Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion

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Controversy regarding genetically modified (GM) plants and their potential impact on human health contrasts with the tacit acceptance of other plants that were also modified, but not considered as GM products (e.g., varieties raised through conventional breeding such as mutagenesis). What is beyond the phenotype of these improved plants? Should mutagenized plants be treated differently from transgenics? We have evaluated the extent of transcriptome modification occurring during rice improvement through transgenesis versus mutation breeding. We used oligonucleotide microarrays to analyze gene expression in four different pools of four types of rice plants and respective controls: (*i*) a γ-irradiated stable mutant, (*ii*) the M1 generation of a 100-Gy γ-irradiated plant, (*iii*) a stable transgenic plant obtained for production of an anticancer antibody, and (*iv*) the T1 generation of a transgenic plant produced aiming for abiotic stress improvement, and all of the unmodified original genotypes as controls. We found that the improvement of a plant variety through the acquisition of a new desired trait, using either mutagenesis or transgenesis, may cause stress and thus lead to an altered expression of untargeted genes. In all of the cases studied, the observed alteration was more extensive in mutagenized than in transgenic plants. We propose that the safety assessment of improved plant varieties should be carried out on a case-by-case basis and not simply restricted to foods obtained through genetic engineering.

food safety evaluation | rice | genetically modified organisms | genetic engineering | γ-irradiation

Plant breeding started thousands of years ago, through the unconscious selection of seeds from plants with higher quality and productivity. After sexual plant reproduction was discovered, in the 17th century, people started to use deliberate interbreeding (crossing) of closely or distantly related species to produce new crops with desirable properties (1). With the discovery, in the beginning of the 20th century, that x-rays induced mutations in the fruit fly *Drosophila melanogaster* and barley, plant breeders and geneticists started to use mutagenesis to rapidly create and increase variability in crop species and ultimately change plant traits. The high efficiency of classical mutagenesis has been widely documented (2), and its global impact for crop improvement has also been evaluated (3). Since the establishment of the joint Food and Agriculture Organization/International Atomic Energy Agency, Division of the Nuclear Techniques in Agriculture (www-infocris.iaea.org/MVD), 1,916 crop and legume varieties were released worldwide (40% γ-irradiated).

Since the 1970s, advances in molecular biology have provided the basis for the development of genetic engineering, leading to the next level of genetic gain in crop cultivars. This technology permits the identification, isolation, and transfer of a gene of interest, originated from any type of organism, to plant cells. Transformed plants are then regenerated from these cells through tissue culture (4).

Contrasting with the readily acceptance of food products obtained through conventional plant breeding, the potential benefits of this new technology have been held largely at bay because of the enormous controversy regarding the food safety of the resulting products (5).

Despite the lack of universal methods for evaluating the potentially hazardous effects of genetic modification, Food and Agriculture Organization and the European Food Safety Authority recommendations call for targeted approaches to evaluate macro-, micro-, and anti-nutrients, toxins, allergens, and secondary metabolites. To increase the chances of detecting unintended effects, some molecular profiling methods have also been proposed (6). One of the mentioned profiling techniques is microarrays. This technology allows for monitoring the expression of thousands of genes simultaneously.

In this study, we used expression microarray analyses to monitor the extension of unexpected transcriptome modifications obtained in rice by conventional plant breeding by γ-irradiation as compared with the ones obtained through genetic engineering. We have analyzed four rice lines (two mutagenized and two transgenic ones) and further compared the stable lines against the recently modified ones.

Results and Discussion

Differentially Expressed Genes Increase with Genetic Instability and from Transgenic to Mutant Lines. Hierarchical clustering (Fig. 1) of the microarray data of transgenic, mutagenized, and control plants showed that duplicate samples always grouped together and modified genotypes always grouped with the respective unmodified controls [see supporting information (SI) Fig. 3 for Pearson's correlation between samples]. Despite the different type of breeding strategy used, genetically stable samples [transgenic single-chain variable fragment (ScFv) and mutant Estrela A] are more closely grouped with their corresponding controls than nonstable ones. Additionally, in nonstable lines, transgenic Nipponbare [Nip. genetically modified (GM)] is more closely related to its control than the line obtained through 100-Gy γ-irradiation. As visible in volcano plots (Fig. 2), 11,267 genes showed differential expression in the nonstable mutagenized rice line, whereas only 2,318 genes were detected in the nonstable transgenic line (despite the inserted gene being a transcription factor). The number of affected genes was strongly

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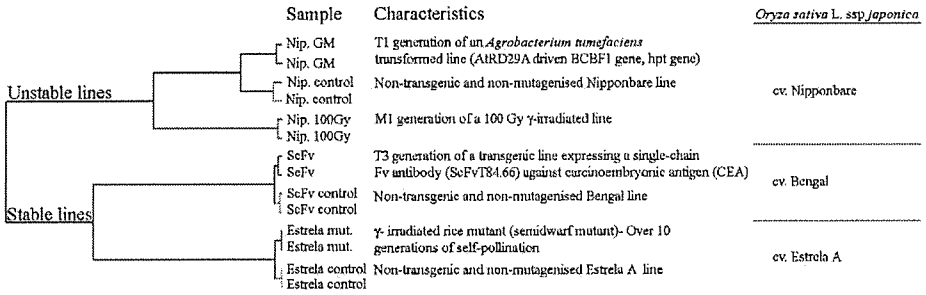

Fig. 1. Plant material used and hierarchical clustering dendrogram of the different samples.

reduced in stable lines (to 51 in the mutant and 25 in the transgenic).

