Molecular insight into the hazards and risks related to genetically modified plants

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European Food Safety Authority

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This talk

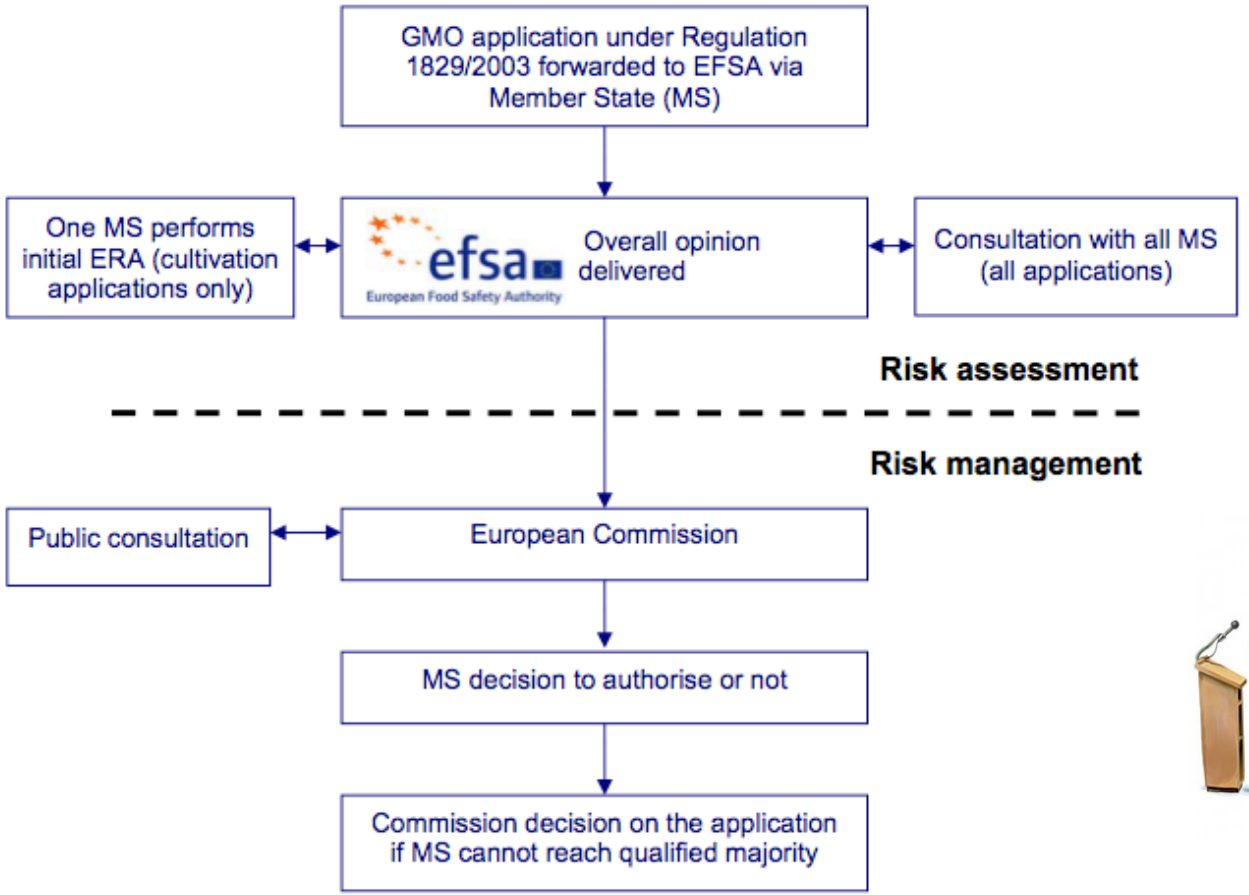
1. What is a hazard ? A risk ?
2. Risk analysis in the EU law : a primer
3. Molecular analysis of GM plants : what, how and why ?
4. The future : new avenues for the genetic modification of plants and possible impacts on risk assessment

Hazard and risk

- **Hazard** : something capable of causing harm, i.e. adverse effects to health or the environment
- **Risk** : a function of the likelihood of the adverse effects and of their severity

The three pilars of Risk analysis :
Risk assessment, risk management, risk communication

Authorisation procedure under Regulation (EC) No 1829/2003 (centralised procedure)



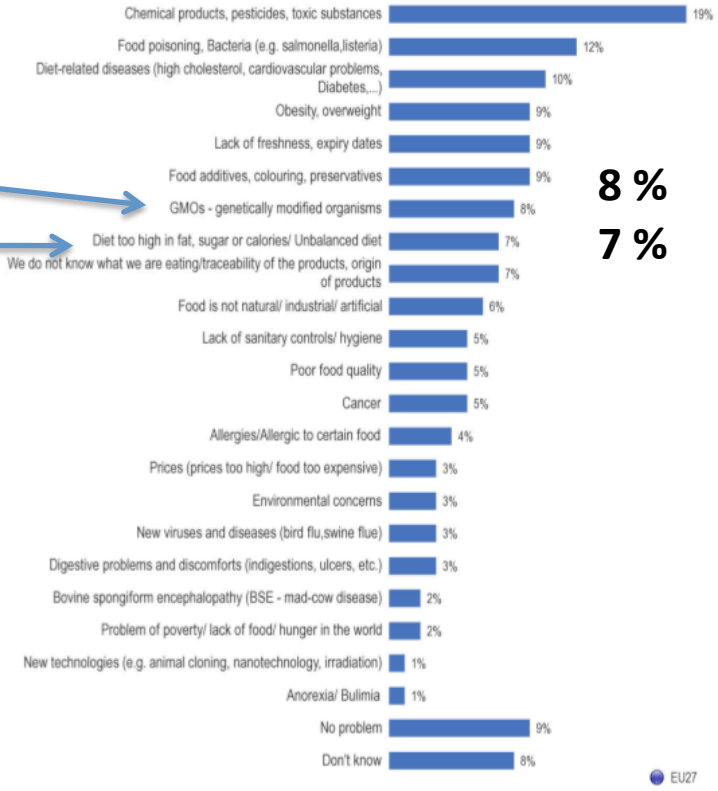
Risk communication :
Scientific risk is not perceived risk.

