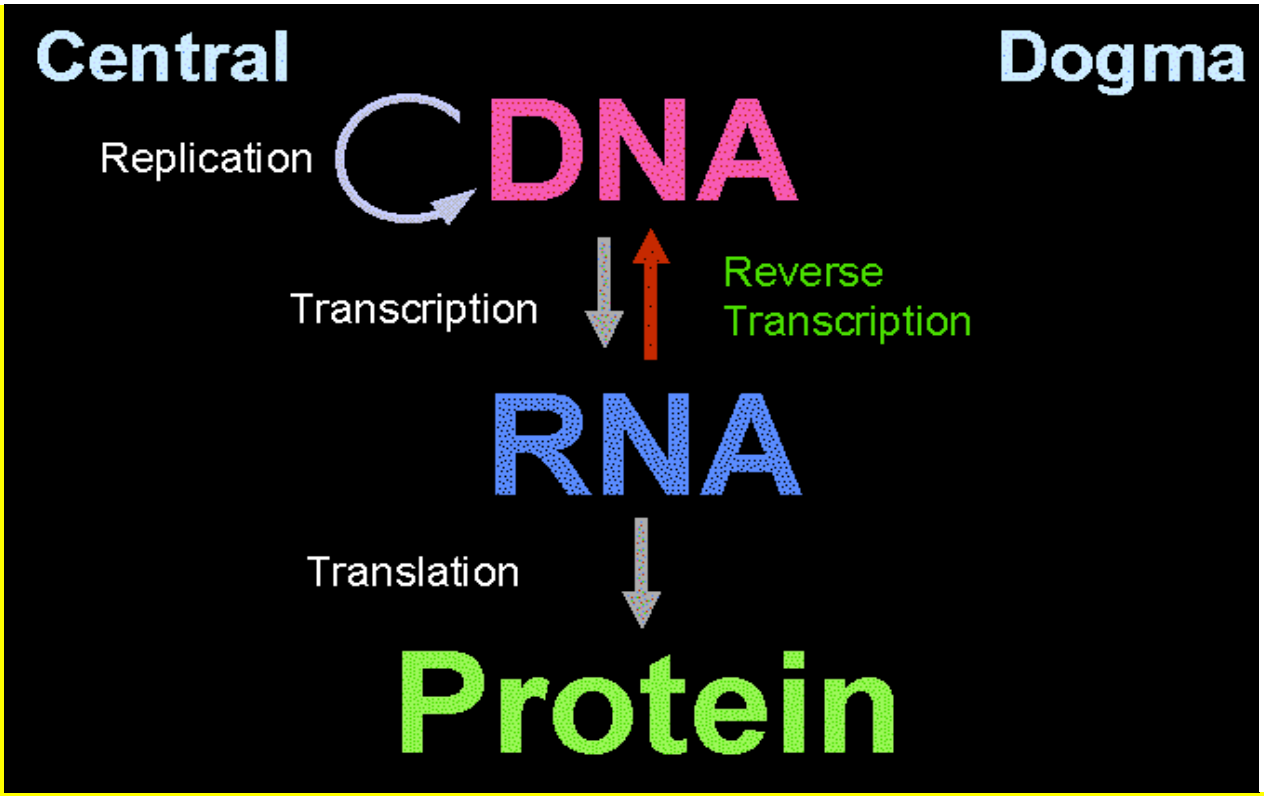
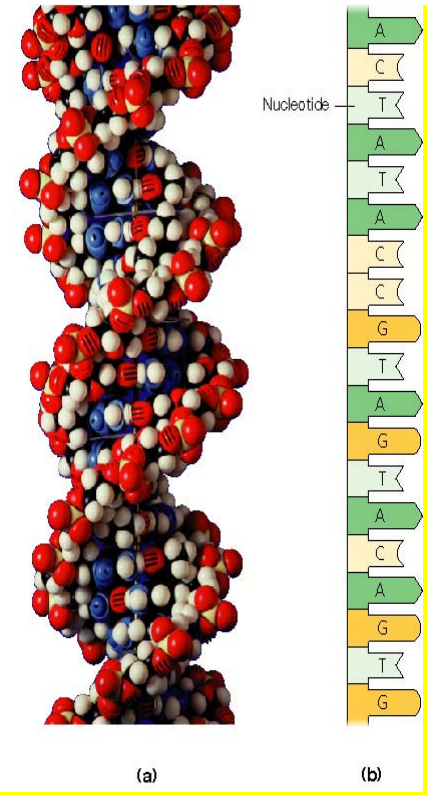




Genetic engineering is a technology derived from the ideology of the "central dogma" of Molecular Biology according to which histories of organisms are pre-determined by genes seen as independent entities just as the components of a machine. On this ground genetic engineers thought that single genes from one genome could be transferred from one specie to another where they would perform the same function without any unintended effect.