The Analyzed Breeding Strategies Cause Stress, and Plants Respond to It by Modifying Transcription for Several Generations. The list of the differentially expressed genes with a cut-off $P < 0.05$ and with >2-fold change (>2 or <−2) after Log2 transformation (identified as high fold change) is shown in Table 1. For nonstable lines, only the top 50 differentially expressed genes are presented (Table 1). For those two experiments we also present a pie chart with all of the differentially expressed genes with a cut-off $P < 0.05$ and high fold change (we only considered genes whose function could be retrieved) separated by functional categories (SI Fig. 4). The genes listed in Table 1 were identified and analyzed for their functions by using Affymetrix, TIGR rice genome annotation, National Center for Biotechnology Information, UniProt, and Pfam internet resources. We found that in all of the experiments, the acquisition of the desired traits is accompanied by modifications in transcript levels of untargeted stress-related genes (genes whose altered transcription cannot be directly related with the introduced transgenes or desired traits are yellow-shadowed in Table 1).

We have also verified that the stressing event is memorized along several generations, although with a decreasing impact in the number of altered transcripts in each new generation (Table 1). This phenomenon of transgeneration memory of stress could be possibly attributed to epigenetic mechanisms and has been reported by others (7).

Although a complete understanding of plant stress response is far from being reached, various papers reporting molecular and biochemical studies suggest the involvement of at least six classes of genes (a–f): class a, genes implicated in stress/defense signaling-signal perception (several types of receptor-like protein kinases, two-component histidine kinases, G protein-coupled receptors, Ca^{2+}-releasing modules), and signal transduction (protein kinases, protein phosphatases, MAP kinases) (8–10); class b, second messengers, such as reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA), and ethylene, which are involved in the regulatory pathways (11); class c, genes implicated in stress response–ROS network (GST, peroxidases) (12) and the systemic acquired resistance (SAR) response (pathogenesis-related genes) (13); class d, genes implicated in protein modification (methylation, isoprenylation, lipidation, ubiquitination) and scaffolds or adapters [these molecules regulate the activity of stress signaling components (8)]; class e, genes encoding transcription factors that are involved in the temporal and spatial regulation of specific stress genes (14); and class f,

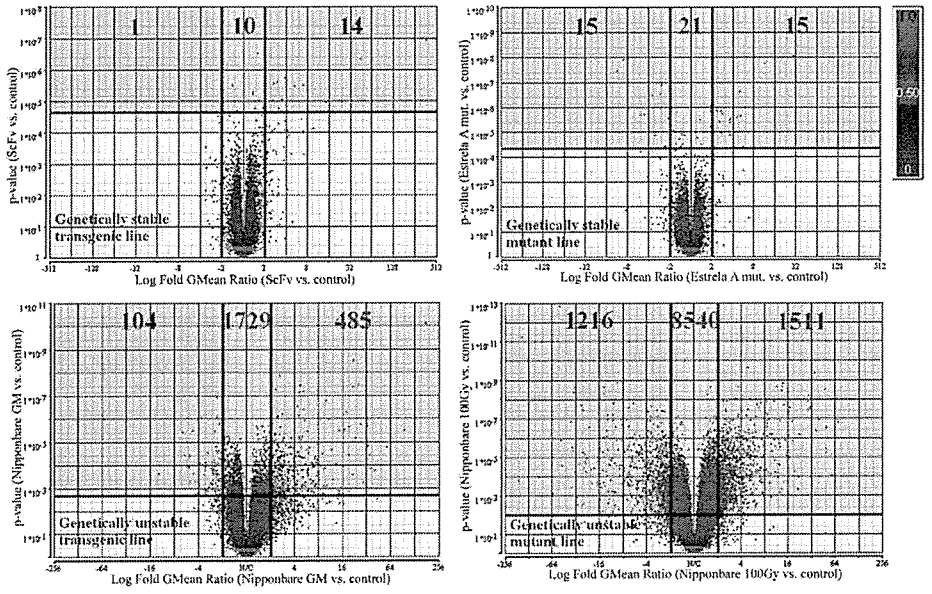

Fig. 2. Volcano plots for differentially expressed genes. Differentially expressed genes appear above the thick horizontal lines. Genes induced >2-fold are on the right of the right vertical lines, and the ones repressed >2-fold are on the left of the left vertical line. The numbers corresponding to the differentially expressed genes induced >2-fold for each experiment (red-shadowed area) are red, and those corresponding to the genes repressed >2-fold (blue-shadowed area) are blue. The green-shadowed area corresponds to differentially expressed genes that were up- or down-regulated <2-fold (green-colored numbers). Blue-colored genes are those with P between 0 and 0.5, and red-colored genes are those with P between 0.5 and 1.

Table 1. Significantly induced and repressed genes (fold change > 2 or < −2) for each experiment

