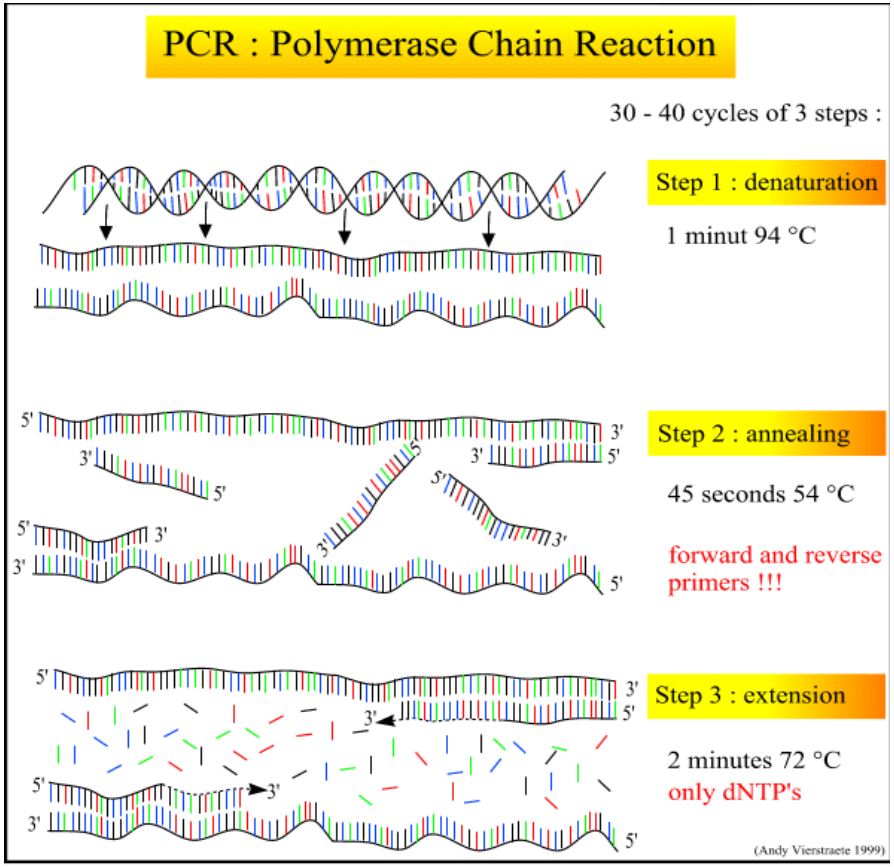


Detection methods

PCR

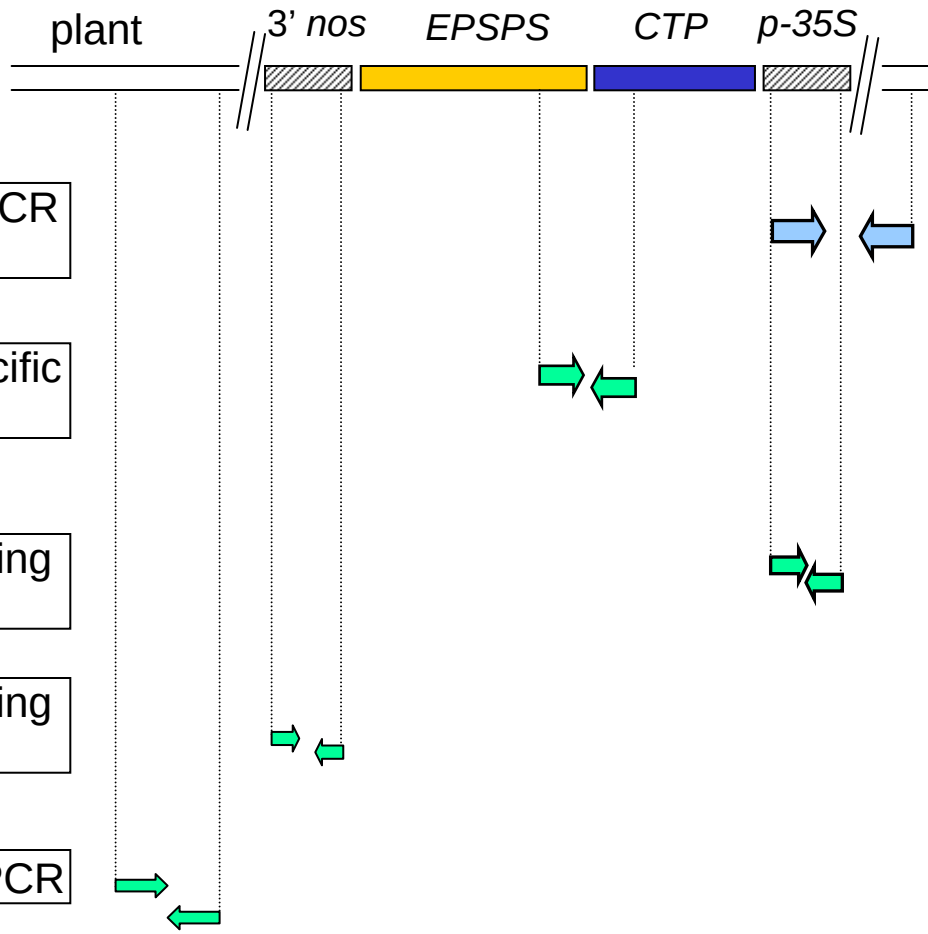


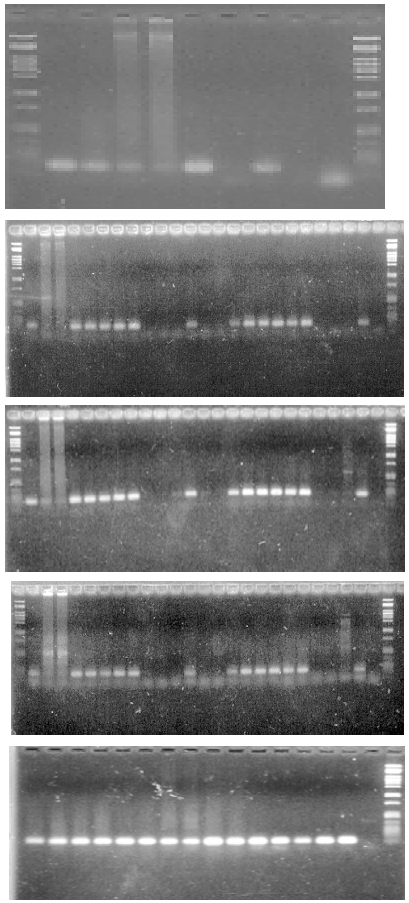




Detection methods

PCR

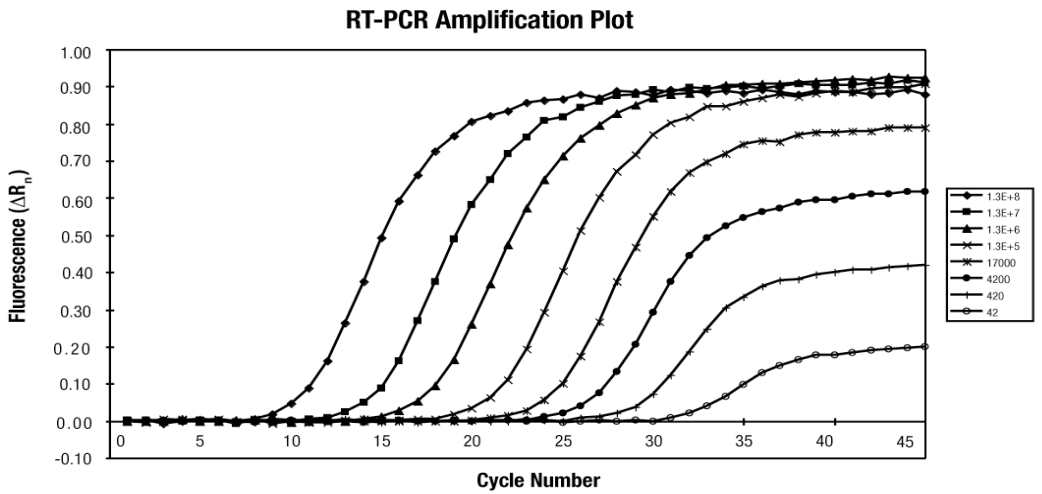


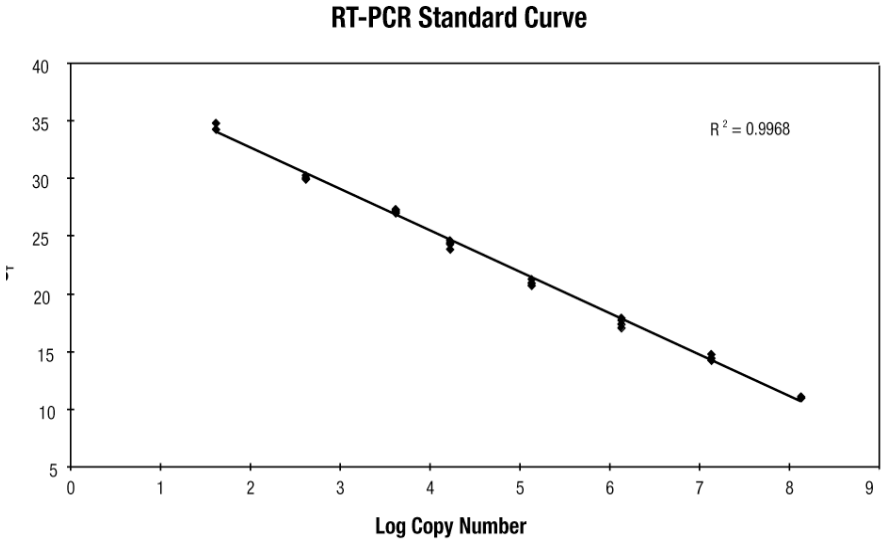


Some technical considerations

Real-time PCR *the technique*

- *Quantification* with real-time PCR:
  - fluorescent signal < TaqMan probe or SYBR Green I dye
  - external calibrator series with well-known amount of target
  - fixing the **F-threshold value** and **threshold cycle numbers**
  - plotting initial target DNA concentration versus C_T value
  - measuring concentration of unknown sample with standard curve