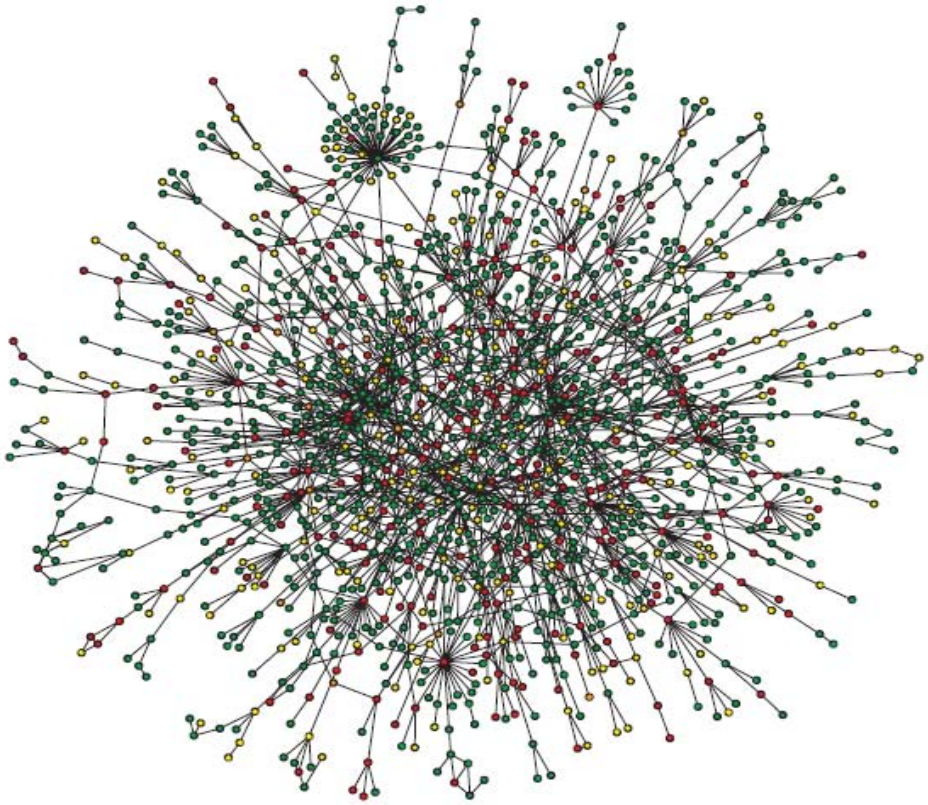
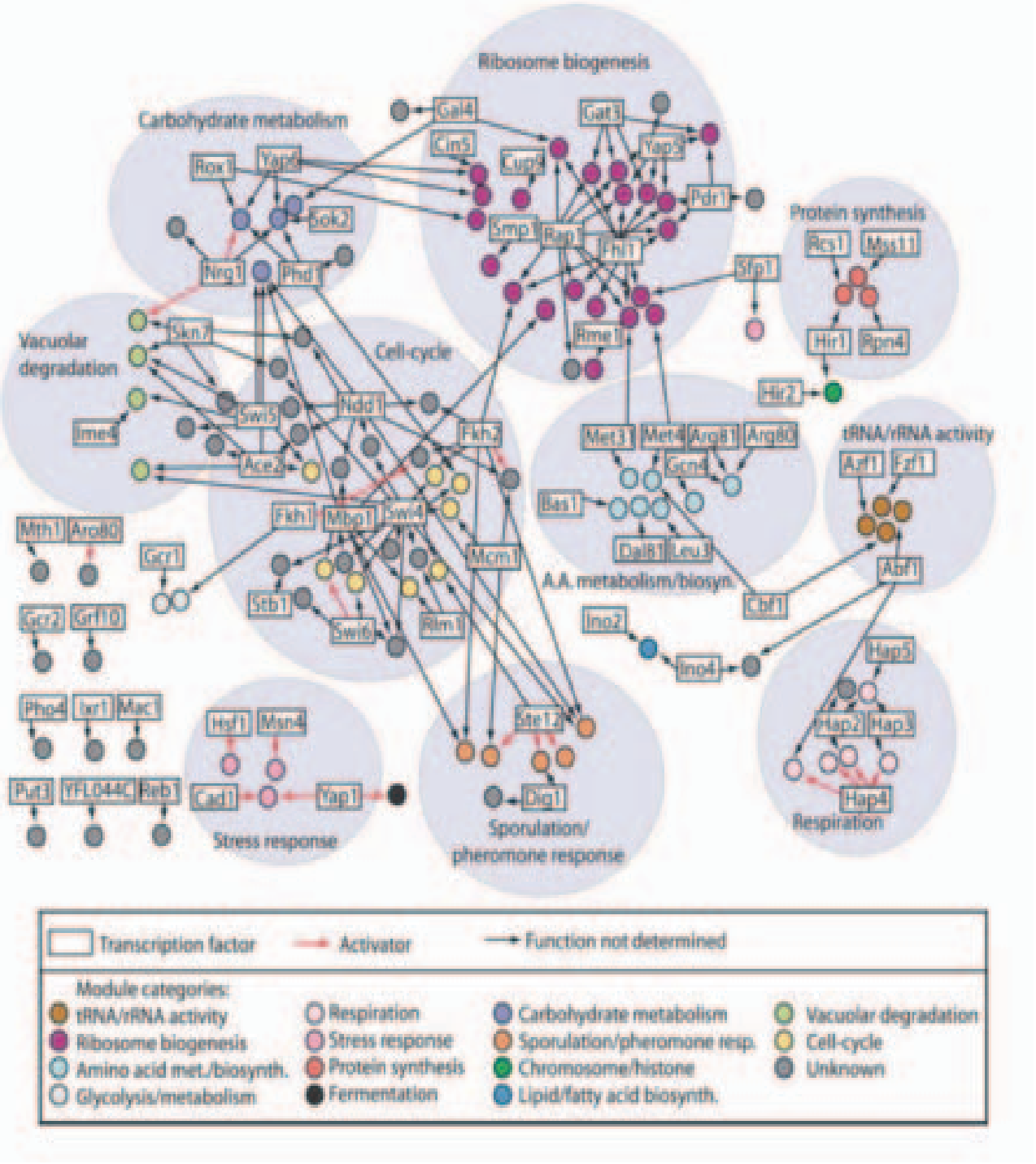


Figure 2 | **Yeast protein interaction network.** A map of protein–protein interactions[18] in *Saccharomyces cerevisiae*, which is based on early yeast two-hybrid measurements[23], illustrates that a few highly connected nodes (which are also known as hubs) hold the network together. The largest cluster, which contains ~78% of all proteins, is shown. The colour of a node indicates the phenotypic effect of removing the corresponding protein (red = lethal, green = non-lethal, orange = slow growth, yellow = unknown). Reproduced with permission from REF. 18 © Macmillan Magazines Ltd.