Putative function	Affymetrix ID	Fold change
Stable ScFv vs. control		
Signal transduction		
C2 domain containing protein	OsAffx.12493.1.S1_at	38.76
Wall associated kinase	Os.15516.1.S1_at	-7.26
Transposons/ Retrotransposons		
Retrotransposon gag protein family	Os.50472.2.S1_x_at	9.94
Retrotransposon protein	Os.21278.1.S1_at	2.66
Transposon protein, putative, Pong sub-class	OsAffx.8070.1.S1_x_at	2.62
Marker gene		
Hygromycin B phosphotransferase	RPTR-Os-K01193-1_at	365.25
Stable Estrela A mut. vs. control		
Signal transduction		
Putative receptor-like kinase	Os.21180.1.S1_at	3.70
Phosphatidylinositol 3- kinase	Os.19018.2.S1_at	-3.24
Transposons/ Retrotransposons		
Retrotransposon gag protein	Os.50755.1.S1_at	5.65
Retrotransposon gag protein	Os.35216.2.S1_x_at	5.14
Retrotransposon gag protein	Os.35216.1.S1_at	3.66
Retrotransposon protein	Os.27592.1.A1_at	-2.42
Stress/defense response/ apoptosis		
Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily	Os.11753.1.S1_at	2.71
Acid phosphatase/vanadium-dependent haloperoxidases related	OsAffx.15718.1.S1_at	-2.08
Regulation of transcription		
Zinc finger, C2H2 type family	OsAffx.28219.1.S1_at	2.35
AP2 domain	Os.55500.1.S1_at	-3.58
Moving, modifying, storing and degrading proteins		
Leucine Rich Repeat family	Os.26855.1.A1_at	-2.09
Zinc finger (C3HC4-type RING finger)	Os.51816.1.S1_at	-2.71
Ubiquitin carboxyl-terminal hydrolase	Os.7411.1.S1_at	-5.27
F-box domain	Os.21626.1.S1_s_at	-15.98
T1 Nipponbare GM vs. control		
Signal transduction		
EF hand- calcium ion binding	Os.27569.1.S2_at	8.37
Putative calcium-dependent protein kinase	Os.12642.1.S1_at	7.88
Protein tyrosine kinase	Os.53763.1.A1_at	-12.57
Transposons/ Retrotransposons		
Transposon protein, putative, CACTA, En/Spm sub-class	Os.3808.4.S1_x_at	19.42
Stress/defense response/ apoptosis		
S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	Os.11812.1.S1_at	19.20
Phenylalanine and histidine ammonia-lyase	Os.10930.1.S1_at	16.27
Trehalose-6-phosphate phosphatase	Os.6092.1.S1_at	12.04
Cysteine proteinase precursor	Os.4181.1.S1_at	14.59
NB-ARC domain containing protein	Os.27538.1.S1_at	11.64
Putative pathogenesis-related protein	Os.50993.1.S1_at	8.56
Regulation of transcription		
Helix-loop-helix DNA-binding domain	Os.6043.1.S1_at	95.47
ZIM motif family protein	Os.46849.1.S1_at	41.23
AP2 domain	OsAffx.17366.1.S1_at	36.24
AP2/ ERF domain	Os.54944.1.S1_at	34.03
CRT/ DRE AP2 domain	Os.51078.1.S1_at	32.59
CBF1 AP2 domain	OsAffx.27442.1.S1_at	23.27
WRKY DNA binding domain	Os29987.2.S1_at	17.79
Putative no apical meristem (NAM) protein	OsAffx.20377.1.S1_x_at	17.01
Helix-loop-helix DNA-binding domain	Os.46956.1.S1_at	10.80
ZIM motif family protein	Os.9923.1.S1_s_at	8.34
Zinc finger, C2H2 type	Os.54232.1.S1_at	8.11
Myb-like DNA-binding domain	Os.10172.1.S1_at	7.62
NB-ARC domain	Os.48915.1.S1_at	7.45
Transport		
Triose-phosphate transporter	Os.32298.1.s1_at	9.43
Unknown function		
Hypothetical protein	OsAffx.26698.1.S1_at	5.24
Hypothetical protein	Os.6148.1.S1_at	3.46
Hypothetical protein	Os.51535.1.S1_at	3.80
Hypothetical protein	Os.50472.2.S1_s_at	14.46
Expressed protein	Os.57052.1.S1_at	7.81
Hypothetical protein	Os.27851.1.A1_a_at	7.05
Expressed protein	Os.55384.1.S1_s_at	3.12
Expressed protein	Os.8480.1.S1_at	2.96
Hypothetical protein	OsAffx.16638.1.S1_at	44.91
Metabolism		
Pyruvate kinase-like protein	Os.18503.1.S1_s_at	289.6
Flavonol 3-O-glucosyltransferase	Os.8143.1.S2_at	30.14
Adenylyl-sulfate kinase	OsAffx.7376.1.S1_at	3.24
Phosphomannose isomerase type I	OsAffx.17841.1.S1_at	2.80
Nitrilase-associated protein	OsAffx.31420.1.S1_at	-11.10
Unknown function		
Expressed protein	Os.14913.1.S1_at	23.93
Expressed protein	Os.22675.1.S2_at	3.66
Expressed protein	Os.9028.1.S1_at	3.59
Expressed protein	OsAffx.19321.1.S1_at	2.70
Hypothetical protein	Os.50431.1.S1_at	2.55
Expressed protein	Os.7793.1.S1_at	-2.02
Hypothetical protein	Os.31112.2.S1_at	-2.32
Expressed protein	Os.7723.1.S1_at	-2.61
Unknown	Os.26863.1.A1_a_at	-4.62
Transmembrane BAX inhibitor motif	OsAffx.31431.1.S1_at	-9.14
Hypothetical protein	Os.18313.1.S1_at	-73.37
Metabolism		
Cytochrome P450	Os.51923.1.S1_at	58.25
Cytochrome P450	Os.14105.1.S1_at	35.87
Hypothetical protein (may have a role in ATPase activation)	Os.32889.1.S1_at	32.45
Putative cytochrome P450	Os.9067.1.S1_at	9.66
Chalcone and stilbene synthases	Os.14616.1.S1_at	8.18
Glycosyl hydrolases family	Os.47778.1.A1_s_at	8.08
NADP-dependent oxidoreductase	Os.15917.1.S1_at	7.47
Unknown function		
Expressed protein	Os.8823.1.S1_at	175.86
Expressed protein	Os.12498.2.S1_at	29.27
Hypothetical protein	Os.55261.1.S1_at	27.28
Hypothetical protein	Os.10660.1.S1_at	25.10
Expressed protein	Os.56018.1.S1_at	23.41
Expressed protein	Os.10266.1.S1_at	22.11
Expressed protein	Os.56053.1.S1_at	21.66
Expressed protein	Os.51546.1.S1_at	21.65
Unknown protein	Os.45902.1.A1_at	19.33
Expressed protein	OsAffx.12383.1.S1_at	14.36
Expressed protein	Os.40325.1.A1_at	13.47
Hypothetical protein	Os.50587.1.S1_at	10.29
Expressed protein	Os.53407.1.S1_at	10.03
Expressed protein	Os.57343.1.S1_at	9.28
Cell structure and biogenesis		
Repetitive proline-rich cell wall protein precursor	Os.17946.1.A1_x_at	7.80
Moving, modifying, storing and degrading proteins		
Germin-like protein subfamily	Os.51532.1.S1_at	9.05
Putative germin A	Os.23557.1.A1_at	8.25
Subtilisin-like serine proteinase	Os.57054.1.S1_at	8.02
RING/C3HC4/PHD zinc finger-like	Os.11365.1.S1_at	7.86