Detection methods: quantification

RealTime - PCR

- Determination of the number of genomes containing a transgenelocus on the total number of genomes
- In RT-PCR quantification the relative amount of target fragment over the number of reference genes is determined
- The target fragment is the part of the transgene locus and the reference gene is an endogenous single copy gene

Protein vs. DNA based methods

- Advantages protein based methods:
  - Cheap, rapid and easy
  - No special equipment needed
  - Formats are simple and user friendly
  - Allows automation and high sample throughput
- Disadvantages protein based methods
  - Complex matrix affects accuracy and precision
  - No constant expression, low expression of protein, certain proteins only expressed in some parts of the plant
  - No distinction between two GM varieties with different genetic construct but same expressed protein
  - Degradation of proteins (thermal treatments, pH changes)

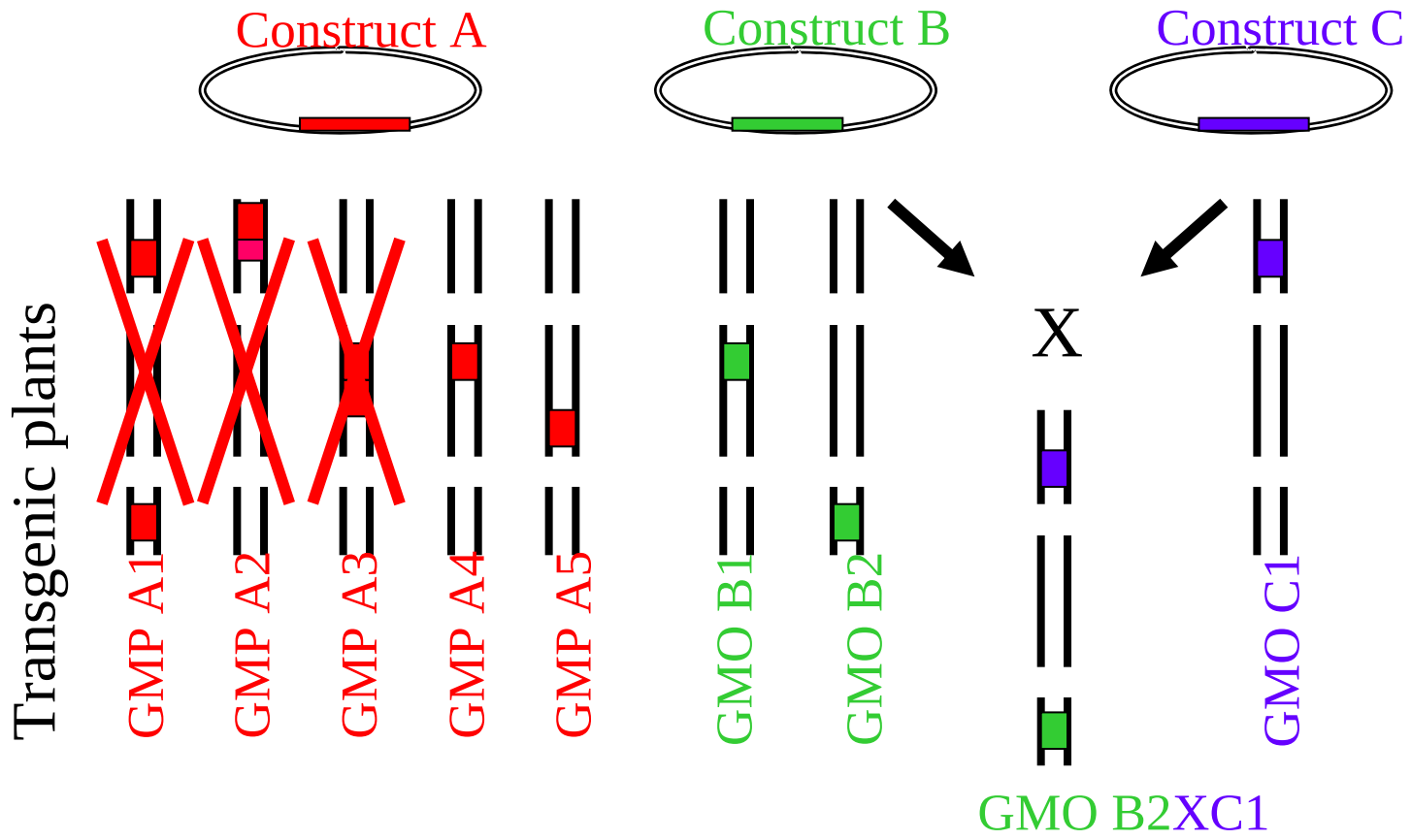
Protein vs. DNA based methods

- Disadvantages DNA based methods
  - Equipment and operation costly
  - Trained staff
- Advantages DNA based methods
  - High specificity and sensitivity
  - Whole genetic information present everywhere in plant
  - Screening, gene-construct and event specific identification possible as well as relative quantification
  - Nucleic acids are very thermostable

Technical considerations

Detection versus identification

Selection of elite events



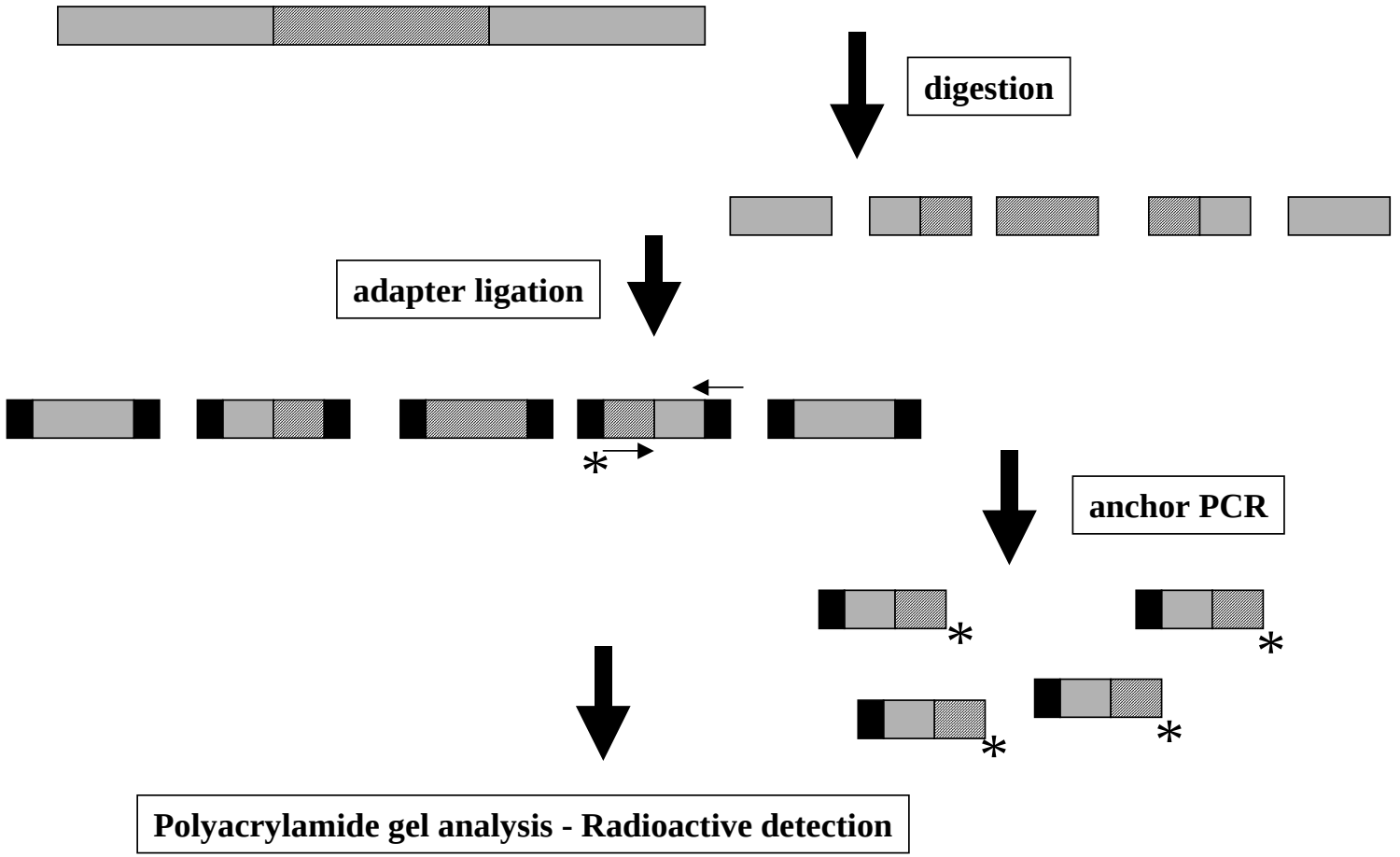
How to quantify stacked gene constructs?