Eurobarometer 2010 on Food-related risks :

« What are all the things that come to your mind when thinking about possible problems or risks associated with food and eating ? »

« GMOs - genetically modified organisms »

« Diet too high in fat, sugar or calories / Unbalanced diet »



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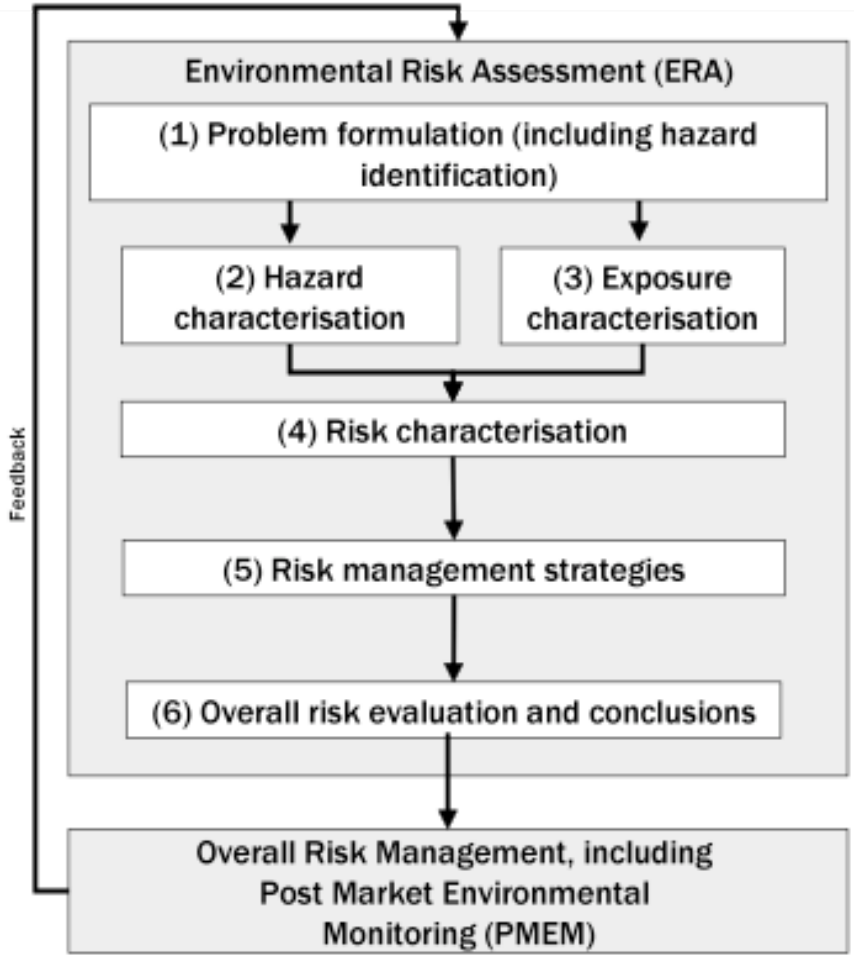
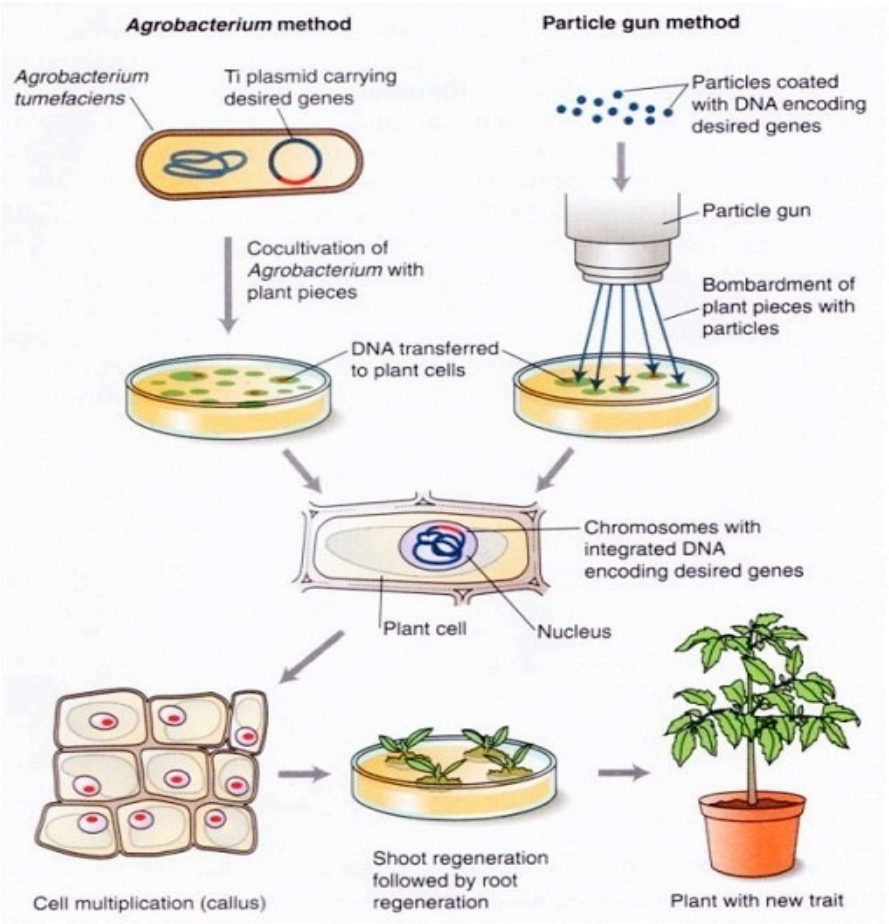


Figure 1: Six steps within the environmental risk assessment (ERA) and relationship to risk management including monitoring according to Directive 2001/18/EC and Regulation (EC) No. 1829/2003.

Molecular characterization of GM plants (but what is a « GMO » ?)

« *an organism, with the exception of human beings, in which the genetically material has been altered in a way that does not occur naturally by mating and/or natural recombination.”*