genes encoding retrotransposons that represent sensitive markers of plant stress (15).

Genes Whose Altered Transcription Could Be Directly Related to the Introduced Genes or Desired Traits. Some of the differentially expressed genes found in Table 1 can be directly associated with the transgenes introduced or with the desired new characteristics of the modified plant (green shadowed). One example of these differentially expressed genes is the hygromycin B phosphotransferase gene, used as a marker gene in the ScFv stable transgenic line (Table 1).

Some of the differentially expressed genes found in the stable

Table 1. (continued)

Putative function	Affymetrix ID	Fold change	Putative function	Affymetrix ID	Fold change
M1 Nipponbare 100Gy vs. control					
Signal transduction			**Metabolism**		
Apyrase	Os.27696.1.S1-at	47.48	Pyruvate, phosphate dikinase, Chloroplast precursor	OsAffx.13147.1.S1_s_at	21.50
Similar to S receptor kinase	Os.49584.1.S1_at	34.95	Photosystem II protein D2	OsAffx.32349.1.A1_at	-23.14
Putative serine/threonine kinase	Os.26226.1.s1_at	22.34	Endo-1,3:1,4-beta-D-glucanase precursor	Os.8582.1.S1_at	-23.68
Protein tyrosine kinase	Os.53763.1.A1_at	-62.99	ent-kaurene oxidase	Os.57506.1.S1_at	-30.18
Transposons/ Retrotransposons			chloroplast ATP synthase	OsAffx.32209.1.A1_at	-33.38
Transposon protein, putative, CACTA, En/Spm sub-class	OsAffx.22999.1.S1_at	-35.25	NADH ubiquinone oxidoreductase	OsAffx.32259.1.A1_at	-35.69
Stress/defense response/ apoptosis			**Unknown function**		
Glutathione S-transferase	Os.49030.1.A1_s_at	57.56	Hypothetical protein	Os.54340.1.S1_at	70.13
NB-ARC domain	Os.4192.1.S1_at	32.64	Expressed protein	Os.27342.1.S1_a_at	50.07
NB-ARC domain	Os.26992.1.S1_at	30.33	BURP domain	Os.37718.1.S1_at	49.60
Late embryogenesis abundant	Os.12551.1.S1_s_at	21.88	Expressed protein	Os.28200.1.S1_x_at	48.84
Phosphoethanolamine methyltransferase	Os.17921.1.S1_at	-25.28	Hypothetical protein	Os.36176.1.S1_at	40.71
Beta-glucosidase aggregating factor	OsAffx.23382.1.S1_at	-26.34	Hypothetical protein	OsAffx.18924.3.S1_at	36.60
Terpene synthase	Os.27751.1.S1_at	-26.92	Expressed protein	Os.52807.1.S1_at	31.56
Peroxidase family	OsAffx.4250.1.S1_s_at	-28.69	Hypothetical protein	Os.54340.1.S1_x_at	29.39
Putative thionin Osthi1	Os.8655.1.S1_at	-31.02	Hypothetical protein	Os.51266.1.S1_at	28.55
Peroxidase 2 precursor	Os.11556.1.S1_at	-33.15	Hypothetical protein	Os.4887.1.S1_at	28.26
Regulation of transcription			Expressed protein	Os.10760.1.s1_at	-20.57
NB-ARC domain	Os.52240.1.S1_at	40.39	Expressed protein	Os.10659.1.S1_at	-21.63
Myb-like DNA-binding domain	OsAffx.15178.1.S1_s_at	-26.16	Hypothetical protein	OsAffx.27752.1.S1_s_at	-27.03
Transport			Expressed protein	OsAffx.28294.2.S1_x_at	-34.29
Probable aquaporin TIP2.1	Os.12345.1.S1_at	-24.53	Expressed protein	Os.9180.1.S1_at	-36.55
Plastocyanin-like domain	OsAffx.5542.1.S1_s_at	-36.71	Expressed protein	Os.10659.1.S1_at	-49.63
ZIP Zinc transporter	Os.19632.1.S1_at	-37.26	Expressed protein	Os.7108.1.S1_at	-63.80
High affinity nitrate transporter	Os.49093.1.S1_at	-48.79	Hypothetical protein	Os.46728.1.S1_at	-66.79
Lipid transfer	Os.47380.1.S1_at	-93.91	Expressed protein	Os.10038.1.S1_s_at	-157.15
Cell structure and biogenesis			Expressed protein	Os.11315.1.S1_at	-174.92
Glycine-rich cell wall structural protein 2 precursor	Os.46669.1.A1_at	-20.16	**RNA processing**		
			Glycine rich RNA binding protein	Os.46902.1.S1_at	-52.90

For T1 Nipponbare GM vs. control and M1 Nipponbare 100 Gy vs. control, only the top 50 differentially expressed genes are presented. Yellow shading indicates the genes are directly or indirectly related with stress response. Green shading indicates the genes' altered expression can be associated with the introduced genes or desired traits. Red shading indicates up-regulated genes, and blue shading indicates down-regulated genes.