Living systems are all organised as networks of molecules, cells, tissues , organisms, ecosystems , the Biosphere. All elements at all levels of the hierarchical organisation are interconnected. For this reason the change of one component always implies changes in the other nodes connected with it with partially unpredictable results. Genetic engineering therefore has a high level of unpredictability.

A gene and metabolic network with a modular structure as all living networks



Inserting a gene from the genome of one organism to that of another often far from the first in phylogenetic terms has several unintended consequences due to the fact that it did not co-evolve with the receiving genotype. If a relevant node is changed there will be pleiotropic effects while, depending to the sequences in which the transgene is transferred, position effects can change regulation

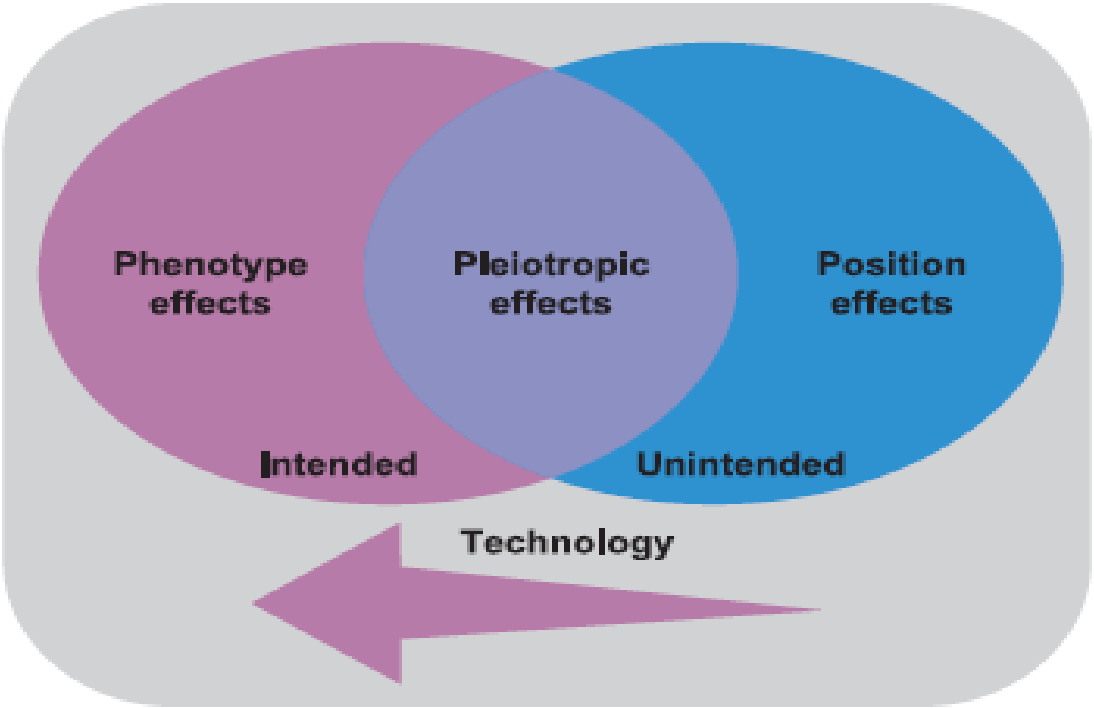


Figure 1 The relationship of the diverse effects of transgene insertion and expression described in the literature. A specific phenotype is the most frequent intended effect conferred by a transgene in a plant. The transgene may also impart a range of phenotypes which constitute the pleiotropic effects of the transgene. These differ from the position effects that modify the phenotype because of the interactions that are induced by processes specific to each insertion site. Both the pleiotropic and position effects may be the unintended effects that are revealed through experimentation with transgenic plants. These need to be understood in order to determine the true phenotype of the transgene. With increased knowledge of the gene and the development of technologies that eliminate or minimize the potential for pleiotropic and position effects, the predictability of achieving the intended phenotype increases and the risk of unintended effects decreases.

The so-called "unintended effects" of genetic engineering are all derived by the dynamic interactions between the inserted construct and the context at different levels of organisation.

-- loosening of developmental constraints during in vitro culture leading to mutations

-- quasi-random location of inserted genes leading to "position effects"

-- production of "fusion RNAs" and putative new proteins

-- "active" re-arrangements and regulation of expression by host organisms

-- Un-predictable interactions with host metabolic networks leading to quantitative and qualitative changes in the transcriptome, proteome and metabolome