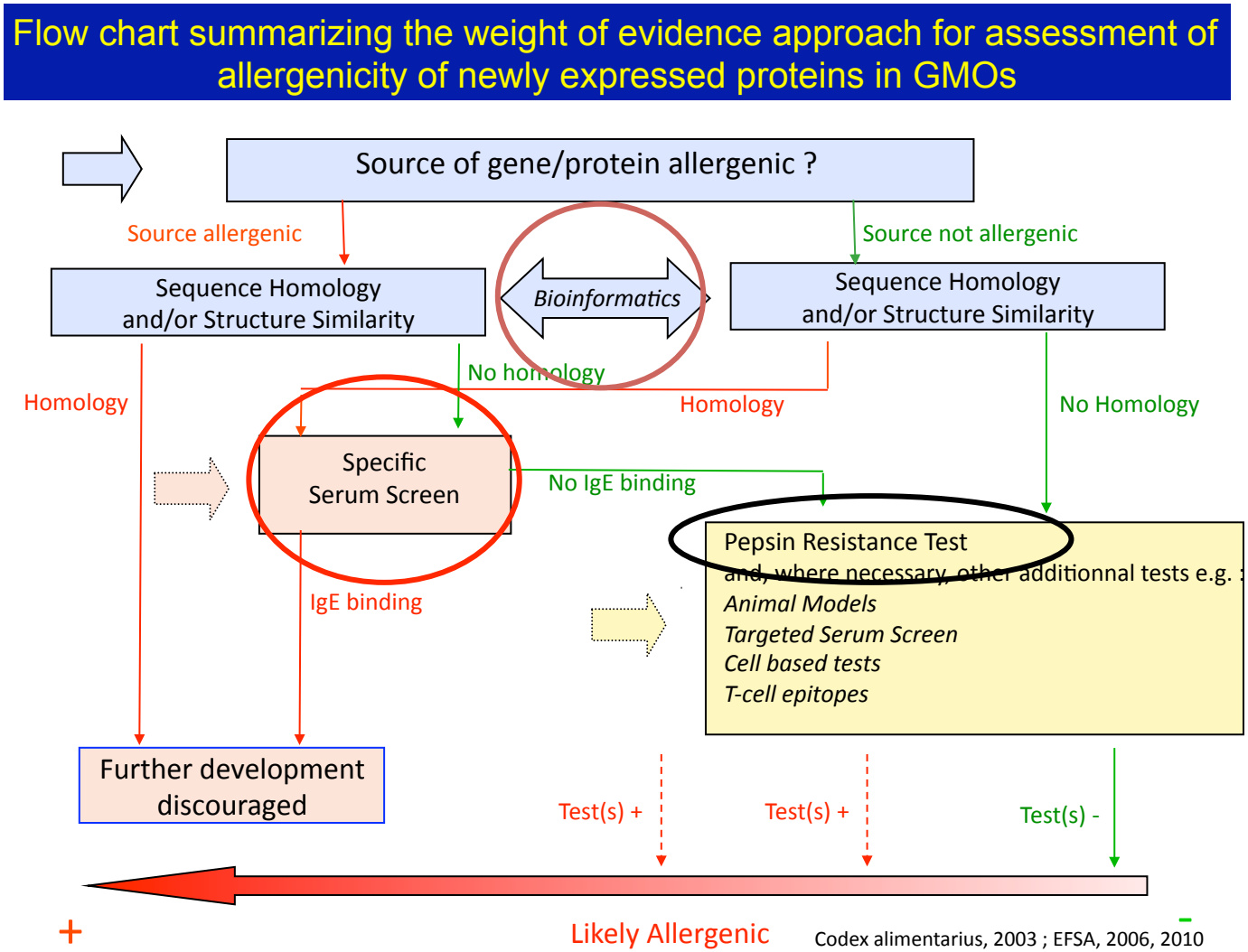
(Directive 2001/18/EC)



Molecular characterization of GM plants : principles

- **The rationale :**
  - If new hazards were due to the genetic modification, analysing the structure and expression of the DNA of the GMP should help in their identification.
  - Molecular characterization is never sufficient to demonstrate a risk : biological data are needed to demonstrate hazards and risks.
- **The aims :**
  - to check for the intended effects at the gene/protein levels : expression of new proteins, up- /down-regulation of endogenous genes
  - to check for unintended effects of the genetic modification : new ORFs potentially coding for peptides with similarity to allergens and toxins, disruption/altered expression of endogenous genes at the insertion site, other hypothesis-driven analyses

Molecular characterization is only part of hazard/risk identification : example of the possible allergenicity of a GMP



(Courtesy of J.-M. Walch, INRA, FR)

Molecular characterization of GM plants : contribution of bioinformatics to hazard identification

- **New proteins** encoded by the transgenes : similarity with allergens, with toxins ?
  - Bioinformatic analysis of the newly expressed proteins : similarity search with known allergens and toxins in public databases

- Unforeseen **peptides encoded by new ORFs** created by the genetic modification ?
  - Bioinformatic search for similarity with known allergens and toxins in public databases

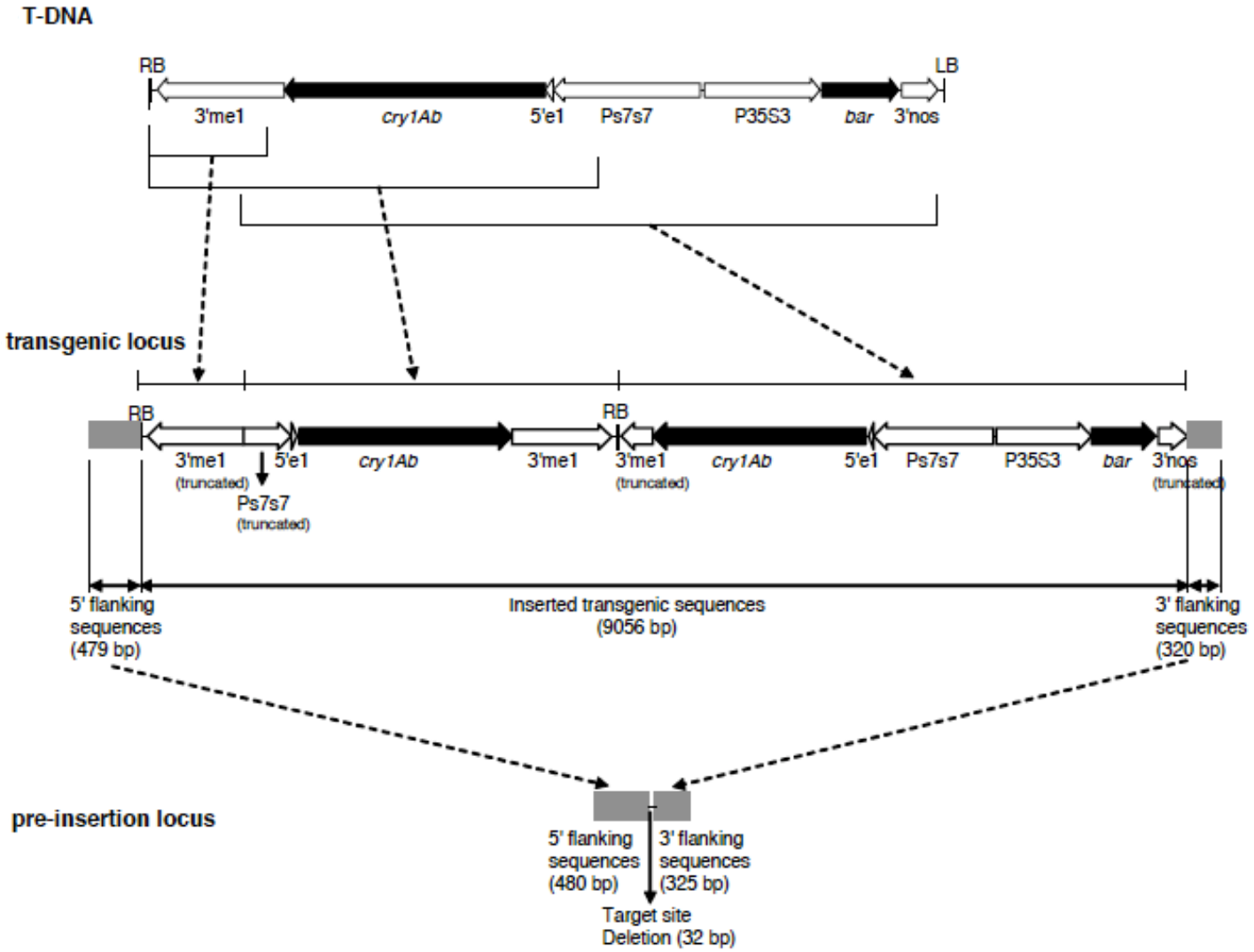
- **Disruption of endogenous genes** at the insertion sites ?
  - Bioinformatic analysis of the insertion locus and search for known endogenous genes