Estrela A line (Table 1) can be eventually related to a reduced indole-3-acetic acid (IAA) content, because the down-regulation of a nitrilase-associated protein was observed in the mutant/dwarfed line (SI Fig. 5*A*). Nitrilases are key enzymes in the biosynthesis of the plant hormone IAA (16), which belongs to the auxin class of plant growth regulators. The enzyme phosphatidylinositol 3-kinase, found in the signal transduction functional group, can also be related to this putative reduced IAA content because the phosphatidylinositol signaling pathway is also involved in plant responses to hormones, like auxins (17). We also found, in this experiment, a group of genes implicated in protein modifications whose altered transcription can be related to the hypothetical reduced IAA content. This group consisted of two proteins involved in ubiquitination: one F-box domain-containing protein and the ubiquitin carboxyl-terminal hidrolase. F-box proteins act as adaptor components of the modular E3 ubiquitin ligase SKP1-CUL1-F-box protein (SCF) complex that functions in phosphorylation-mediated ubiquitination. Protein ubiquitination is a precise strategy for regulating gene function, driving tagged proteins for degradation via the proteasome, and it is suggested as an important control system in desiccation tolerance (18). The down-regulation of these two proteins could be explained by the decreased auxin content because auxin regulates transcription by promoting the degradation of a family of transcriptional repressors known as Aux/IAA proteins, this degradation depending on a ubiquitin protein ligase named SCF(TIR1). In the presence of auxin, the F-box protein TIR1 binds to the Aux/IAA proteins, resulting in their ubiquitination and consequent degradation (19).

The unstable transgenic line Nipponbare GM contains one copy of the barley CBF1 gene (*BCBF1*). C-repeat binding factors (CBFs) specifically interact with the *cis*-acting dehydration-responsive element-DRE (core motif:G/ACCGAC) and control the expression of many stress-inducible genes (20). Although *BCBF1* gene is under the control of a stress-inducible promotor (*AtRD29A*), preliminary experimental results obtained within our team (unpublished data) reveal a leaky expression of the *BCBF1* gene in rice, even in the absence of stress conditions. For this reason, in this particular case, the differential expression of the stress-related genes found in our experiments may be either caused by the stress imposed by the *Agrobacterium*-mediated genetic modification or, at least in part, by the introduced *BCBF1* transcription factor. To clarify this point we decided to analyze the promoter (2 kb upstream of the ATG start codon) of the top 50 differentially expressed genes to search for DRE core motifs. From this study we found that almost all of the top 50 genes (90%) contain several DRE core motifs in their promoter regions (green shadowed in Table 1). Therefore, it seems that the differential expression of these genes may be related mainly to the specific transgene integrated. This result highlights the importance of carefully studying transformants carrying inserted genes coding for transcription factors.

Genes Implicated in Stress/Defense Signaling (Class A). All of the differentially expressed genes found in the signal transduction category, and not related to the transgene's introduction or desired traits, could be related with stress/defense. Thus, in Table 1 we observe in this functional group a wall-associated kinase and a C2 domain-containing protein. In plants, many protein kinases and phosphatases are involved in environmental stress responses (8–10). The C2 domain is a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins

involved in signal transduction or membrane trafficking and thought to be involved in binding calcium-dependent phospholipids (21). This domain has been correlated with stress signaling (22). In the stable mutagenized line we found two signal transduction-associated proteins, both also already characterized as stress/defense associated (Table 1): a receptor-like kinase (9, 10) and a phosphatidylinositol 3-kinase (17). Finally, concerning the unstable mutagenized line (Table 1) we found, in this category, an apyrase (23), a S receptor kinase (9, 10), a putative serine/threonine kinase (24), and a protein tyrosine kinase (25).

Genes Implicated in Stress/Defense/Apoptosis (Class C). In this functional group we found four genes potentially involved in the ROS network, (one GST and three peroxidases) (12), three NB–ARC domain-containing proteins (26), one glyoxalase (27), one late embryogenesis abundant protein (28), one phosphoethanolamine methyltransferase (29), one β-glucosidase (30), one terpene synthase (31), and one putative thionin (32).

Genes Implicated in the Regulation of Transcription (Class E). All of the differentially expressed genes found in this category, and not related with the transgenes' introduction or desired traits, could also be related with stress/defense. Thus, we found one AP2 domain, one zinc finger of the C_2H_2 type family, one WRKY DNA binding domain, a helix–loop–helix DNA-binding domain, a NB-ARC domain, and a Myb-like motif. All of these domain-containing proteins were previously associated with stress response (10, 14, 26, 33).

Transposons/Retrotransposons (Class F). All of the tested plants showed detected alteration in the transcription of genes encoding transposons/retrotransposons. As stated above, these genes are sensitive markers of plant stress (15).