Some technical considerations

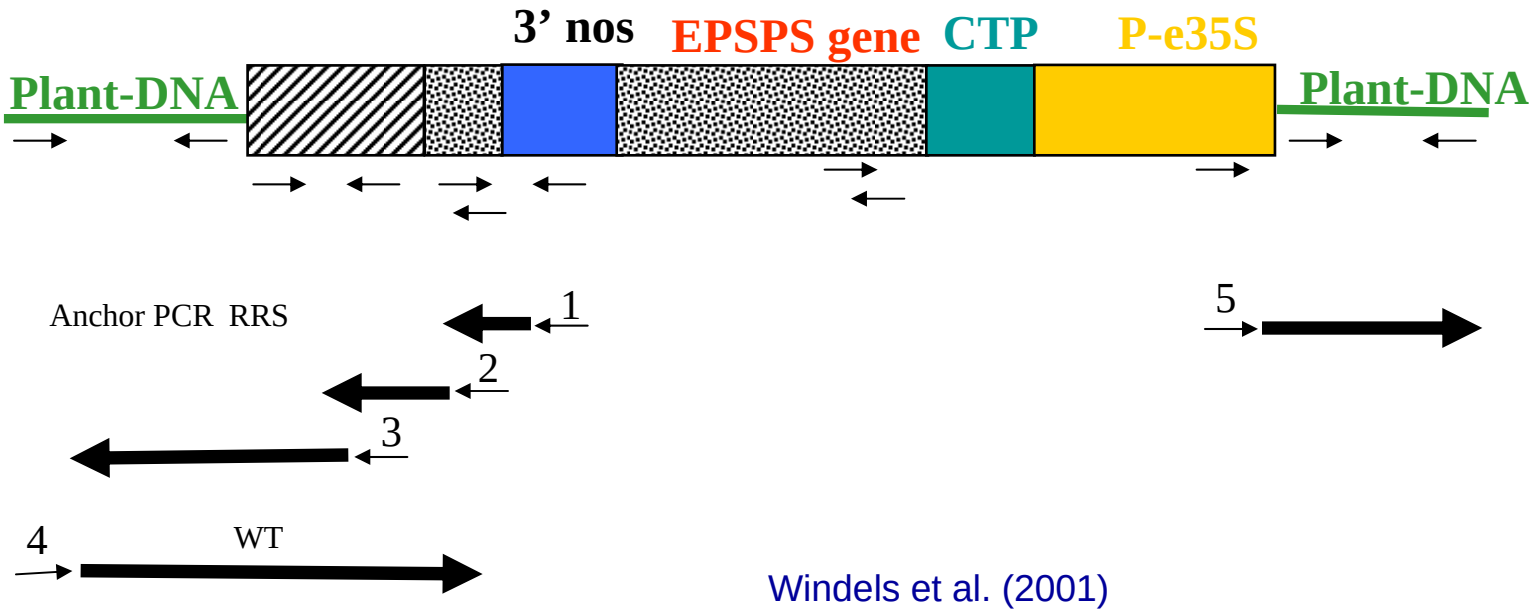
Where to collect information for control purposes?

- Published data:
  - Scientific papers
  - EFSA website
  - JRC, ENGL, CRL website
- From the applicant
- Characterisation of junction regions of the inserted fragments

Anchor PCR

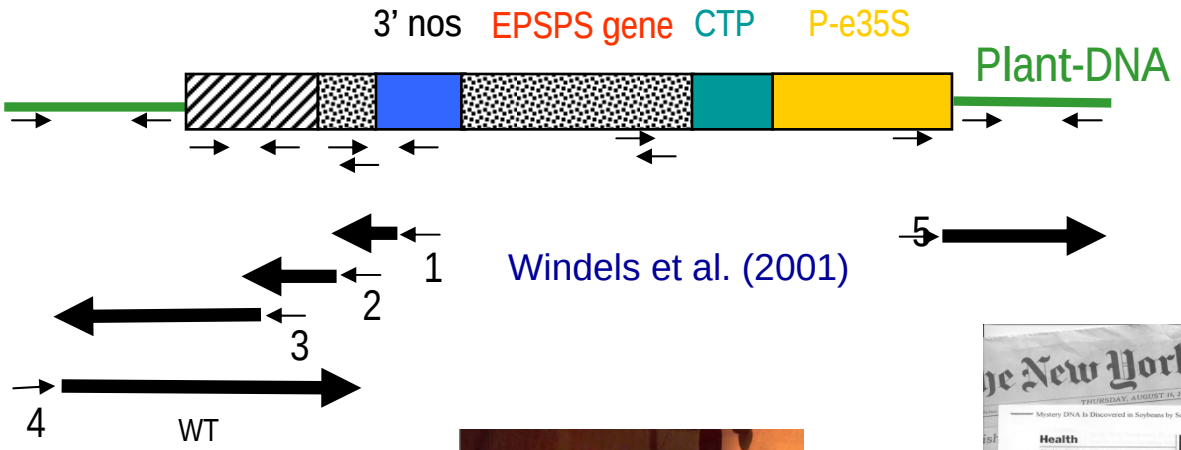

digestion
adapter ligation
anchor PCR
Polyacrylamide gel analysis - Radioactive detection

RR soya: a case study



Windels et al. (2001)

RR soya: a case study



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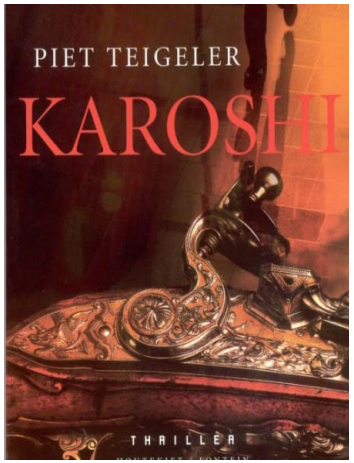
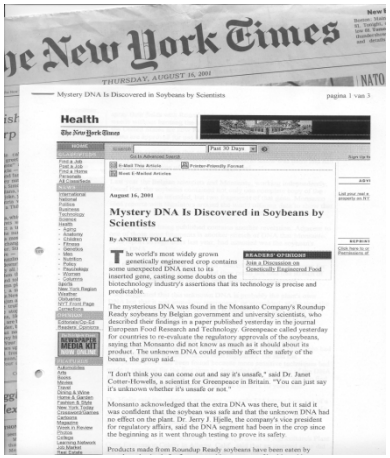
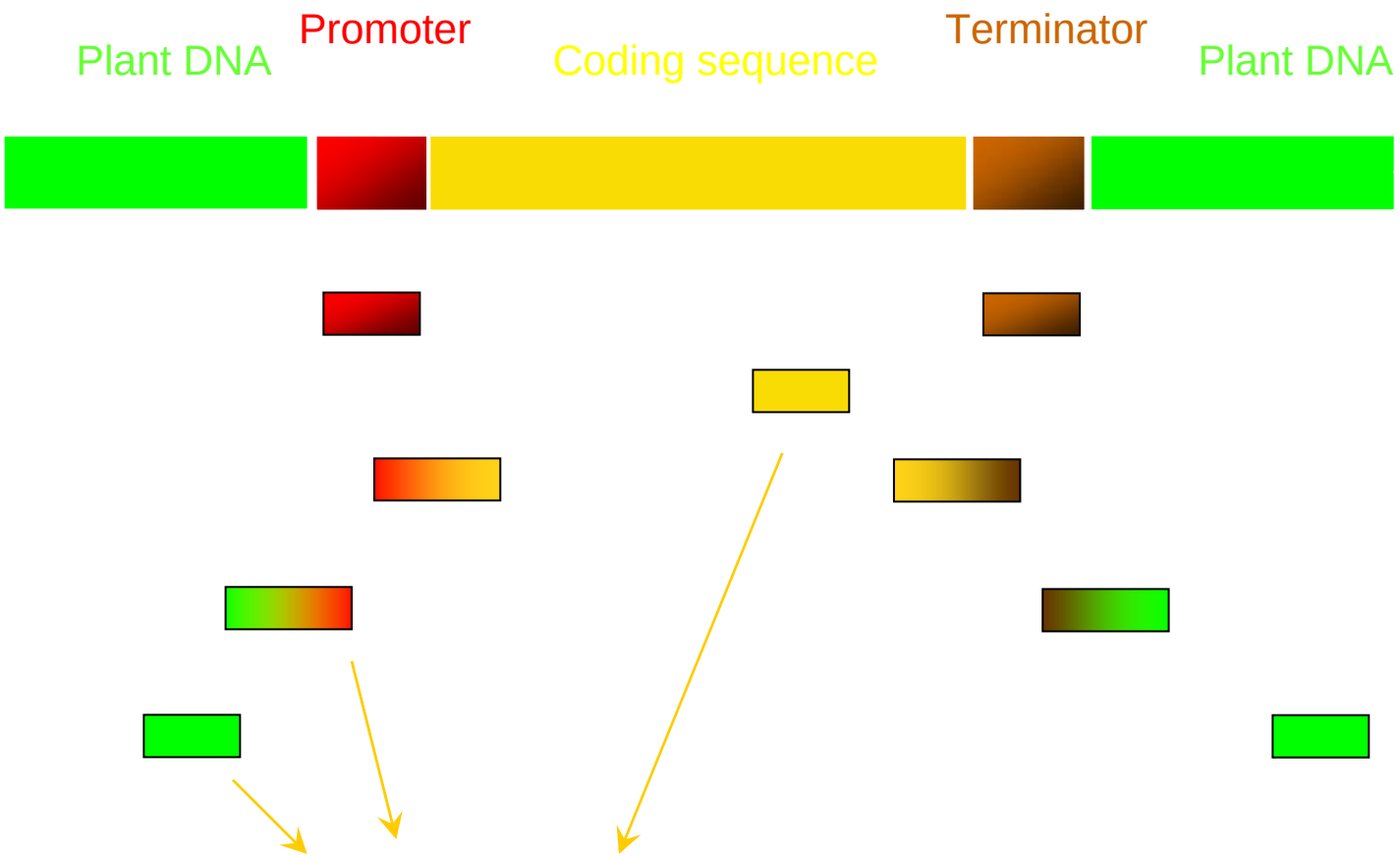