- Recombinogenic sequences on the T-DNA promoting **horizontal gene transfers** ?
  - Bioinformatic analysis of similarities with microbial genomes (likelihood of homologous recombinations), presence of site-specific recombinogenic sequences on the T-DNA

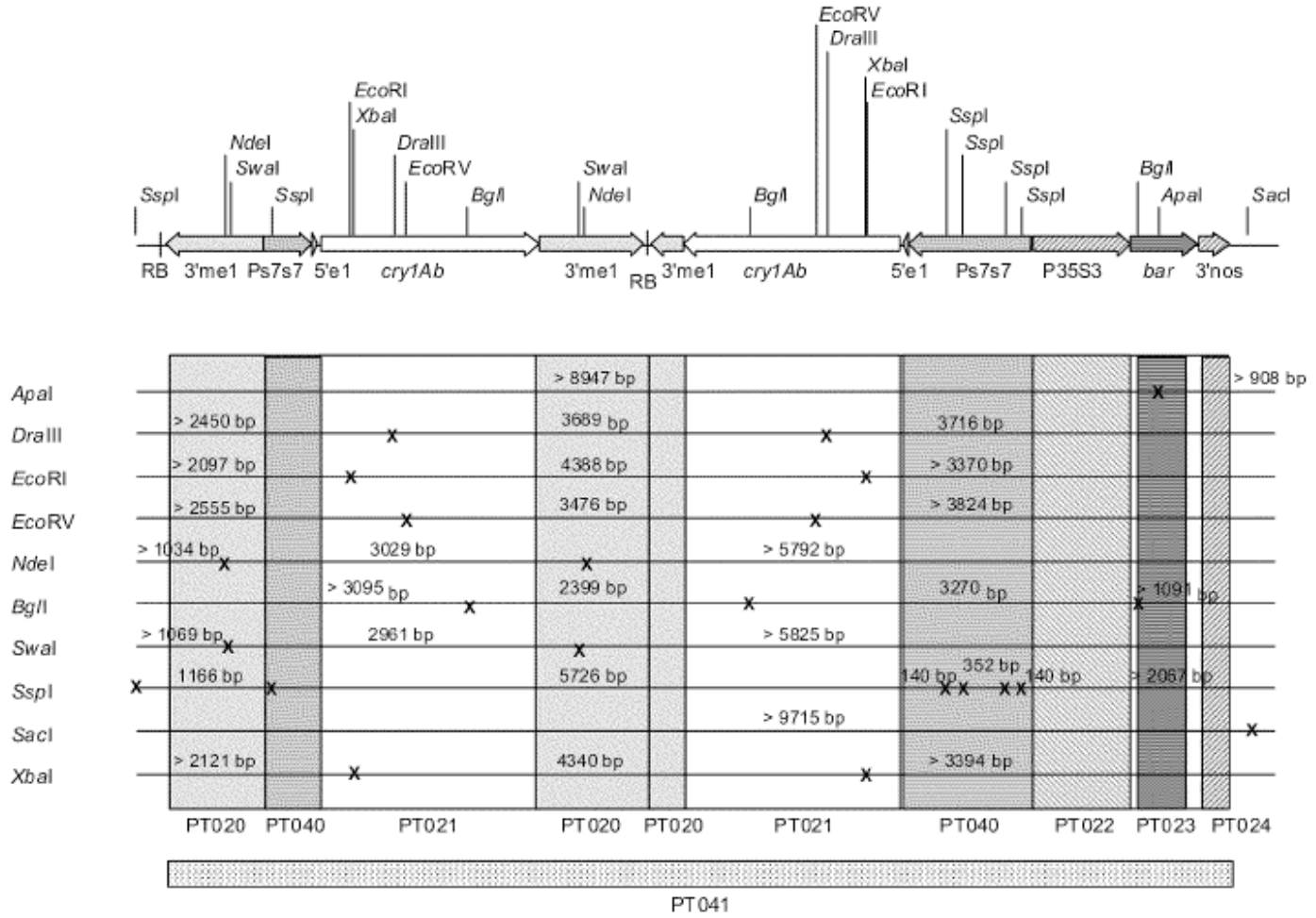
The modified plant genome : analysis of the structure of the insert

- **The rationale** :
  - Authorization will bear on the «transformation event », *i.e.* the new DNA in its insertion locus (but possibly in multiple genetic backrounds).
  - This event needs to be precisely defined for the purposes of risk assessment (task of EFSA) and of risk management (*e.g.* detection methods, task of COM JRC- Ispra).
- **The aims** :
  - To determine the number and structure of all detectable inserts, complete or partial.
  - To determine the sub-cellular location of the inserts
  - To determine the flanking regions of the recipient genome

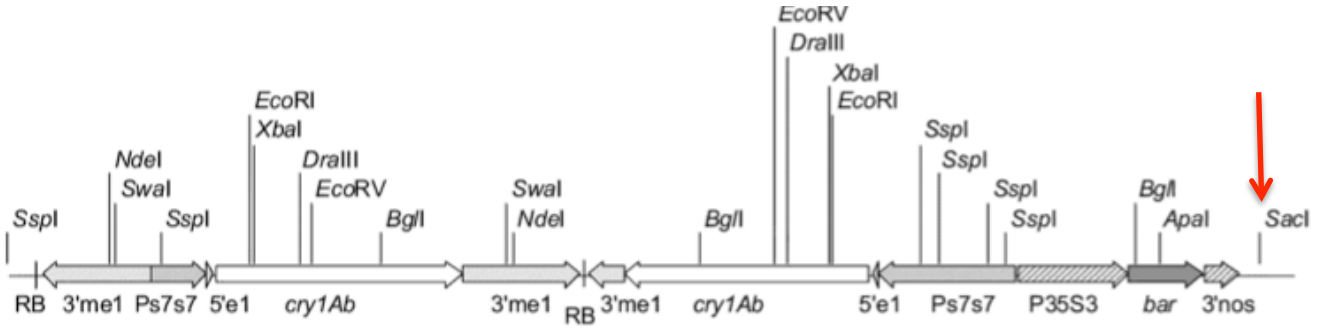
Analysing the transgenic locus by DNA sequencing : example