-- Interaction of the GMO with the agro-ecosystem

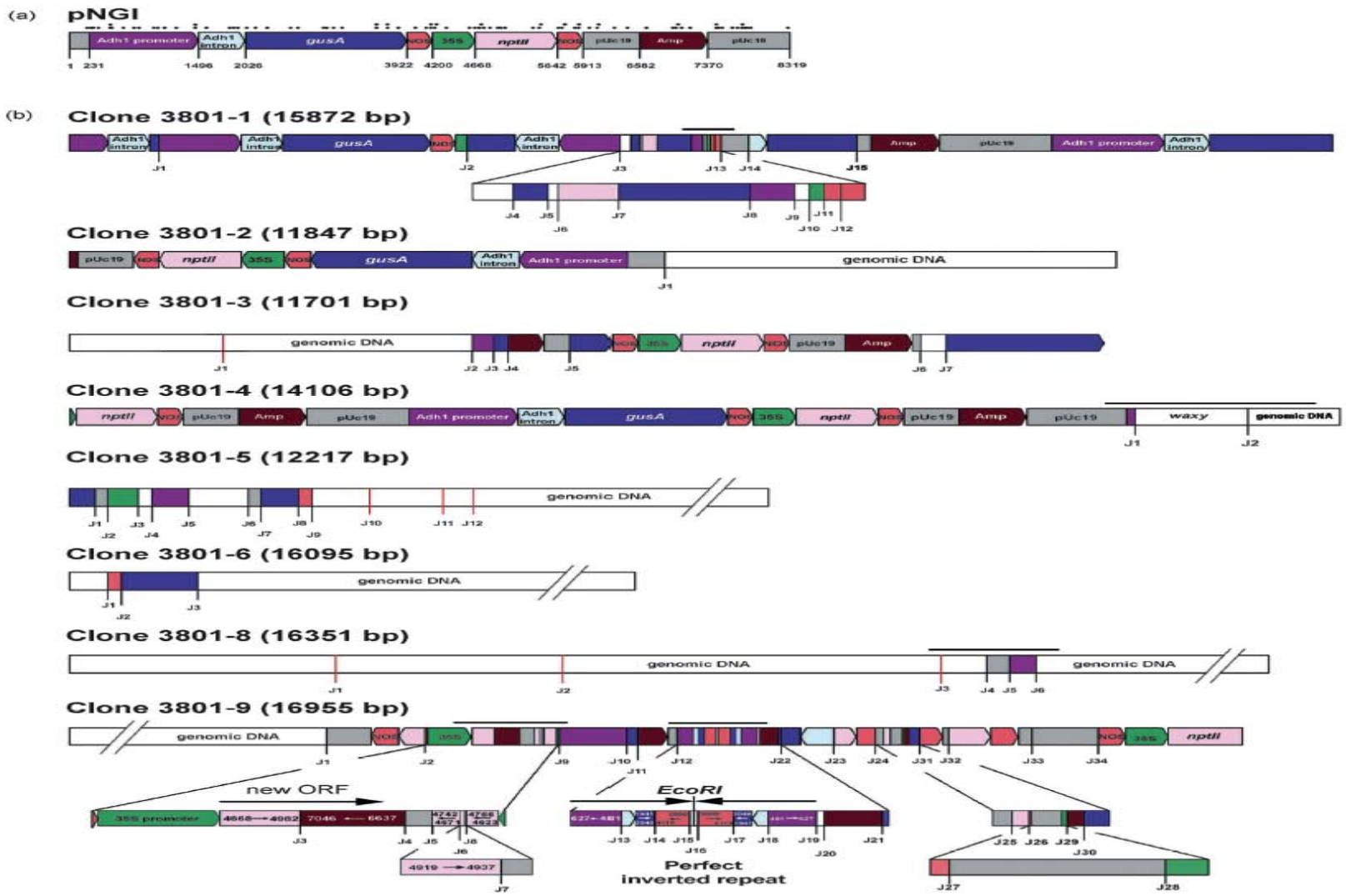
-- Effects on health of humans and animals

EFSA guidelines are not sufficient for a correct monitoring of unintended effects in these fields

Table 1. Location, activity and stability of transgenes

Line	Chromosome	Position	Transgene copies[a]	Expression[b]	Stability
1	2A	Subtelomeric	5	Medium	Stable
2	6B	Intercalary	>10	High	Unstable
3	6B	Intercalary	2	Medium	Stable
4a	5D?	Intercalary	≈5	High	Stable
4b	1D	Telomeric	≈2	Low	Stable
5	1D	Intercalary	>9	na	Stable
6	4A	Telomeric	5	Medium	Stable
7	2B	Telomeric	≈5	Low	Stable
8	6A	Intercalary	1	Medium	Stable

In a transformation experiment it is impossible to predict where in the genome will be inserted disrupting the pre-existing sequence. EFSA guidelines suggest obsolete techniques for this analysis



It is impossible to predict whether and how the transgene construct will be modified. Here we show:
The original construct (a) and nine different re-arranged sequences found in a single transformed oat line EFSA guidelines do not necessarily imply the analysis for the presence of unintended fragments

If one looks at authorised GMPs present in the market one finds a number of unintended changes in the genome. We shall take two insect resistant Maize events and particularly MON 810 to show them .

Transformed line or transformation event	Transformation method*	USDA application number and other relevant references	Date of approval for deregulation	Superfluous DNA at or near the transgene locus	Sequence analysis of DNA flanking the inserted transgene
LLCotton25 Herbicide tolerant	*Agrobacterium*-mediated transformation	02-042-01p	March 2003	Polylinker sequence	n.d.
Newleaf® Plus RBMT22-82 Potato Virus resistant and Colorado Potato Beetle resistant	*Agrobacterium*-mediated transformation	99-173-01p	July 2000	Three independent insertion events; one event also included plasmid sequences	n.d.
CZW-3 Squash Virus resistant	*Agrobacterium*-mediated transformation	95-352-01p	June 1996	Selectable marker gene	n.d.
Maize MON863 Corn rootworm protected	Particle bombardment with gene cassette	01-137-01p	October 2002	Selectable marker gene and a fragment of a superfluous gene	Yes, but sequence is not publicly available
63-1 Papaya **55-1 Papaya** Virus resistant	Particle bombardment with whole plasmid	96-051-01p Fitch *et al.* 1992	September 1996	Both lines included selectable marker genes and plasmid DNA sequences	n.d.
Roundup Ready® Soybean 40-3-2 herbicide tolerant soybean line	Particle bombardment with whole plasmid	93-258-01p Windels *et al.* 2001	May 1994	Superfluous transgene sequences and unknown DNA sequences	n.d. (probable deletion and/or rearrangement at insertion site)
Maize YieldGard® MON810 Lepidopteran insect resistant	Particle bombardment	96-017-01p Hernandez *et al.* 2003	March 1996	none	n.d. (probable deletion and/or rearrangement at insertion site)

Unintended modifications of MON 810 maize

a) Loss of two fragments of the Bt (insect resistance) construct, Hernandez et al.(2003) leading to a second patent after discovery

b) Insertion of the construct in a relevant gene (ubiquitin ligase) and synthesis of completely new RNA products possibly leading to unknown proteins. Bogani et al. (2008)

c) Significant changes in the metabolic structure putatively affecting the nutritional value (Motto et al 2006)