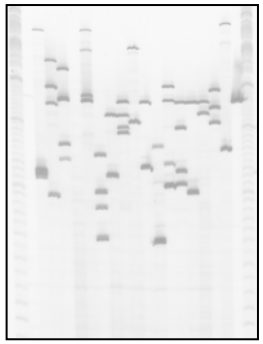
Detection versus identification



Identification from scratch to practise

Development of an analytical control platform, independent from the stakeholders economically involved

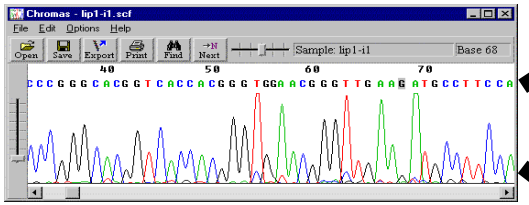
Anchor-PCR



1

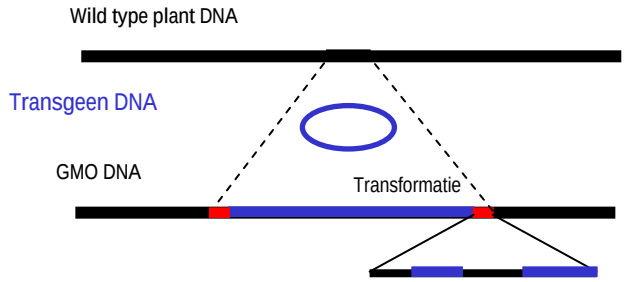
Identification

DNA sequenering



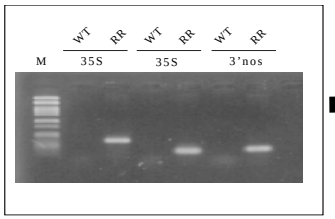
2

Characterisation GMO's



3

GMO routine test

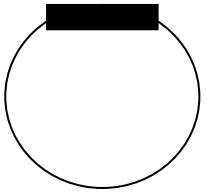


6

NRL and Accreditation

4

Cloning GMO specific DNA fragments



Production of calibrators for GMO quantification

5

EU legislation: 1829/2003/EC en 2001/18/EC

ILVO

European Commission TAIEX
Technical Assistance Information Exchange Instrument (TAIEX), DG Enlargement

Quantification

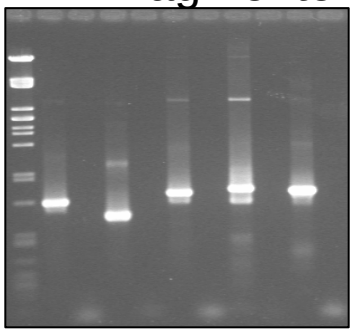
- How to understand the labelling legislation and how to implement this interpretation in a real analysis?
- Internal standard - Reference genes
- Reference material

Reference material - standard

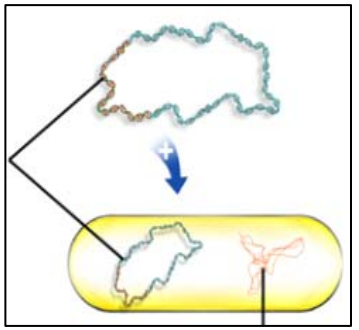
- What can be used as a standard?
  - Mixed grains or seeds
  - Milled grains or seeds
  - DNA (genomic or cloned fragments)
  - Proteins
- Should a standard be universal, for whatever method
- Which material to use as wild type?
- Availability

Constructie van plasmide DNA merkers

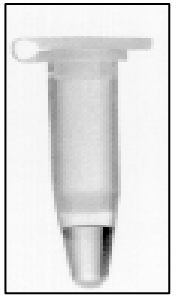
Amplificatie van DNA fragmenten



Klonering en transformatie



Bereiding van Zuiver plasmide DNA



Berekening van DNA kopij aantallen

nanogram → **kopijen**

Verdunningsreeks in aantal kopijen

Eur Food Res Technol (2001) 213:417–424
DOI 10.1007/s002170100405

ORIGINAL PAPER

Isabel Taverniers · Pieter Windels
Erik Van Bockstaele · Marc De Loose

Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products

ILVO

European Commission TAIEX
Technical Assistance Information Exchange Instrument (TAIEX), DG Enlargement

Reference material - standard

Development of an analytical control platform, independent from the stakeholders economically involved

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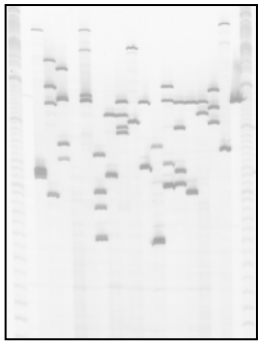

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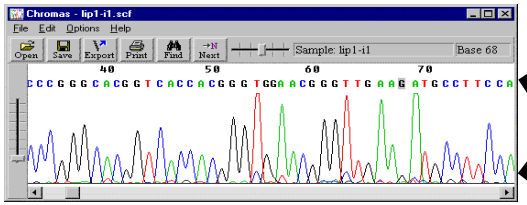

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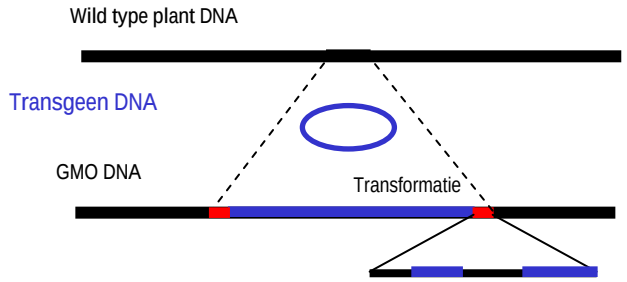

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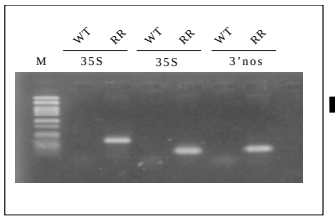

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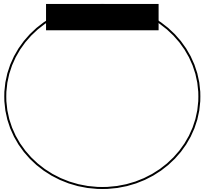

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EU legislation: 1829/2003/EC en 2001/18/EC

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European Commission TAIEX
Technical Assistance Information Exchange Instrument (TAIEX), DG Enlargement

Analytical difficulty is largely determined by the status of the sample to be analysed

Analysis

Event specific quantification

Total quantification

Identification

Detection

Seed

pure product

ixed product

End product

Stacked events

Product

EU authorised Part C

Authorised in non-EU

EU authorised Part B

Unknown/unauthorized

owing number of events that are authorised

Legal status

Analytical difficulty is largely determined by the status of the sample to be analysed