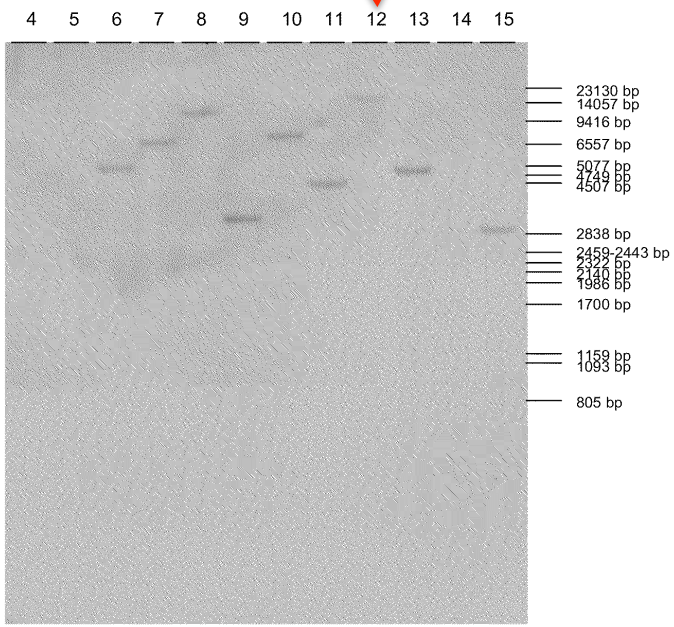


Southern blot analysis is extensively used for analysing insert structure.



Southern analysis of insert structure and number





Lane 4: *Gossypium hirsutum* elite event [illegible] *Apa*I digested
Lane 5: *Gossypium hirsutum* elite event [illegible] *Dra*III digested
Lane 6: *Gossypium hirsutum* elite event [illegible] *Eco*RI digested
Lane 7: *Gossypium hirsutum* elite event [illegible] *Eco*RV digested
Lane 8: *Gossypium hirsutum* elite event [illegible] *Nde*I digested
Lane 9: *Gossypium hirsutum* elite event [illegible] *Bgl*I digested
Lane 10: *Gossypium hirsutum* elite event [illegible] *Swa*I digested
Lane 11: *Gossypium hirsutum* elite event [illegible] *Ssp*I digested
Lane 12: *Gossypium hirsutum* elite event [illegible] *Sac*I digested
Lane 13: *Gossypium hirsutum* elite event [illegible] *Xba*I digested
Lane 14: *Gossypium hirsutum* wild type va[illegible] 66 – *Xba*I digested
Lane 15: *Gossypium hirsutum* wild type va[illegible] 66 – *Xba*I digested + an equimolar amount of pTDL008 – *Xba*I digested

Figure 5: Southern blot analysis – P35S3 probe

NB : SacI allows insert number determination.

Southern analysis of the absence of the vector backbone : checking for the absence of (*e.g.*) antibiotic resistance marker genes

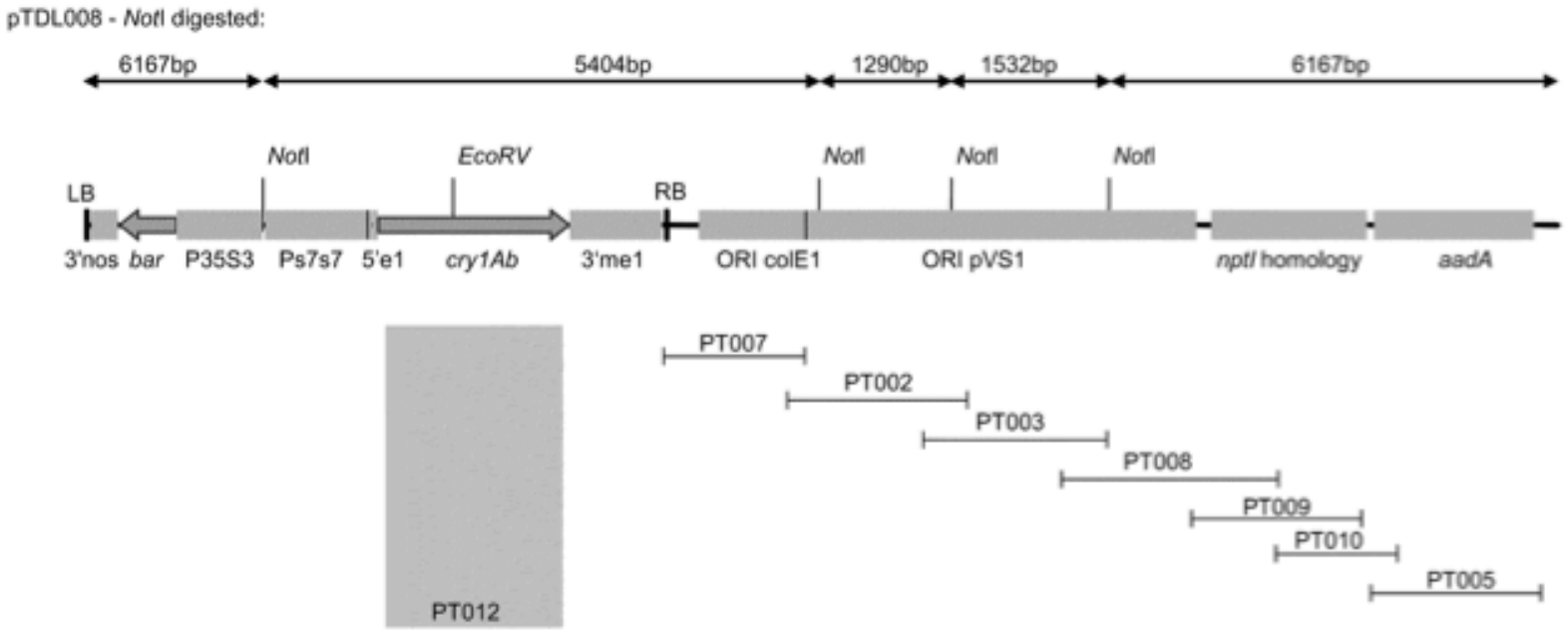


Figure 2: Schematic drawing of pTDL008 with indication of relevant restriction sites and position of the probes used.