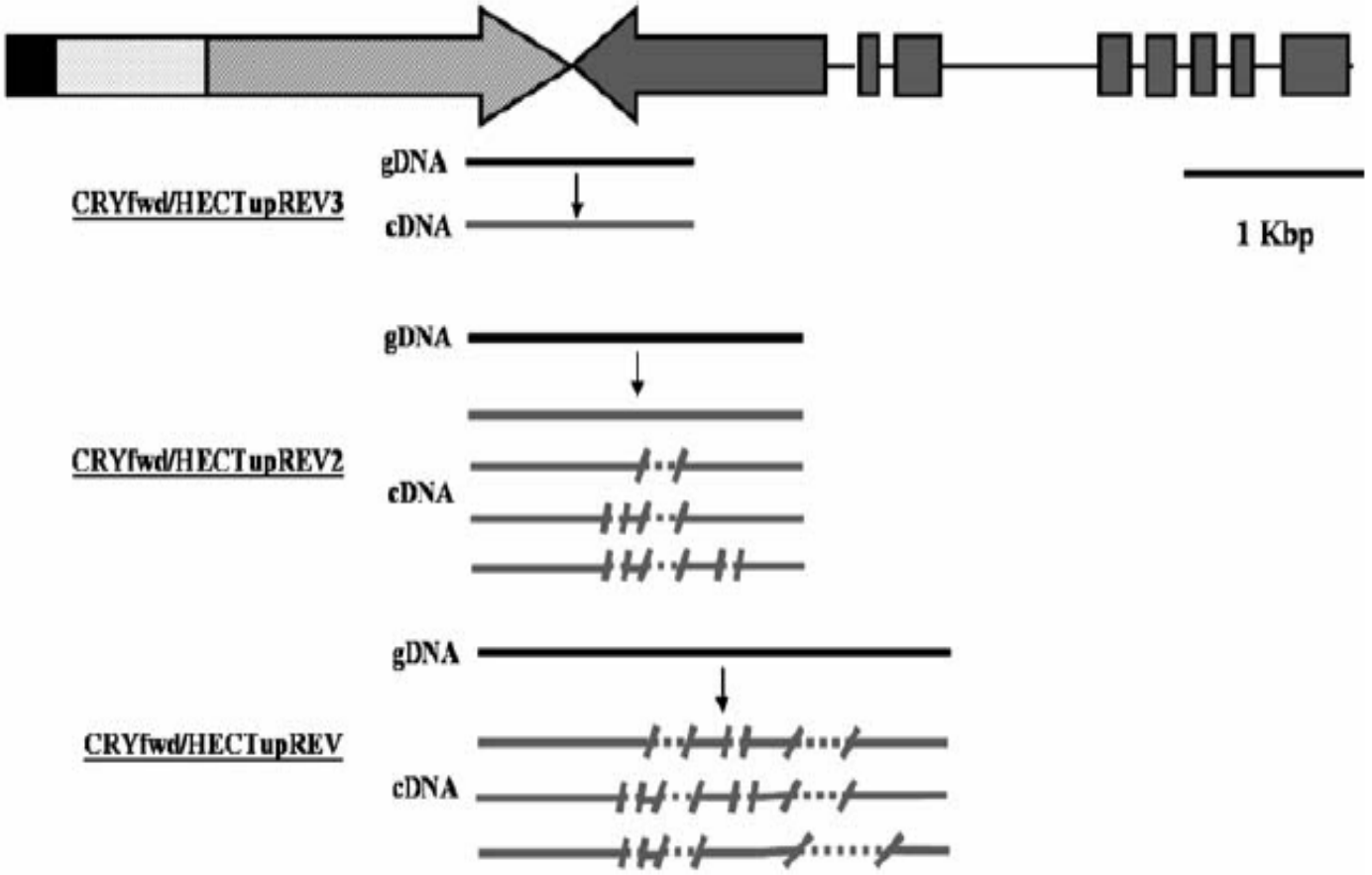


Fig. 4 Scheme for the MON810 3′ insertion site. PCR fragments obtained from both genomic DNA (gDNA, black bars) and RNA (cDNA, grey bars), with the primer pair used are shown. Black box: CaMV35S promoter; dotted box: hsp70 intron; light grey arrow: *cryIA(b)* truncated gene; dark gray arrow: truncated exon 8 of the putative HECT E3 ubiquitin-ligase gene

The production of fusion RNAs , putatively leading to new proteins, has been shown also in transgenic soybean. ***According to EFSA guidelines no analyses of the transcriptome through cDNA studies nor microarray studies on gene expression patterns in transgenic and so-called near isogenic lines are requested . Only computational studies on possible fusion proteins are required and expression profiles are limited to the inserted sequence thus disregarding the possible interactions with the pre-existing genetic context.***

Differences between MON810 and control for more than 100 proteins. No proteome analysis is asked in EFSA guidelines

research articles Journal of proteome research

Proteomics as a Complementary Tool for Identifying Unintended Side Effects Occurring in Transgenic Maize Seeds As a Result of Genetic Modifications

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To improve the probability of detecting unintended side effects during maize gene manipulations by bombardment, proteomics was used as an analytical tool complementary to the existing safety assessment techniques. Since seed proteome is highly dynamic, depending on the species variability and environmental influence, we analyzed the proteomic profiles of one transgenic maize variety (event MON 810) in two subsequent generations (T05 and T06) with their respective isogenic controls (WT05 and WT06). Thus, by comparing the proteomic profiles of WT05 with WT06 we could determine the environmental effects, while the comparison between WT06 and T06 seeds from plants grown under controlled conditions enabled us to investigate the effects of DNA manipulation. Finally, by comparison of T05 with T06 seed proteomes, it was possible to get some indications about similarities and differences between the adaptations of transgenic and isogenic plants to the same strictly controlled growth environment. Approximately 100 total proteins resulted differentially modulated in the expression level as a consequence of the environmental influence (WT06 vs WT05), whereas 43 proteins resulted up- or down-regulated in transgenic seeds with respect to their controls (T06 vs WT06), which could be specifically related to the insertion of a single gene into a maize genome by particle bombardment. Transgenic seeds responded differentially to the same environment as compared to their respective isogenic controls, as a result of the genome rearrangement derived from gene insertion.