Analysis

Event specific quantification

Total quantification

Identification

Detection

Seed

Grain

Raw, pure product

Raw, mixed product

End product

Product

EU authorised Part C

Authorised in non-EU

EU authorised Part B

Unknown/unauthorized

Legal status

Cost ⟷ Risk

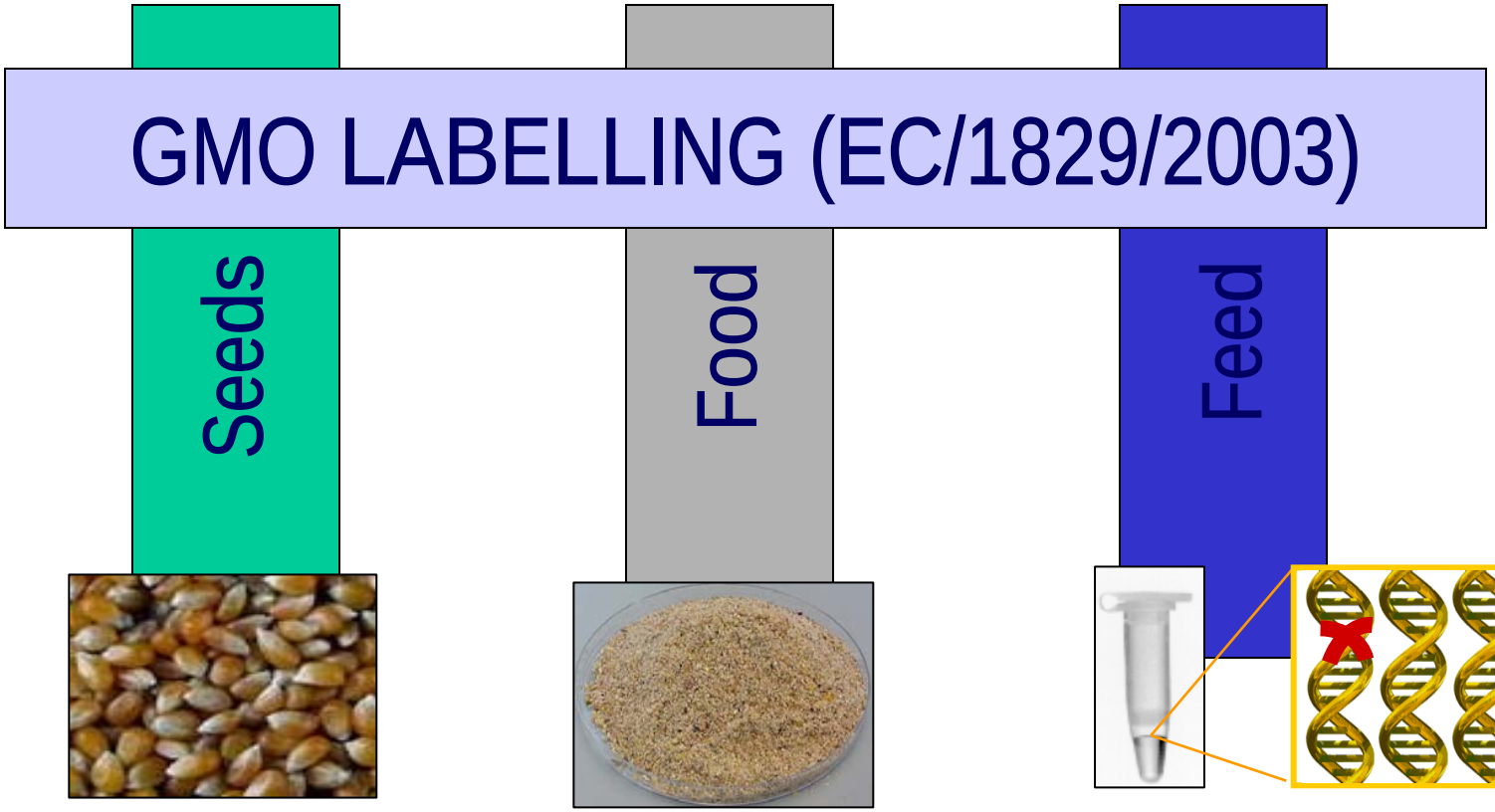
Results have to be scientifically correct

Technical considerations

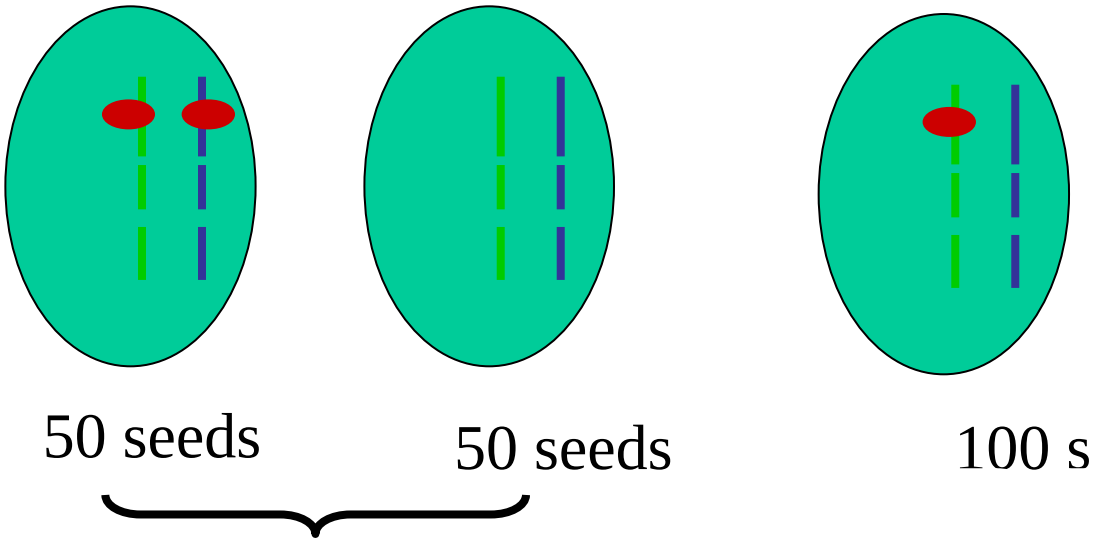
- Transferability of analytical data throughout the production chain
- Which unit to be used to express the experimental result?

- Number of transgenic seeds:total number of seeds (seeds for sawing)
- Number of transgenic grains/beets/fruits:total number grains/beets/fruits (harvested products)
- Number of transgenic particles: total number of particles (milled, raw, pure or mixed products)
- Number of transgenic proteins:total number of proteins (isolated proteins from seeds, grains or derived products)
- Number of transgenic haploid genomes:total number of haploid genomes (isolated DNA from seeds, grains or derived products)

How to implement GMO detection across the food production chain

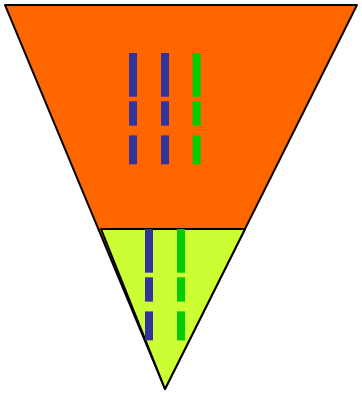


Consideration on genetics



- When in a quantitative analysis a powder is used the same result will be obtained
- But the impact of a seed being homozygous for the transgene locus will be double when we consider the the quality of the grains

Genome distribution in the maize seed



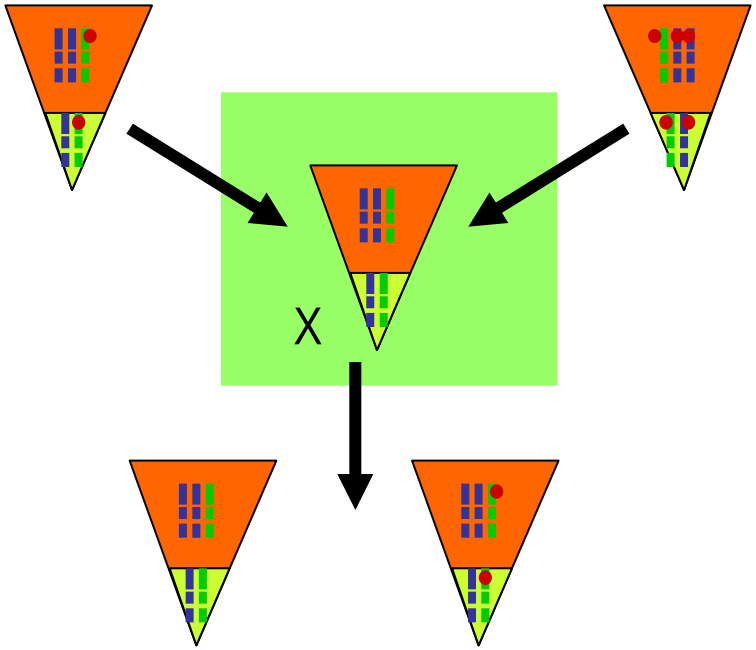
What is the number of nuclei in the endosperm and in the embryo

- cells in embryo are small
- cells in endosperm are larger, but might contain more DNA due to endoreduplication

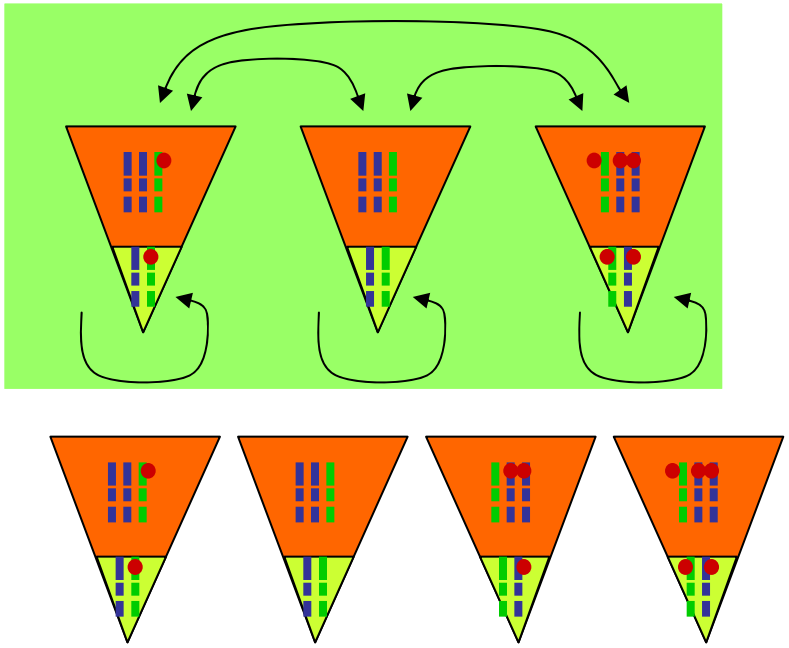
Experiments:

- Determine the amount of DNA in the embryo versus the endosperm
- Compare results based on real-time PCR versus individual seeds

Impact on interpretation of data



Risk of underestimating the number of seeds



?????