Other Genes That Could also Be Indirectly Related to Stress. We could find in Table 1 some genes whose altered expression can be also indirectly related to stress. Thus, concerning the stable Estrela A mutagenized line (Table 1) the up-regulation of adenylylsulfate kinase can be related to gluthatione-based detoxification of methylglyoxal because this enzyme is involved in the sulfate assimilation pathway required for glutathione production (34). The up-regulation of a putative flavonol 3-O-glucosyltransferase could also be related to stress. This enzyme catalyzes the transfer of glucose from UDP-glucose to a flavonol, one of the last steps in anthocyanin pigment biosynthesis. Anthocyanins are produced by various plants as a result of stress and in senescing foliage as a consequence of the autumn hostile environment (35). Finally, the up-regulation of both pyruvate kinase and phosphomanose isomerase may also be related to stress. Pyruvate kinase is involved in glycolysis, and phosphomanose isomerase catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate, also a component of the glycolytic pathway. The stress induction of glycolysis transcripts has been reported in other studies (36). Regarding the unstable Nipponbare 100-Gy line (Table 1), we also found, in the different functional groups, some genes already associated with stress/defense responses, specifically to NaCl-stress response: several aquaporin and lipid transfer proteins (37), one high-affinity nitrate transporter (38), one glycine-rich cell wall protein (24), and one endo-1,3–1,4-β-D-glucanase (39). The altered expression of the photosynthesis-associated genes encoding photosystem II protein D2 and chloroplast ATP synthase is consistent with the already known effect of γ-irradiation on the photosynthetic activity (40). Pyruvate phosphate dikinase up-regulation, NADH ubiquinone oxidoreductase down-regulation, and down-regulation of the photosynthesis-related genes may be a response to oxidative stress and a way of limiting mitochondrial ROS production while keeping the electron transport chain relatively oxidized (41).

The pie charts we obtained for the genetically unstable lines (SI Fig. 4) are strikingly similar to the one obtained for *Arabidopsis* under various stress conditions (9). This similarity also supports our statement about the relation between genetic modification and stress response.

In conclusion, we have demonstrated that:

(*i*) DNA microarray technology should be considered as a powerful profiling tool for studying altered gene expression induced by different breeding strategies. However, changes in transcriptome do not necessarily correlate with risk. Proteomic studies should thus be performed to provide data on the nature of proteins.

(*ii*) Transcript profile of the stable lines was less altered than that of unstable ones and tested GM plants showed fewer genetic alterations than mutagenized ones. This last difference remains well known for the tested stable lines despite the higher number of self-pollinations for the mutant stable line as compared with the transgenic (10 vs. 3). Although these results may be specific to the particular mutagenized and transgenic plants examined here, they show that transgenic plants may have fewer changes than mutagenized ones.

(*iii*) The improvement of a plant variety through the acquisition of a new desired trait or modification of a previous one (either by genetic engineering or mutagenesis) causes stress and thus has a broad impact on gene expression.

(*iv*) Even several generations after the breeding event, the plant still maintains the "memory" of that incident and responds accordingly.

(*v*) Similar phenotypes do not obligatorily mean similar transcript profiles, which was evident for the unstable mutant line (SI Fig. 5*B*). However, we cannot rule out that under certain environmental conditions different morphology would not become evident.

Finally, we believe that safety assessment of improved plant varieties should be carried out on a case-by-case basis and not simply restricted to foods obtained through genetic engineering.

Materials and Methods

Plant Materials. Two genetically stable *Oryza sativa* L. ssp. *japonica* lines: a γ-irradiated rice mutant (cv. Estrela A) and a well characterized transgenic rice line (cv. Bengal) were used as well as controls (Fig. 1). The stable mutant was obtained in 1988 by γ-irradiation, had already gone >10 generations of self-pollination, and had a mature average height ≈45 cm lower than the wild type (SI Fig. 5*A*). The stable transgenic line, which was already in the third generation of self-pollination after transformation, expresses a ScFV antibody (ScFvT84.66) against carcinoembryonic antigen, a well characterized tumor-associated marker antigen (42).

We have also used two genetically unstable rice lines: the M1 generation of a 100-Gy γ-irradiated line (98% survival after mutagenesis) and the T1 generation of an *Agrobacterium*-transformed transgenic line (both cv. Nipponbare) containing one copy of the *BCBF1* gene driven by the *AtRD29A* promoter from *Arabidopsis* and one copy of the *hpt* II gene (Fig. 1). We used seeds from the same self-pollinated panicle for control and irradiation/transgenesis. The nonstable mutant line chosen for this experiment was the one showing a phenotype more similar to that of the nonirradiated control (SI Fig. 5*B*).

In the case of the transgenic lines, stability was based on the stable inheritance of the introduced transgenes in the homozygous progeny. Regarding the mutagenized plants we have defined as genetically stable plants those that, after mutagenesis, had already gone through several cycles of self-pollination while maintaining the desirable traits.

Seed Treatment and Seedling Growth. Seeds were manually peeled and immersed for 30 min at 50°C in 0.1% Benlate (fungicide). After washing in distilled sterilized water, seeds were surface-disinfected with 70% (vol/vol) ethanol for 1 min and then with a solution of 2% sodium hypochloride with traces of Tween 20, for 30 min, at room temperature. After thorough washing with distilled sterile water seeds were kept overnight in the final wash and then soaked in Yoshida's medium (43) for germination in the dark for 2 days at 28°C. Seedlings were further grown at 28°C for 10 days under a 12-h

photoperiod regime. Yoshida's medium used for the transgenic lines was supplemented with 30 mg/liter of hygromicin B. Twelve-day-old seedlings were frozen in liquid nitrogen and kept at −80°C until RNA extraction.

RNA Extraction and Microarrays. Two pools of six whole seedlings were prepared for each condition under test, and RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was kept at −80°C and sent to the Affymetrix core facility (Instituto Gulbenkian de Ciência, Oeiras, Portugal), where quality-control analysis was carried out before cDNA synthesis from the mRNA [with appropriate oligo(dT) primers], labeling (through synthesis of cRNA with incorporation of biotinylated ribonucleotide analogs), and hybridization to the GeneChip Rice Genome Array (Affymetrix). This array contains probes to query 51,279 transcripts representing two rice subspecies (48,564 *japonica* transcripts and 1,260 transcripts of *indica* subspecies).