Molecular characterization of the expression of the insert

- Determination of the **levels of the newly expressed proteins** (in a range of tissues depending on the scope of the application)
- In case of stacked events (typically obtained by crossing GMPs), control of **absence of interactions** between the combined events (*i.e.* changes of protein levels in the stack as compared with the single events)
- Phenotypic data confirming generational **stability** of the trait / expression of the inserted genes
- **Methods** : typically ELISA

Summary : Example of molecular data sets in a dossier submitted to EFSA

Type of analysis	Parameters analyzed	Appendix Ref.	Relevance in Chapter D
Southern blot hybridization	Copy number, Vector backbone sequences	Moens and Criel, 2008 M-311245-05-1	2(a); 2(b); 6(a)
	Insert stability in multiple generations		2(d); 5
	Insert stability in different environments		2(d); 5
	Insert stability in different genetic backgrounds		2(d); 5
Polymerase Chain Reaction	Sequence information	Moens and De Pestel, 2008 M-311249-01-1	2(d); 2(e);
	Flanking sequence determination	Moens and De Pestel, 2008 M-311249-01-1 Moens, 2009 M-349640-01-1	2(d); 7.8
BLAST sequence similarity research	Integration site	Moens, 2011 M-356281-02-1 Capt, 2011a M-350086-03-1 Ranjan, 2011 M-411811-01-1	2(c); 2(d); 2(e); 7.8
	Similarities between flanking sequences and known genes		2(d); 7.8
	Open Reading Frame research		2(d); 3(c); 7.8
Northern blot hybridization	Cryptic expression analysis	Moens, 2010b M-356282-02-1	3(c); 7.8
	Transgene expression		3 (b); 3(d)
ELISA[1]	Cry1Ab and PAT expression analysis	Currier and Massengill, 2008 M-312372-01-1 Hunt and Robinson, 2009 M-356278-01-1 Martone, 2010 M-327148-04-1	3 (a-d)

New techniques for the molecular characterization of GM plants ?
The usefulness of Next Generation Sequencing under discussion

ORIGINAL RESEARCH

The Use of Next Generation Sequencing and Junction Sequence Analysis Bioinformatics to Achieve Molecular Characterization of Crops Improved Through Modern Biotechnology

David Kovalic,* Carl Garnaat, Liang Guo, Yongpan Yan, Jeanna Groat, Andre Silvanovich, Lyle Ralston, Mingya Huang, Qing Tian, Allen Christian, Nordine Cheikh, Jerry Hjelle, Stephen Padgette, and Gary Bannon

THE PLANT GENOME ■ NOVEMBER 2012 ■ VOL. 5, NO. 3

Food Anal. Methods
DOI 10.1007/s12161-013-9673-x

Next-Generation Sequencing as a Tool for Detailed Molecular Characterisation of Genomic Insertions and Flanking Regions in Genetically Modified Plants: a Pilot Study Using a Rice Event Unauthorised in the EU

Daniela Wahler · Leif Schauser · Joachim Bendiek · Lutz Grohmann

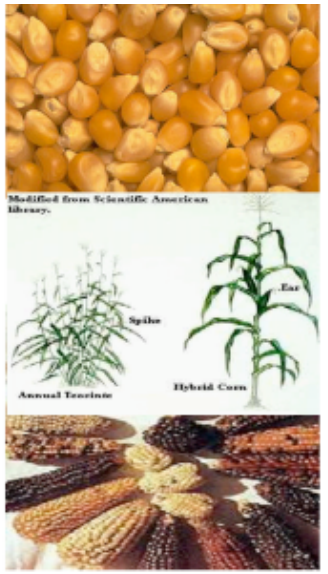
New techniques for the molecular characterization of GM plants ?
High throughput technologies ('omics') for detecting unintended effects ?

FP 6 "Safe Foods" www.safefoods.nl

"Omics" and sources of biological variation
Breeding, environment, cultural practices etc.

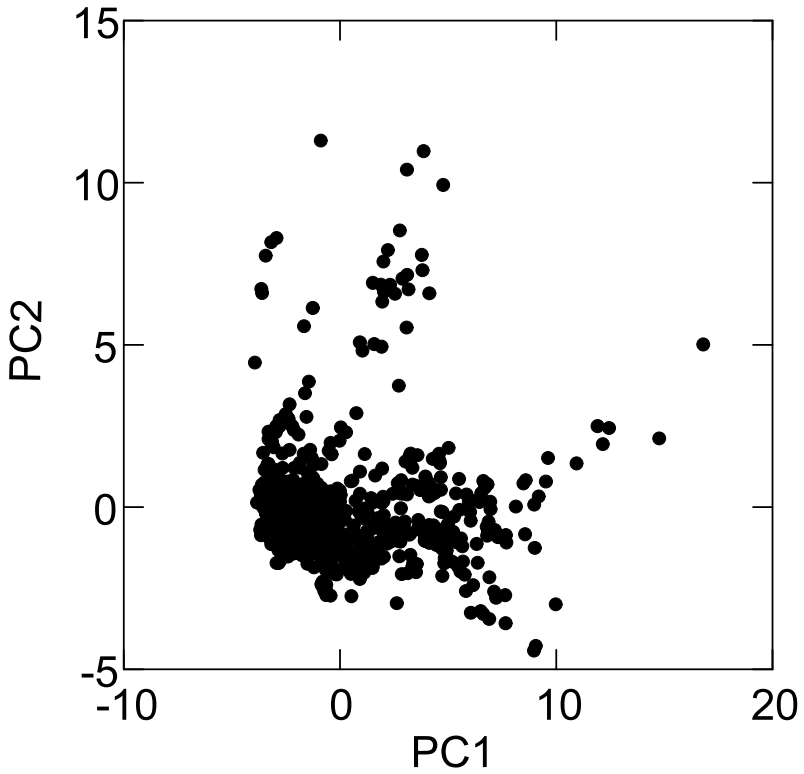
Safe Foods

Potato Maize



(Courtesy of Pr H. Davies, Jame Hutton Institute, UK)