Many factors can have an effect on the genetic drift and on the composition of the lot

Unit to be used to express the experimental result

COMMISSION RECOMMENDATION

on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003:

Percentage of GM DNA: the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes.

⟹ Quantitative experimental result expressed as copy number

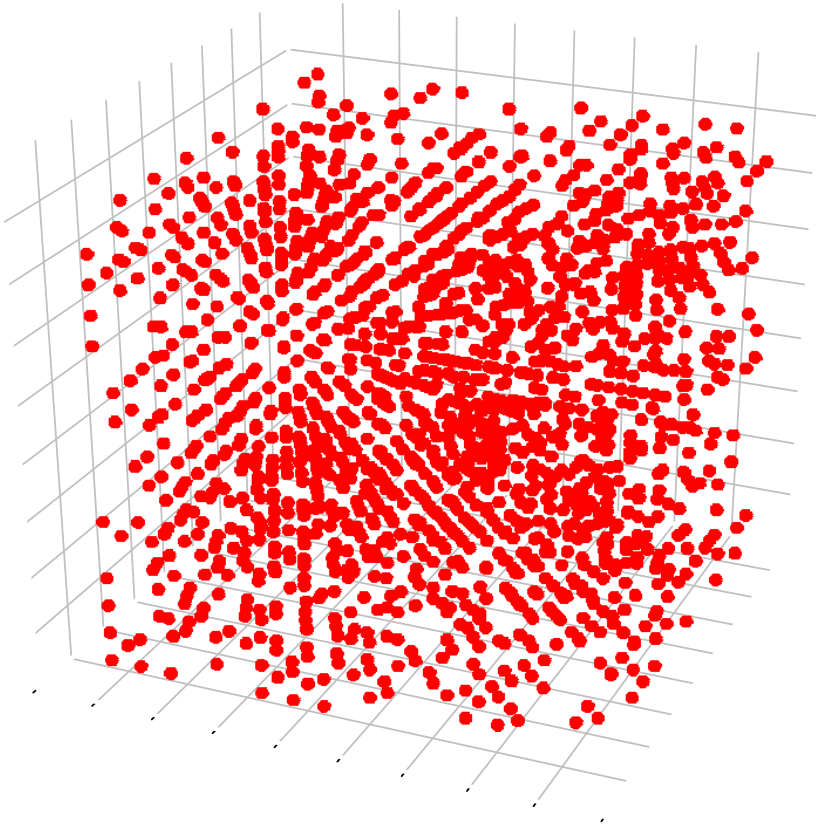
Importance of representative sampling

Might be responsible for errors much bigger than those linked to the analysis method