Data Analysis. Microarrays data analysis was performed with Partek Genomics Suite software. Affymetrix CEL files were imported by using the Robust Multichip Average method, which involves four steps: background correction of the perfect match values, quintile normalization across all of the chips in the experiment, Log2 transformation, and median polish summarization. The logged data were used for hierarchical cluster analysis and statistical analysis. Hierarchical cluster analysis was performed by using Pearson's dissimilarity product moment correlation coefficient and Ward's algorithm.

For the identification of differentially expressed genes we used ANOVA and a false discovery rate with a 0.05 threshold.

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RESEARCH ARTICLE Open Access

Do transgenesis and marker-assisted backcross breeding produce substantially equivalent plants? - A comparative study of transgenic and backcross rice carrying bacterial blight resistant gene Xa21

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Abstract

Background: The potential impact of genetically modified (GM) plants on human health has attracted much attention worldwide, and the issue remains controversial. This is in sharp contrast to the broad acceptance of plants produced by breeding through Marker Assisted Backcrossing (MAB).

Results: Focusing on transcriptome variation and perturbation to signaling pathways, we assessed the molecular and biological aspects of substantial equivalence, a general principle for food safety endorsed by the Food and Agricultural Organization and the World Health Organization, between a transgenic crop and a plant from MAB breeding. We compared a transgenic rice line (DXT) and a MAB rice line (DXB), both of which contain the gene *Xa21* providing resistance to bacterial leaf blight. By using Next-Generation sequencing data of DXT, DXB and their parental line (D62B), we compared the transcriptome variation of DXT and DXB. Remarkably, DXT had 43% fewer differentially expressed genes (DEGs) than DXB. The genes exclusively expressed in DXT and in DXB have pathogen and stress defense functions. Functional categories of DEGs in DXT were comparable to that in DXB, and seven of the eleven pathways significantly affected by transgenesis were also perturbed by MAB breeding.

Conclusions: These results indicated that the transgenic rice and rice from MAB breeding are substantial equivalent at the transcriptome level, and paved a way for further study of transgenic rice, e.g., understanding the chemical and nutritional properties of the DEGs identified in the current study.

Keywords: Transgenesis, Marker-assisted backcrossing, *Substantial equivalence*, Transcriptome profile, *Xa21*

Background

The primary objective of breeding in agriculture is to develop plants of desired genotypes or traits, such as high yields and resistance to adverse environmental impact. Marker-assisted backcrossing (MAB) and transgenesis (aka genetic modification or GM) are two widely adopted plant breeding techniques. As a conventional technique, MAB breeding has been used to develop new crop cultivars of, e.g., baley, maize and rice [1-4]. The basis of MAB breeding [5] is to transfer a specific allele at the target locus in a donor line to a recipient line while selecting against donor introgression across the rest of the genome. In most cases, the recipient line used for backcrossing has a large number of favorable attributes but is deficient in a few characteristics. Since MAB breeding to certain degree mimics or replicates natural selection, novel cultivars produced through MAB breeding have been regarded as genetically safe. However, MAB breeding is laborious,

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requiring several backcrossings and a large number of individual plant screenings, typically on the order of thousands. It typically takes a great deal of luck to produce a product of a desirable trait.

Transgenesis is an effective means for improving crop genetic makeup for deriving favorable traits. Breeding by transgenesis has several advantages over breeding by MAB. Transgenesis is a direct means for introducing a gene or genotype to a genome in order to produce a target trait. As a result, the potential to produce plants with favorable traits increases dramatically. For example, by transfering a gene that encodes a plastidial adenyalte kinase into a potato variety, the transgenic potato displays wild-type growth and developmental phenotype but also has an increased yield and starch concentration [6]. By introducing the hybrid cellulase gene *cel-hyb1* into a spring barley variety through Agrobacterium-mediated transformation, the selected marker-free transgenic barley produces a high level of cellulase (1,4-β-glucanase) in developing grains, suggesting that the transgenic barley has the potential for producing a large quantity of cellulase for commercial use [7]. The nutritional value of Golden Rice is improved with increased pro-vitamin A content by introducing genes encoding phytoene synthase (*psy*) in combination with the Erwinia uredovora carotene desaturase (*crtI*) into rice [8]. Through an Agrobacterium-mediated genetic transformation system, *Xa21*, a rice bacterial blight resistance gene, has been introduced into five Chinese rice varieties and as a result, the transgenic rice plants exhibit a high resistance to bacterial blight [9].

While transgenesis offers immense opportunities to curtail the severe threat of food shortage the expanding world population is facing, there are considerable public concerns over the use of transgenesis for crop improvement. Indeed, it remains extremely controversial whether or not transgenic crops have an adverse impact on human health. At the center of this controversy is the issue of whether or not insertion of a transgene into the host plant genome or manipulation of an allele in the host genome may affect the expression of other genes and ultimately lead to unintended phenotypes. Unfortunately, it is technically challenging to address this issue because accurate prediction of phenotypes based on genotypic variation and/or gene expression alteration remains a research topic.

Due to the difficulty, efforts of evaluating the safety of transgenic crops has been geared toward assessing the substantial equivalence between transgenic plants and wildtype or conventionally bred plants, like plants from MAB breeding [10]. *Substantial equivalence* has been introduced as a standard by the Organization for Economic Cooperation and Development (OECD) and has been endorsed by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) [11]. However, the standard is based on comparative analysis and offers only a general principle. No specific molecular, biological, chemical or nutritional basis has been established to precisely specify the degree of substantial equivalence [12,13]. It thus leaves widely open the study of various aspects of equivalence, ranging from molecular, biological, and chemical to nutritional equivalence, between a transgenic plant and a wildtype or plant produced by MAB breeding. Nevertheless, it has been agreed that to be considered substantially equivalent, the characteristics of a transgenic plant must be within a natural range of variation [14], a guideline we follow in our study.