Maize Metabolomics



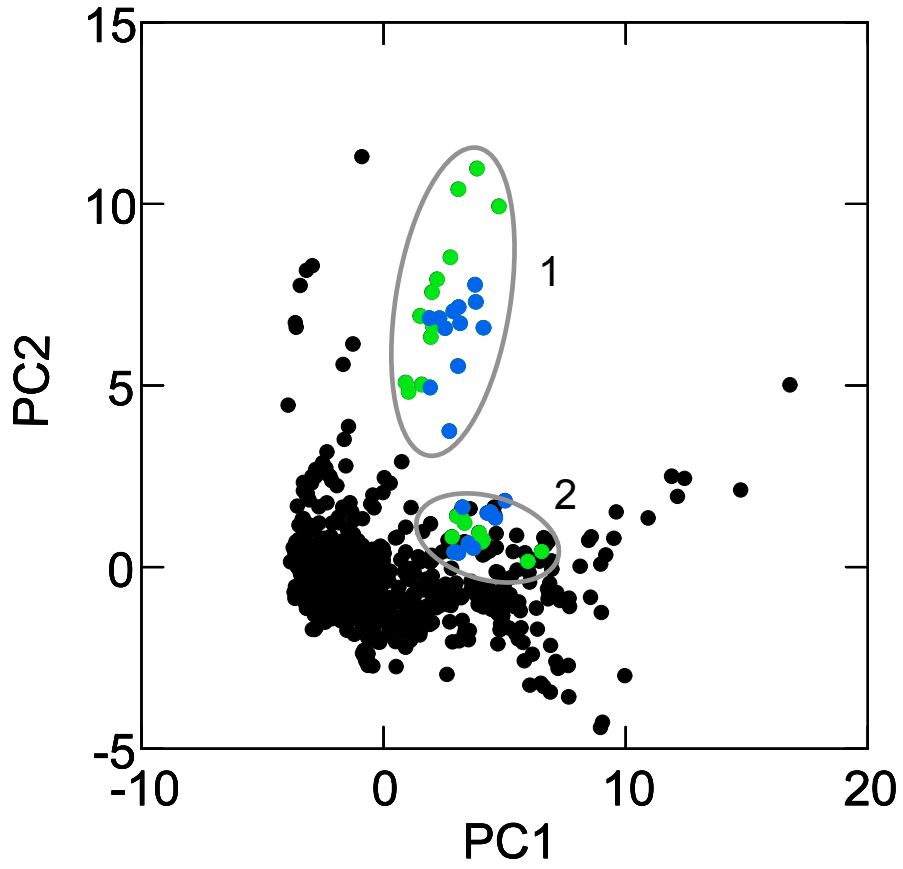
Pooled samples from all experiments

Dot is one anaylsis

Germany + South Africa

(Courtesy of Pr H. Davies, Jame Hutton Institute, UK)

Maize Metabolomics GM vs Non GM



Locations

1 Neuhof
2 Pfaffenhofen

- **isogenic maize**
- **Bt-maize**

Engel et al TUM
unpublished

(Courtesy of Pr H. Davies, Jame Hutton Institute, UK)

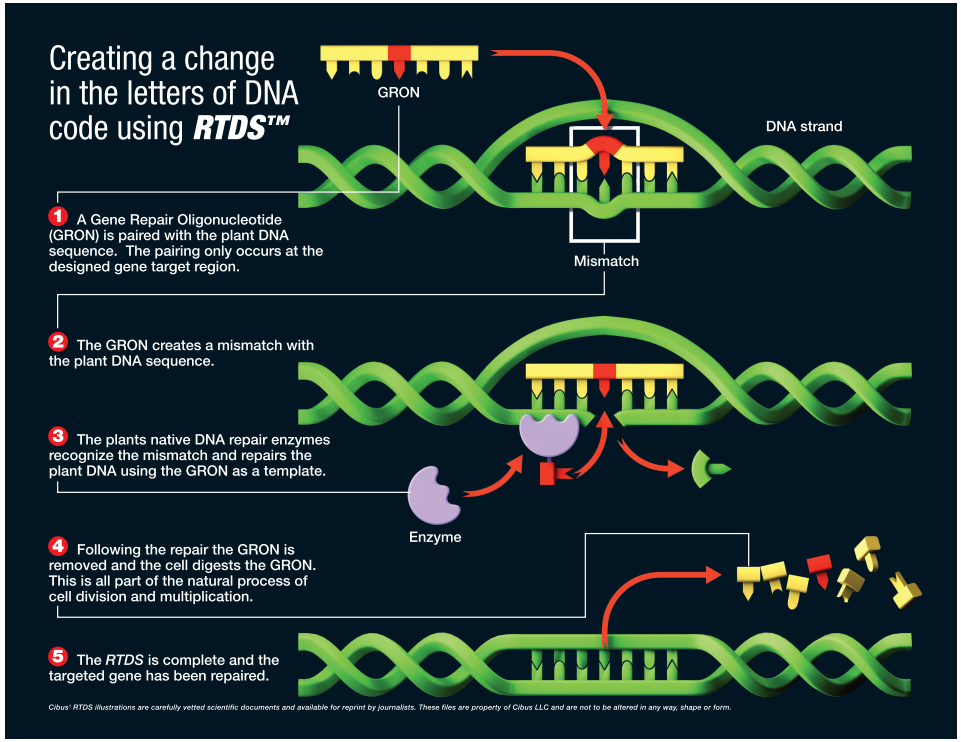
The future : new avenues for the genetic modification of plants (and possible impacts on risk assessment)

- New « breeding » techniques are being developed for the targeted genetic modification of plants.
- They do not necessarily involve addition of transgenes.
- Whether they will be considered as GMOs in the sense of the EU law is still unclear.
- The EFSA GMO Panel has issued scientific opinions on how to implement / adapt existing guidelines for their risk assessment, for specific new breeding techniques (cis-/ intragenesis, site-specific nucleases-mediated DNA modifications).

« New plant breeding techniques »

1. Zinc finger nuclease (ZFN) technology
2. Oligonucleotide directed mutagenesis (ODM)
3. Cisgenesis and intragenesis
4. RNA-dependent DNA methylation (RdDM)
5. Grafting (on GM rootstock)
6. Reverse breeding
7. Agro-infiltration (agro-infiltration “sensu stricto”, agro-inoculation, floral dip)
8. Synthetic genomics

Oligonucleotide-mediated site-specific mutation



Environ. Biosafety Res. (2009)
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DOI: 10.1051/ebr/2009007

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Commentary

Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge?