A metabonomic study of transgenic maize (*Zea mays*) seeds revealed variations in osmolytes and branched amino acids

Cesare Manetti*, Cristiano Bianchetti, Lorena Casciani, Cecilia Castro, Maria Enrica Di Cocco, Alfredo Miccheli, Mario Motto and Filippo Conti

Table 3. *ANOVA results for selected signals from control (c) and transgenic (t) maize seed spectra*

Metabolite (signal)	*F*-value[a]	Order
Acetate (βCH_3)	0.3	
Ala (βCH_3)	14.6**	t <c
α-Glucose (C1H)	12.6**	t >c
Asn (βCH_2)	18.6***	t <c
β-Glucose (C1H)	17.2**	t >c
Choline [$N(CH_3)_3$]	105.6***	t <c
Dimethylamine (CH_3)	4.0	
Ferulic acid (HF)	4.5	
Formate (CH)	0.2	
GABA (αCH_2)	28.8***	t >c
Gln (βCH_2)	18.5***	t >c
Glu (γCH_2)	1.5	
His (C2H, ring)	9.2**	t <c
Ile (γCH_3)	2.4	
Melibiose (Gal1H)	6.6*	t >c
Pyruvate (CH_2)	3.4	
Succinate (α-βCH_2)	44.5***	t >c
Sucrose (F1H)	7.1*	t >c
Thr (γCH_3)	0.3	
Trigonelline (HA)	0.3	
Tyr (C2, H6, ring)	1.9	
Val (CH'_3)	0.5	

[a]*,**,***, Significant at the .05, 0.01, and 0.001 probability levels, respectively.

Significant differences in 11 out of 22 compounds analysed between MON810 and control (50%). No metabolome analysis in EFSA guidelines)

Metabolome analysis with up to date methods is not required by EFSA procedures , research being limited to compositional studies only related to a small number of compounds. The criteria of MIAME (Minimum information about a mnicroarray experiment) and MIAPE (Minimum information about a proteomics experiment) are not even mentioned in EFSA guidelines

Using Metabolomics To Estimate Unintended Effects in Transgenic Crop Plants: Problems, Promises, and Opportunities

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FUTURE PROSPECTS

The MIAME and MIAPE standards are now requirements for publishing microarray and proteomic experiments in many journals. Hopefully, similar standards will be applied to metabolomic data as well. As more and more of these datasets are deposited in publicly accessible databases, meta-analyses that integrate multiple levels of information will allow us to ask many different systems biology questions. The adoption of controlled vocabularies for gene, trait, and phenotypic ontologies will further assist these meta-analyses. The benefit of this ability to leverage large collections of data should be obvious to the scientific community. Likewise, the identification of genetically informative populations has been very effective to address important biomedical and agronomic questions, such as the identification of cancer risk factors and genes important for carotenoid biofortification in staple crops.[89,90] If these genetically informative populations are studied using metabolomic, genomic, and proteomic methods, this should provide an immediately useful but also durable resource. From this base of knowledge, the range and identity of unintended effects to composition and quality of transgenic foods can be assessed in the most complete manner, and help inform consumers, regulators, and other stakeholders in their decision-making.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2008, 56, 11533–11539 11533

Intestinal and Peripheral Immune Response to MON810 Maize Ingestion in Weaning and Old Mice

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This study evaluated the gut and peripheral immune response to genetically modified (GM) maize in mice in vulnerable conditions. Weaning and old mice were fed a diet containing MON810 or its parental control maize or a pellet diet containing a GM-free maize for 30 and 90 days. The immunophenotype of intestinal intraepithelial, spleen, and blood lymphocytes of control maize fed mice was similar to that of pellet fed mice. As compared to control maize, MON810 maize induced alterations in the percentage of T and B cells and of $CD4^+$, $CD8^+$, $\gamma\delta$T, and $\alpha\beta$T subpopulations of weaning and old mice fed for 30 or 90 days, respectively, at the gut and peripheral sites. An increase of serum IL-6, IL-13, IL-12p70, and MIP-1β after MON810 feeding was also found. These results suggest the importance of the gut and peripheral immune response to GM crop ingestion as well as the age of the consumer in the GMO safety evaluation.

KEYWORDS: MON810; transgenic maize; mice; intestinal immune response; lymphocytes subpopulations

Significant differences in the peripheral immune response of rats fed with MON810 from control. No analysis of immune response in EFSA guidelines

Modifications in hepatocytes have been observed in long term experiments on rats fed with transgenic soybean. EFSA guidelines involve short term feeding trials on a limited number of rats

In the literature, the reports on the effects of a genetically modified (GM) diet are scanty and heterogeneous; in particular, no direct evidence has so far been reported that GM food may affect human or animal health.
Hepatocytes represent a suitable model for monitoring the effects of a GM diet, the liver potentially being a primary target. In a previous study, we demonstrated that some modifications occur in hepatocyte nuclei of mice fed on GM soybean. In order to elucidate whether such modifications can be reversed, in the present study, 3 months old mice fed on GM soybean since their weaning were submitted to a diet containing wild type soybean only, for one month. In parallel, to investigate the influence of GM soybean on adult individuals, mice fed on wild type soybean were changed to a GM diet, for the same time. Using immunoelectron microscopy, we demonstrated that a one-month diet reversion can influence some nuclear features in adult mice, restoring typical characteristics of controls in GM-fed animals, and inducing in control mice modifications similar to those observed in animals fed on GM soybean from weaning. This suggests that the modifications related to GM soybean are potentially reversible, but also that some modifications are inducible in adult organisms in a short time.