Importance of representative sampling

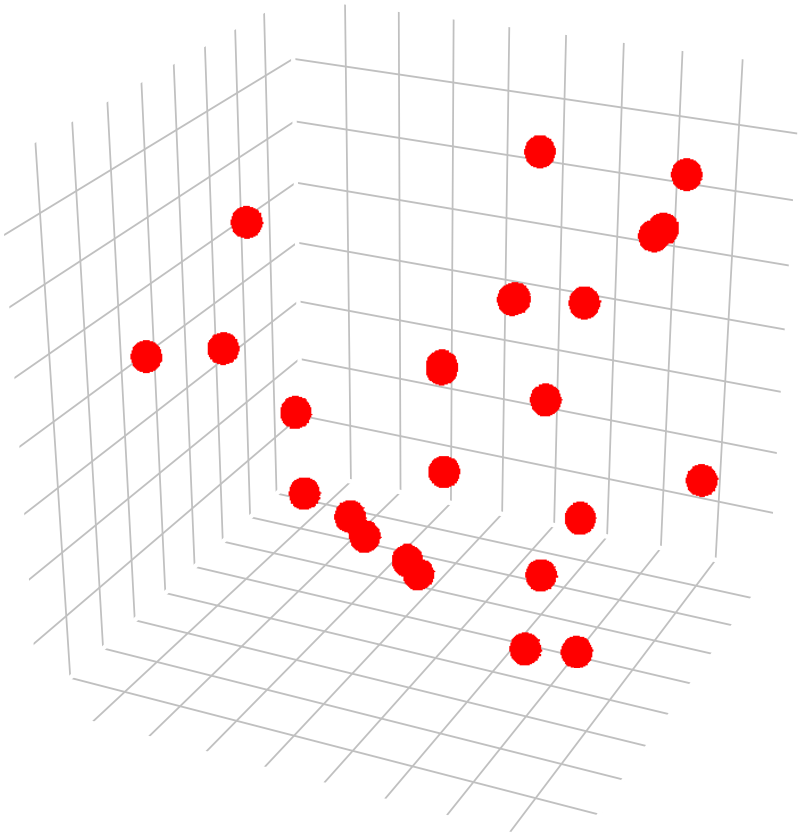
% impurity = 1%

Stratification = high

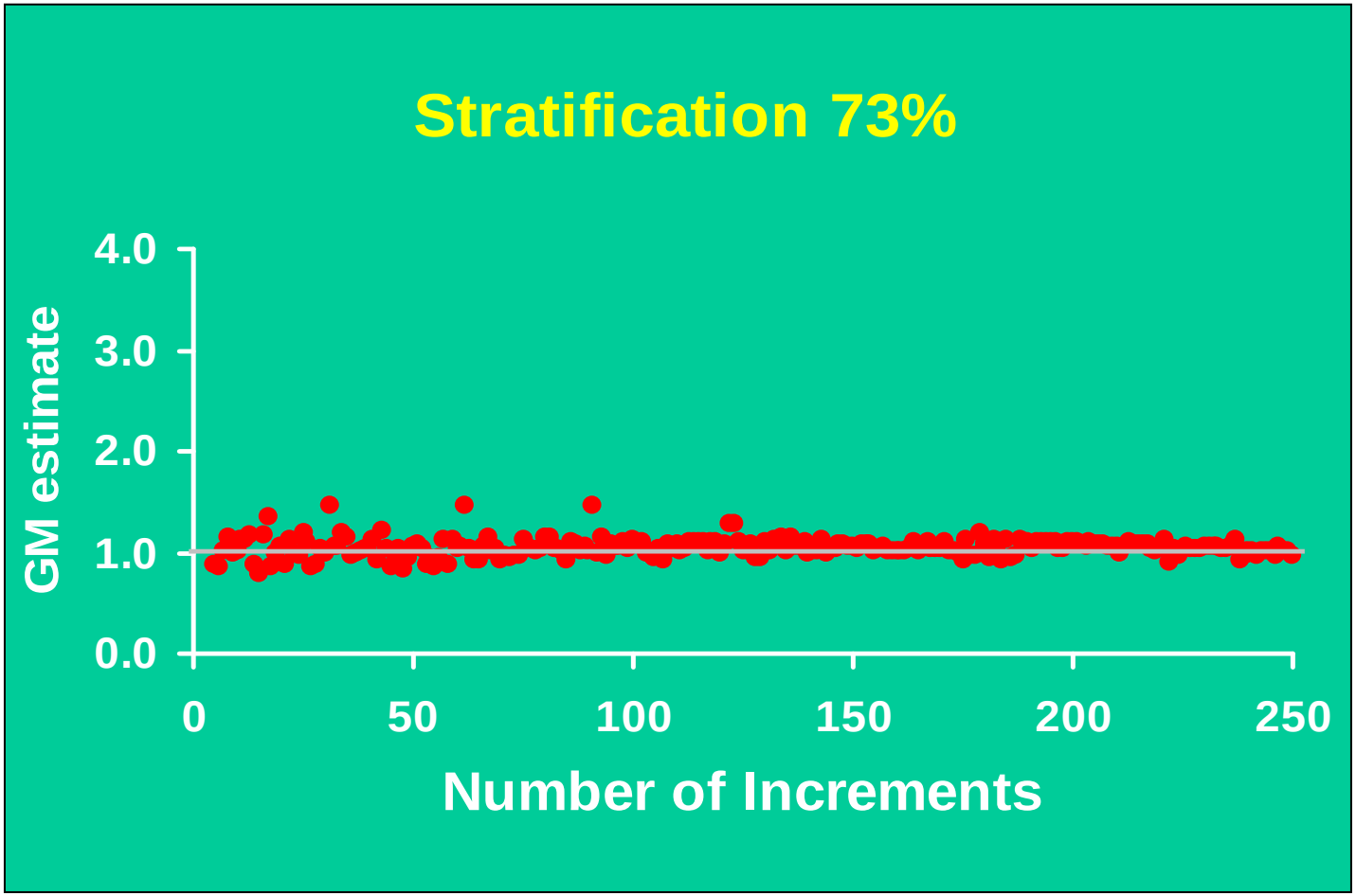


% impurity = 1%

Stratification = low



Importance of representative sampling



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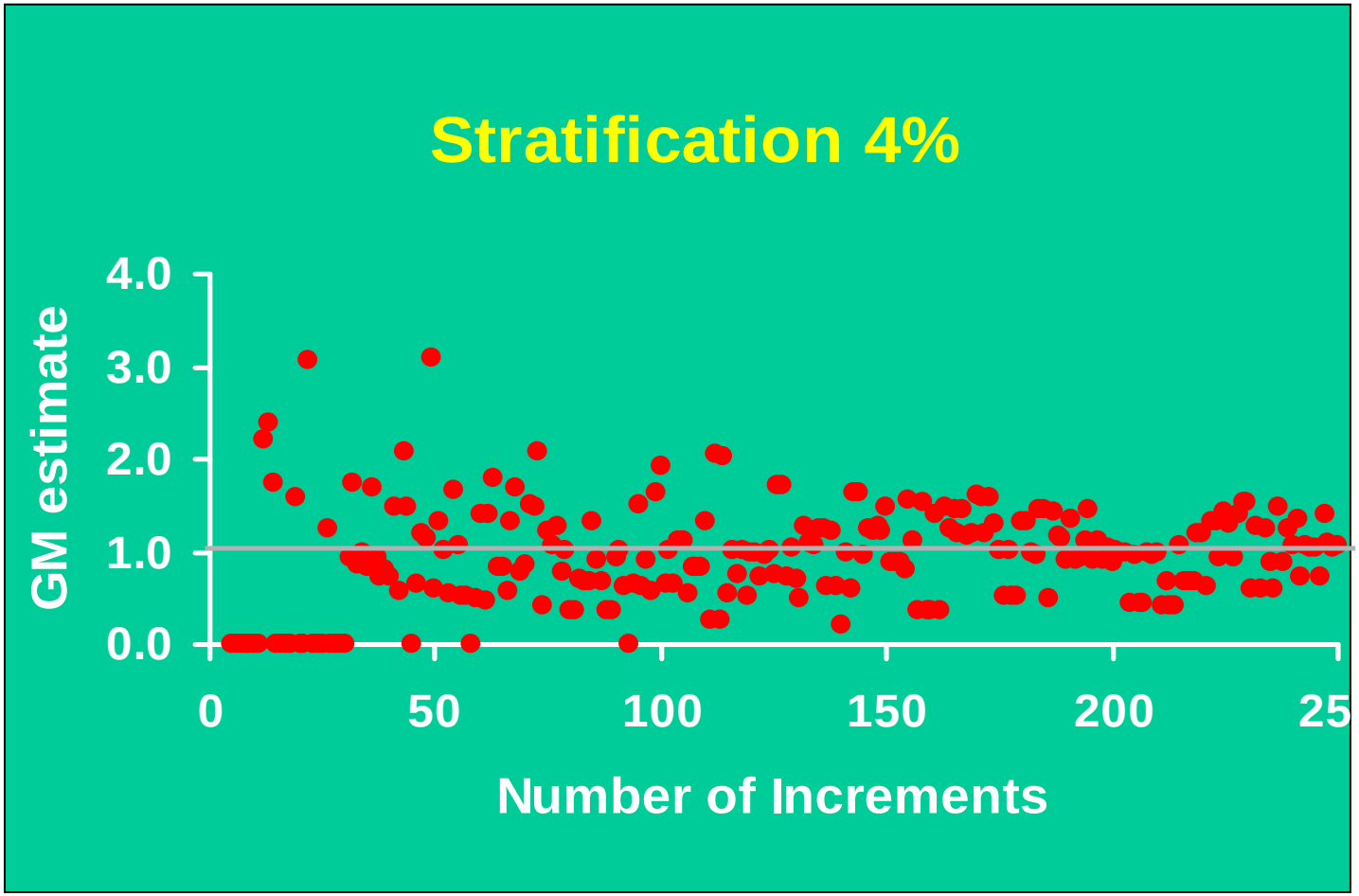
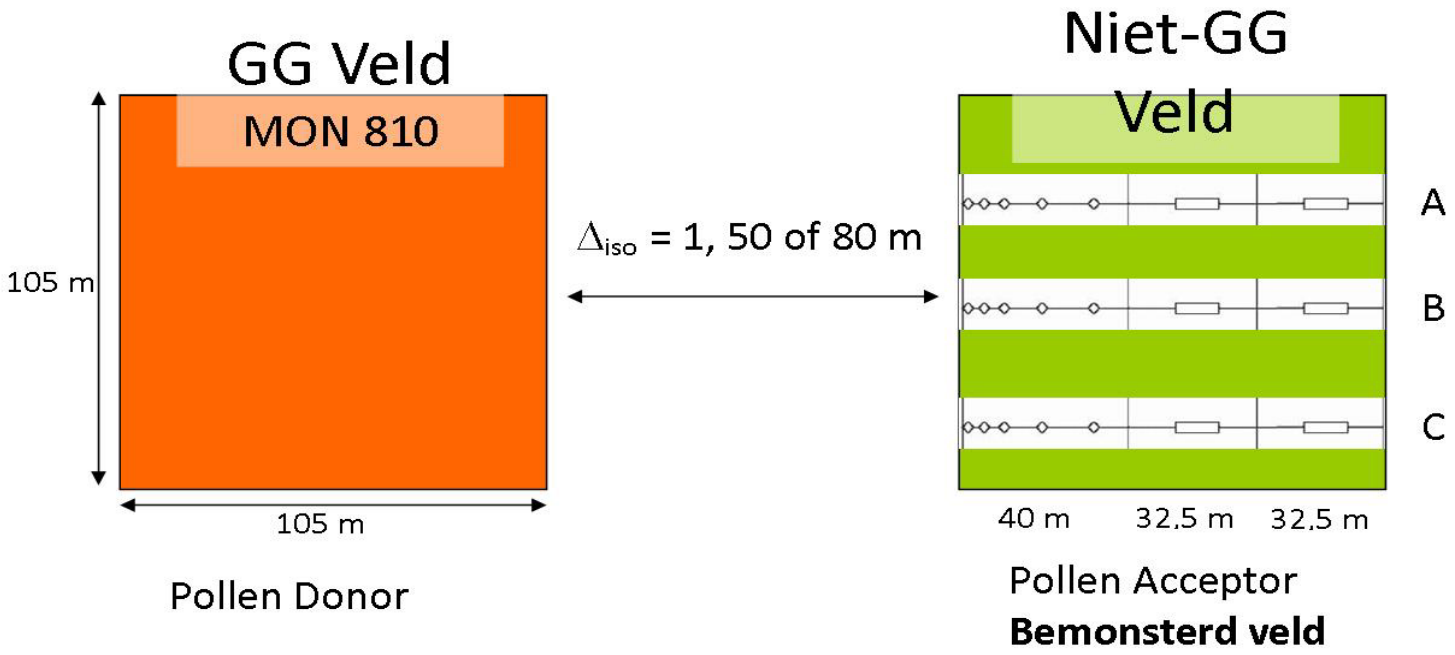


Sampling in the context of co-existance

EXPERIMENTAL FIELD TRIAL

DETAILED DETERMINATION OF OUTCROSSING

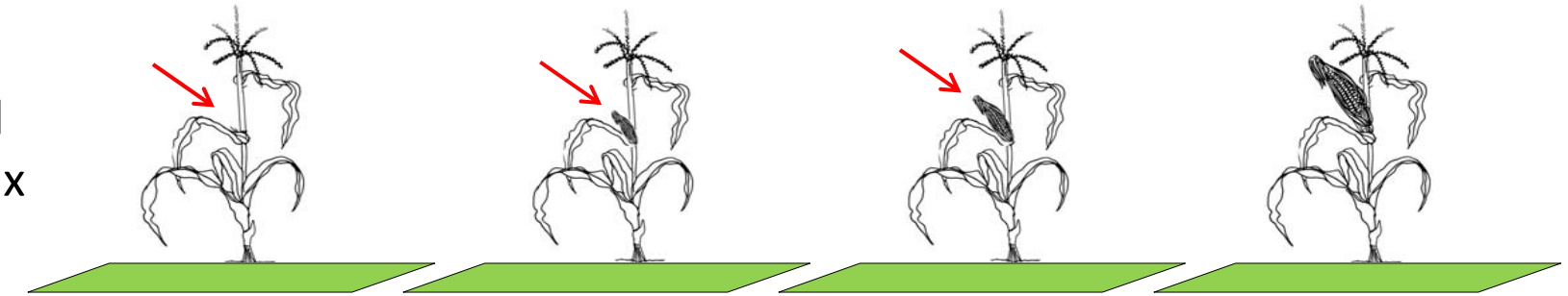


Sampling in the context of co-existance

EFFECT ONTWIKKELINGSSTADIUM VAN DE PLANT OP
AANDEEL EMBRYO/KORREL EN AANDEEL KORREL & KOLF/PLANT

% GGO geoogst product ~ aandeel korrel en kolf in product ~ tijdstip van bemonstering:

Maïsveld
variëteit x



Tijdstip 1
(**na bestuiving**)

Tijdstip 2
(groeiende kolf)

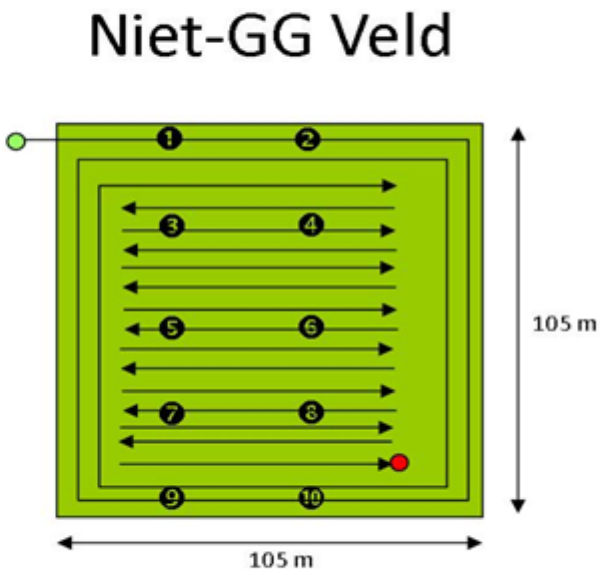
Tijdstip 3
(groeiende kolf)

Tijdstip 4
(**voor oogst**)

Sampling in the context of co-existance

BEPALING GGO GEHALTE OP BASIS VAN BEMONSTERING TIJDENS OOGST

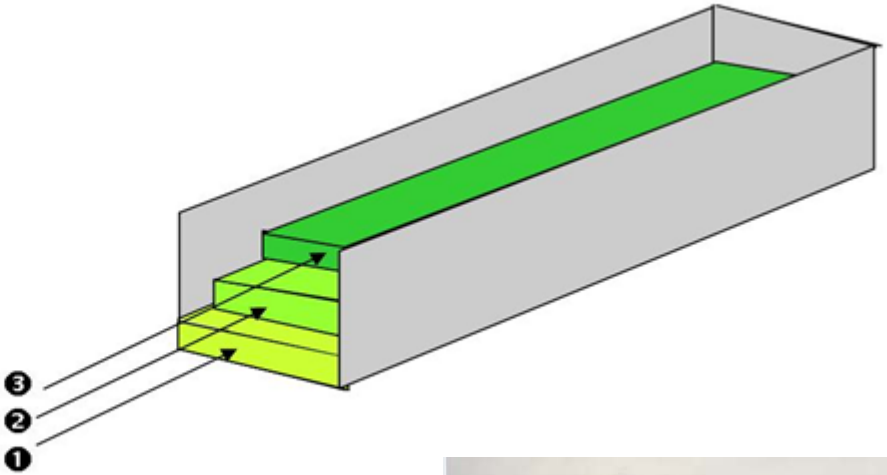
Percentage kruisbestuiving in niet-GG velden op verschillende afstanden van GG veld



Procedure staalname tijdens oogst+ analyse

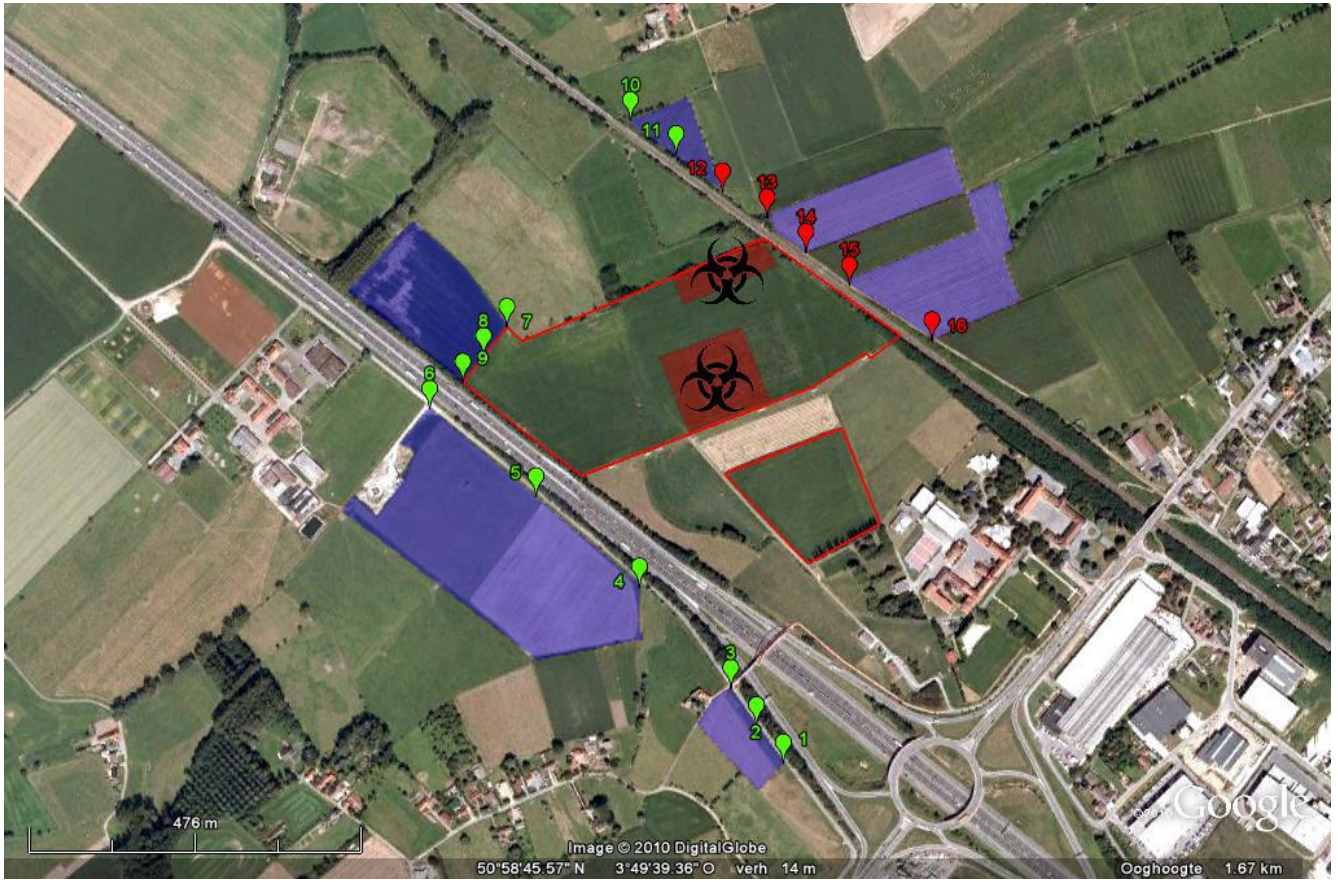
Sampling in the context of co-existance

BEPALING GGO GEHALTE OP BASIS VAN BEMONSTERING OP DE PLAATS VAN EERSTE OPSLAG



Procedure staalname na de oogst + analyse

Importance of representative sampling



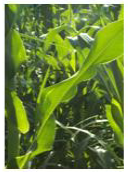
Sampling in the context of co-existance

Importance of representative sampling

Official sampling in the presence of a bailiff

For daily enforcement, and for farmers this is far too expensive. Therefore different srategies are currently under evaluation

Sampling in the context of co-existance

1


Bemonsteren bladmateriaal, kolven en korrels



Bemonsteren geoogste snijmals en CCM

2

Tijdelijke bewaring bij –80°C

3

Vriesdrogen van grote hoeveelheden stalen, in bulk

4

Verpoederen van gevriesdroogde stalen

5

Poeder klaar voor analyse

6

Georganiseerde bewaring vacuümverpakte poeders bij –20°C

Sampling and PCR analysis on site

General considerations and conclusion

At the moment GMO detection, identification and quantification in EU is focusing on amplification based methods

- Methods need to be validated
- Laboratories for routine testing are equipped for PCR based methods
- Screening for common elements is used to reduce costs
- The method is applicable throughout the agricultural food/feed/non-food/feed production chain

General considerations and conclusion

- The complexity of the analytical strategy and the difficulty of the experiment will largely be influenced by:
- The status of the GMO: lab environment, breeding program or commercial application
- The level in the production chain to be controlled: seed production, primary harvest, raw product, processed product, final product
- The information that is available
- The question to be addressed
- For what purpose the data will be used: academic experiment, quality control, enforcement, disputes etc.

Thank you for your attention

29 & 30 September 2010

Enlargement/Networking Workshop on Harmonisation of GMO Analysis

Institute for Agricultural and Fisheries Research

Technology and Food Science Unit

www.ilvo.vlaanderen.be

Agriculture and Fisheries Policy Area