Didier BREYER[1]*, Philippe HERMAN[1], Annick BRANDENBURGER[2], Godelieve GHEYSEN[3], Erik REMAUT[4,5], Patrice SOUMILLION[6], Jan VAN DOORSSELAERE[7], René CUSTERS[8], Katia PAUWELS[1], Myriam SNEYERS[1] and Dirk REHEUL[9]

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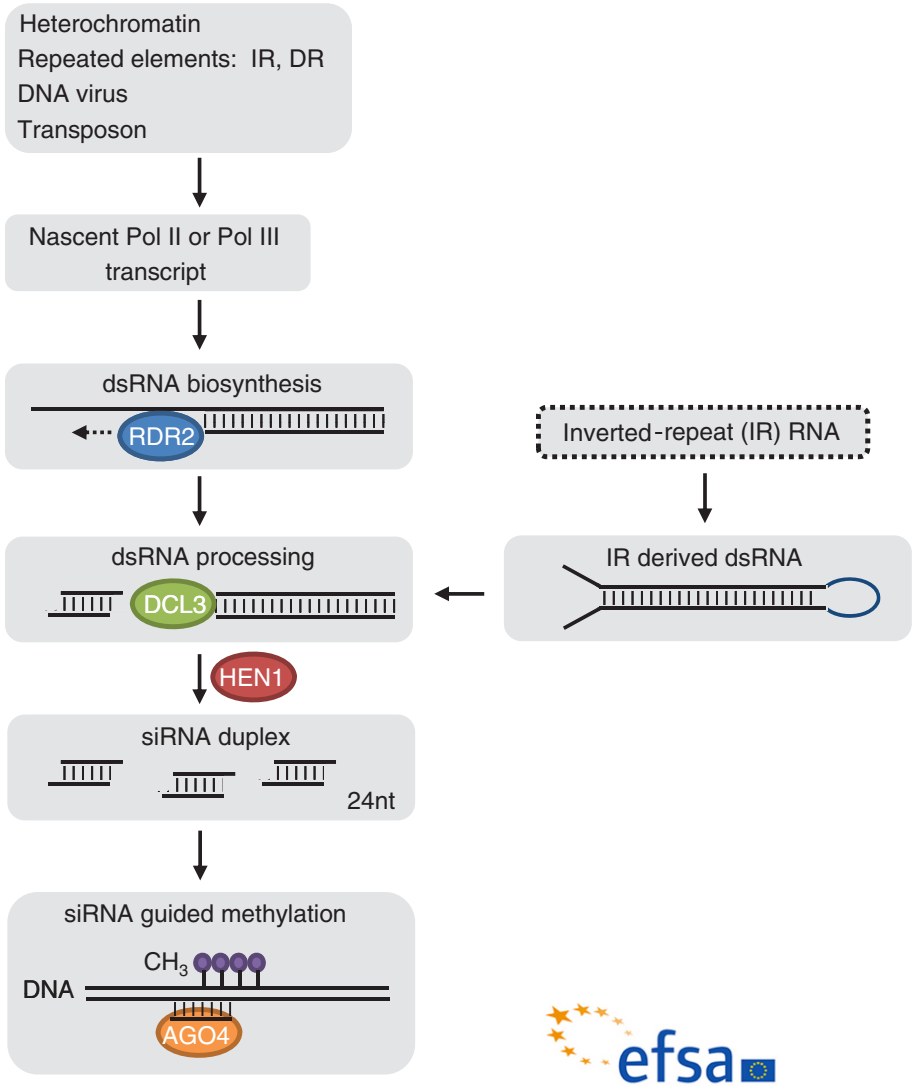
In the European Union, the definition of a GMO is technology-based. This means that a novel organism will be regulated under the GMO regulatory framework only if it has been developed with the use of defined techniques. This approach is now challenged with the emergence of new techniques. In this paper, we describe regulatory and safety issues associated with the use of oligonucleotide-mediated mutagenesis to develop novel organisms. We present scientific arguments for not having organisms developed through this technique fall within the scope of the EU regulation on GMOs. We conclude that any political decision on this issue should be taken on the basis of a broad reflection at EU level, while avoiding discrepancies at international level.

Keywords: GMO / EU regulation / gene modification / oligonucleotide / new techniques / mutagenesis / risk assessment

« Paramutations » :
RNA-mediated transcriptional gene silencing

Principle : small interfering RNAs are transiently delivered to plant cells, where they cause methylation and silencing of chosen gene promoters.

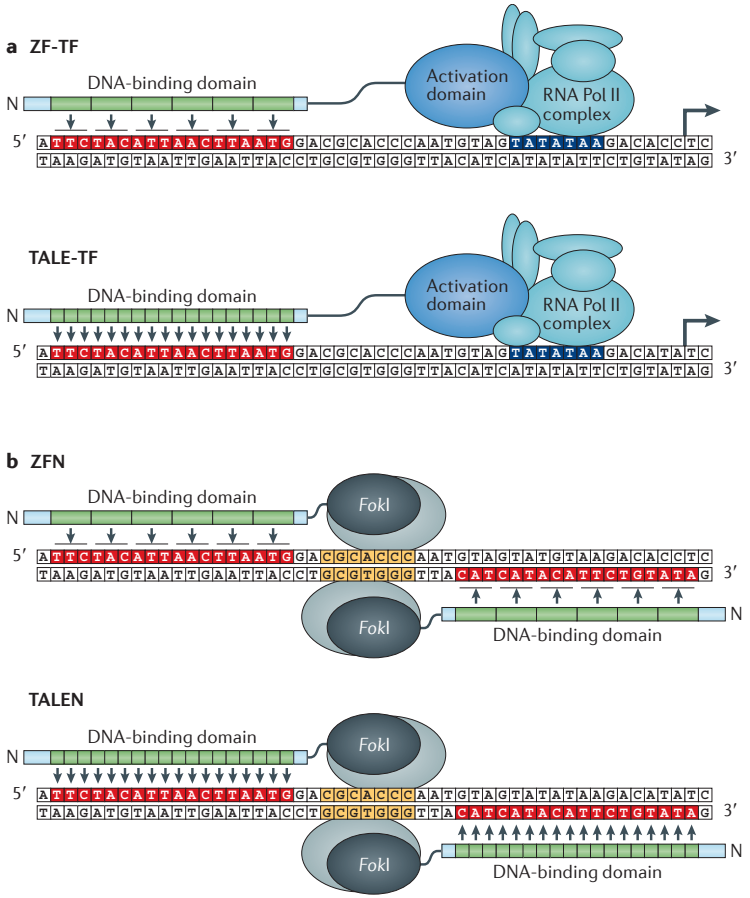


(Frizzi and Huang 2010)

Targeted modifications of the genome using recombinant site-specific nucleases

- *Zn finger*-Nucleases, TALEN (*Transcription Activator-Like Effectors Nucleases*), *RNA-guided Nucleases (CRISPR/Cas), etc.*

- *Double-strand breaks* are introduced in specified loci, allowing sequence editing, replacement and insertion of DNA.



(Liu et al. Nature Genetics, November 2013)

Conclusions

1. Molecular characterization (MC) contributes to hazard and risk identification, but must be complemented by biological evidence.
2. Both intended and uninteded effects are addressed.
3. Beyond the basic requirements of MC, case-by-case assessment may request further, hypothesis-driven analyses.
4. New molecular techniques are emerging for the characterization of GMPs.
5. New breeding techniques are emerging for the genetic modification of plants, challenging the current risk assessment approach.