Key words: cell nucleus, liver, genetically modified soybean, electron microscopy.

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At variance with EFSA suggestions data coming from long term trials in feeding experiments should be analysed with proper statistical techniques to obtain statistical significant meaning

International Journal of Biological Sciences
2009; 5(7):706-726

Research Paper

A Comparison of the Effects of Three GM Corn Varieties on Mammalian Health

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Abstract

We present for the first time a comparative analysis of blood and organ system data from trials with rats fed three main commercialized genetically modified (GM) maize (NK 603, MON 810, MON 863), which are present in food and feed in the world. NK 603 has been modified to be tolerant to the broad spectrum herbicide Roundup and thus contains residues of this formulation. MON 810 and MON 863 are engineered to synthesize two different Bt toxins used as insecticides. Approximately 60 different biochemical parameters were classified per organ and measured in serum and urine after 5 and 14 weeks of feeding. GM maize-fed rats were compared first to their respective isogenic or parental non-GM equivalent control groups. This was followed by comparison to six reference groups, which had consumed various other non-GM maize varieties. We applied nonparametric methods, including multiple pairwise comparisons with a False Discovery Rate approach. Principal Component Analysis allowed the investigation of scattering of different factors (sex, weeks of feeding, diet, dose and group). Our analysis clearly reveals for the 3 GMOs new side effects linked with GM maize consumption, which were sex- and often dose-dependent. Effects were mostly associated with the kidney and liver, the dietary detoxifying organs, although different between the 3 GMOs. Other effects were also noticed in the heart, adrenal glands, spleen and haematopoietic system. We conclude that these data highlight signs of hepatorenal toxicity, possibly due to the new pesticides specific to each GM corn. In addition, unintended direct or indirect metabolic consequences of the genetic modification cannot be excluded.

Key words: GMO, toxicity, GM corn, rat, NK 603, MON 810, MON 863

Plant and Soil **266**: 69–75, 2004.

Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi

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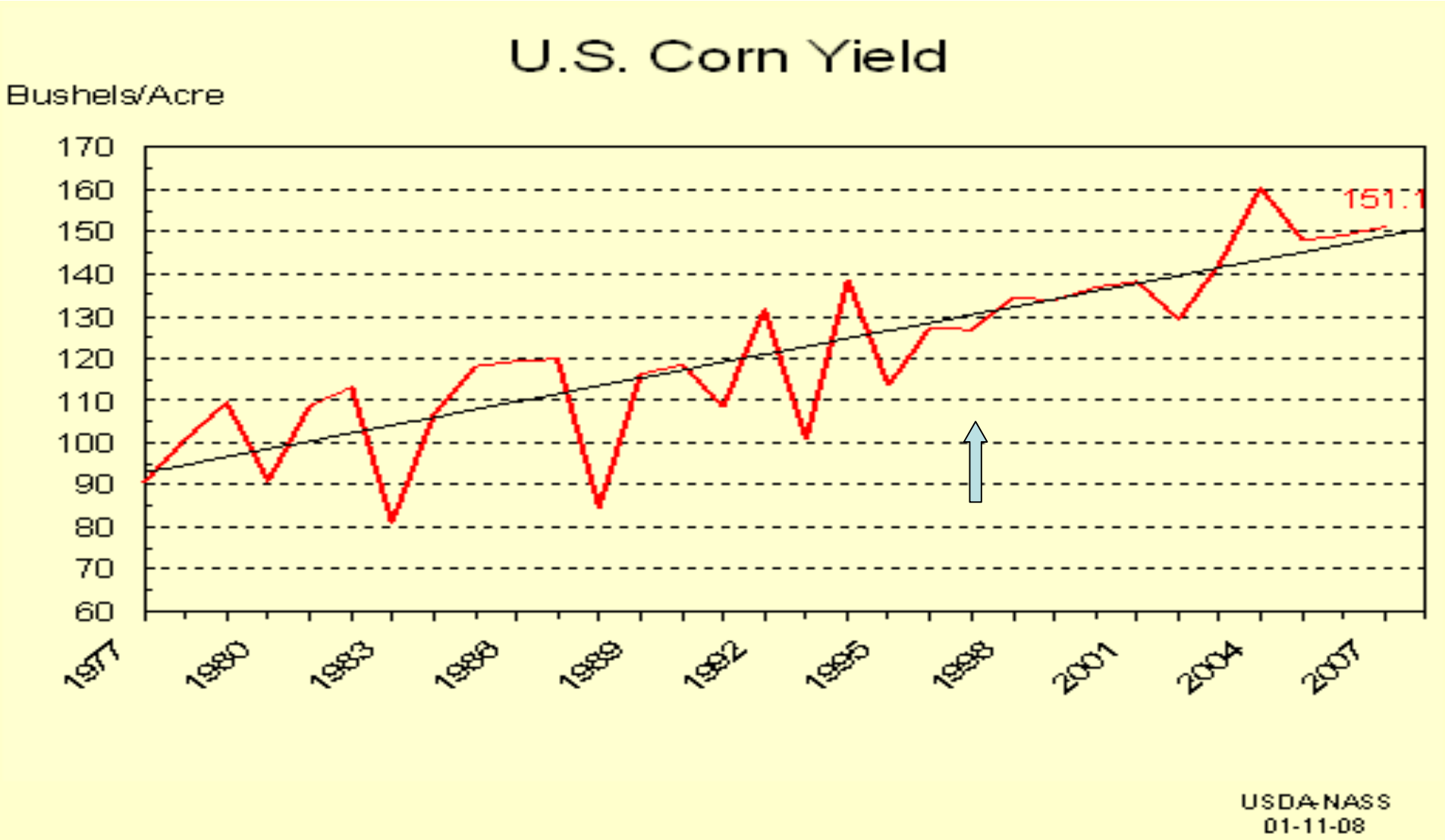
Key words: arbuscular mycorrhizal fungi, *Bt* toxin, environmental impact, plant defensins, root exudates of transgenic plants, test system

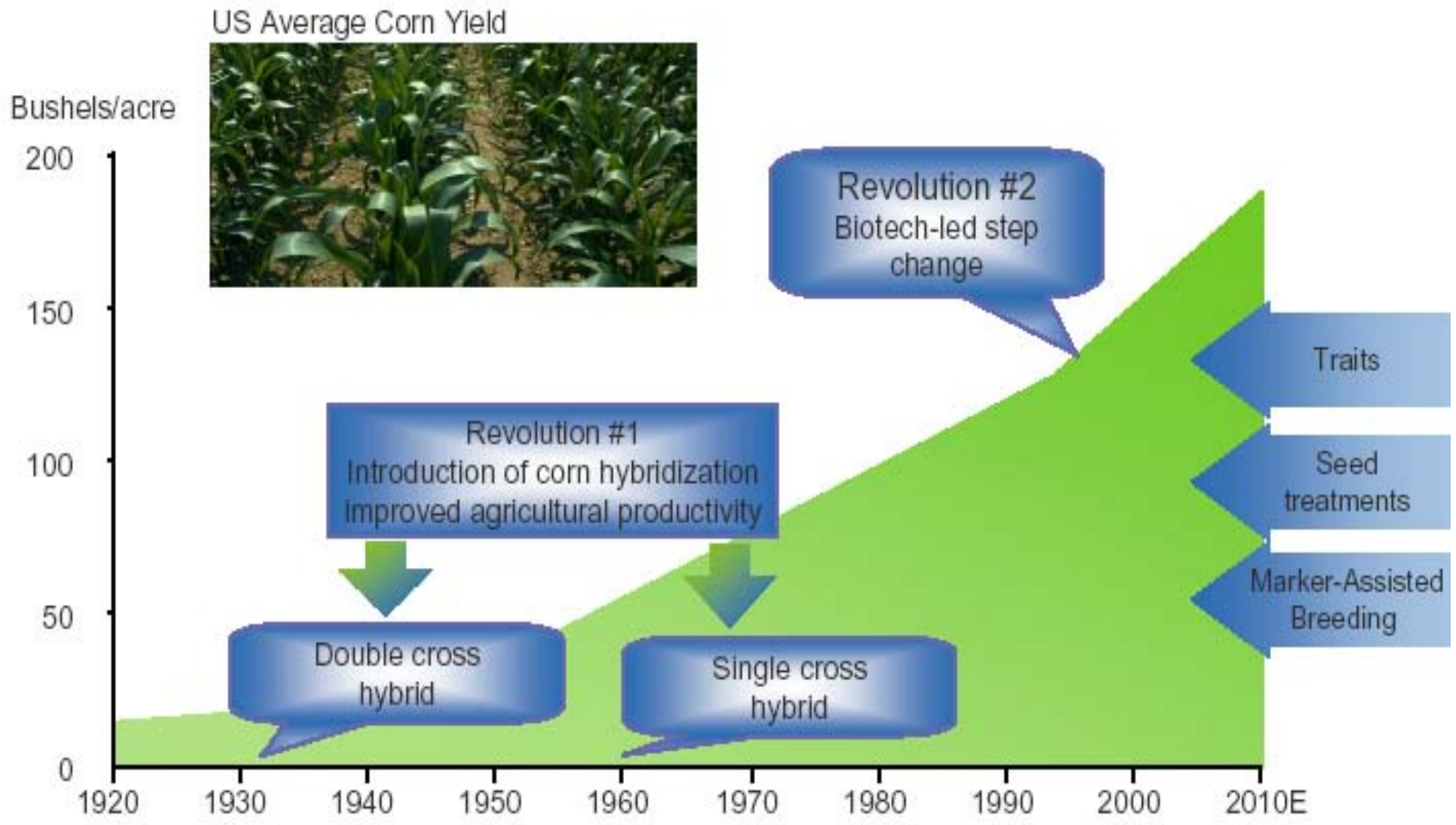
Abstract

We developed an experimental model system to monitor the impact of generically modified (GM) plants on arbuscular mycorrhizal (AM) fungi, a group of non-target soil microorganisms, fundamental for soil fertility and plant nutrition. The system allowed us to study the effects of root exudates of both commercial *Bt* corn and aubergine plants expressing Dm-AMP1 defensin on different stages of the life cycle of the AM fungal species *G. mosseae*. Root exudates of *Bt* 176 corn significantly reduced pre-symbiotic hyphal growth, compared to *Bt* 11 and non-transgenic plants. No differences were found in mycelial growth in the presence of Dm-AMP1 and control plant root exudates. Differential hyphal morphogenesis occurred irrespective of the plant line, suggesting that both exuded *Bt* toxin and defensin do not interfere with fungal host recognition mechanisms. *Bt* 176 affected the regular development of appressoria, 36% of which failed to produce viable infection pegs. Our experimental model system represents an easy assay for testing the impact of GM plants on non-target soil-borne AM fungi.

An analysis of the effect of transgenic plants on soil microflora and particularly on mycorrhizae-plants symbiosis have shown significant unintended affects but EFSA does not recommend this area of of genotype-environment interactions



An unintended effect: the pace of improvement of maize yield did not change after 1996, the year of introduction of GMOs into the market according to official USDA data.



The same data as in Fig 1 presented by Syngenta, one of the leading companies in the field showing a non existent "jump" in production since the introduction of GMOs in 1996.