Rice is an essential staple crop for the world population and a model plant for basic and applied research. Rice bacterial leaf blight (BLB), caused by bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating rice diseases throughout the world. Utilization of BLB resistant genes in breeding is the most effective and economical strategy for controlling BLB [15]. *Xa21*, the first-cloned BLB resistant gene from *Oryza longistaminata*, has received much attention because of its broad spectrum of resistance to BLB [16]. The gene has also been widely used in BLB resistance breeding through both the transgenic and MAB strategies [17-21]. Therefore, transgenic rice and MAB rice carrying *Xa21* offer an excellent opportunity to assess the possible substantial equivalence of transgenic and MAB rice as well as rice in natural environments.

In order to pave the way for future studies of the safety of transgenic crops, we focused on the molecular and biological aspects of substantial equivalence of transgenic rice. We adopted a systems-biology perspective and examined the transcriptome variation of transgenic rice. Specifically, we incorporated *Xa21* into the three-line maintainer line D62B through transgenesis and MAB breeding. Adopting Next Generation (Next-Gen) sequencing, we profiled the transcriptomes of four rice plants: the *Xa21* transgenic line (named as DXT), the *Xa21* MAB breeding line (named as DXB), the untransformed recipient D62B, and another rice variety MH86 (restorer line). We then analyzed transcriptome variation of the two rice plants carrying *Xa21* in reference to that of D62B and transcriptome change between D62B and MH86. This transcriptome analysis was further enhanced by a pathway analysis to understand the pathways that might be disturbed in the two rice plants carrying *Xa21*.

Results

A system for comparative study of transgenic and MAB rice

In order to compare transgenic and MAB plants, transgenic and MAB rice plants carrying *Xa21* using the parental line D62B were constructed. To generate the transgenic rice, *Xa21* was introduced into D62B through *Agrobacterium*-mediated transgenesis. The transgenic rice plants were selected from the T_0, T_1 and T_2 generations by molecular and resistance analysis. The homozygous, single copy, and

marker-free transgenic line, or DXT for short, was obtained from the T_2 generation. To confirm the results, Southern hybridization analysis was performed using the restriction endonuclease *Pvu*II to digest genomic DNA from the transgenic and parental plants. The details for developing the transgenic rice was described in our early report [22]. The sample used for analysis was the T_9 generation of DXT with stable agronomic traits.

Xa21 was introgressed into the parental line D62B to produce the MAB breeding line using IRBB21 as the donor. IRBB21 was bred by transfering *Xa21* into IR24 through backcrossing [23]. Six backcrossing generations were made because it is usually necessary to take a minimum of six backcrossing generations in order to recover the phenotype of recurrent parent lines and eliminate donor chromosome fragments linked to the target gene [5]. A backcrossed line with homozygous *Xa21* and similar phenotype with the recipient D62B was obtained in BC_6F_2 generation and named DXB.

In order to facilitate direct in-field screening and molecular analysis of transgenic and MAB plants that showed consistent agronomic traits similar to that of their parental line, the transgenic line (DXT), the MAB line (DXB) and their parental line (D62B) were grown in the same fields in the breeding process. D62B can thus serve as an ideal control for the comparison of DXT and DXB. In order to introduce a reference to natural variation, another rice varieties MH86, an *indica* restorer line in the three-line breeding system was also included in the profiling experiments. Since rice carrying *Xa21* confers robust resistance to most strains of *Xoo* at adult stages, the RNA samples were extracted from adult leaves of the four rice lines for transcriptome profiling.

The transgenic rice and MAB rice were phenotypically similar

The morphological characteristics of DXB, DXT, D62B, and MH86 were examined in the rice fields. DXT and DXB were morphologically similar to their parental line under visual inspection. The major agronomic traits of DXT and DXB, listed in Table 1, were also scored in the fields. The results showed that the main agronomic traits of DXT and DXB were consistent with or comparable to that of D62B, except with respect to the focal trait of BLB resistance. Both DXT and DXB were highly resistant to nine Philippine races of *Xoo* but D62B was susecptible to the all *Xoo* races (Figure 1). Highly phenotypic similarity among DXT, DXB, and D62B suggested that both transgenic and MAB breeding strategies had very little impact on the morphological characteristics and main agronomic traits of D62B other than the expected BLB resistance.

In contrast, there were evident morphological differences between the two distinct rice varieties D62B and MH86. The sheath and blade of MH86 were light green but that of D62B were reddish dark green. Moreover, D62B showed a narrower blade, thinner and shorter stem, and a shorter growth period than MH86.

Transcriptome profiling using Next-Gen sequencing

In order to investigate transcriptome variations of the transgenic and MAB rice, digital gene expression profiles of DXT, DXB, D62B and MH86 were obtained using Next-Gen sequencing. The 5.8 to 6.1 million raw sequencing reads (Table 2) for the samples have been deposited into NCBI/GEO (accession number SRA061839). Preprocessing of these raw data (see Methods) gave rise to 5.7 to 6.0 million qualified reads for down-stream analysis. The qualified reads were mapped to the cDNA sequences of *Oryza Sativa Nipponbare* with a stringent criterion, giving 12.1 to 13.3 thousand unigenes per sample (Table 2). A statistical analysis (see Methods) of all the genes mapped in the four samples detected 15,268 unigenes, among which 10,362 were common to all four rice plants. Based on their abundance, measured in reads per kilobase of exon model per million mapped reads (RPKM), 508 (4.9%), 3,786 (36.5%) and 6,068 (58.6%) of the 10,362 common genes were expressed at high (RPKM >100), medium (10 < RPKM ≤100) and low (RPKM ≤10) levels, respectively. The results indicated that the majority of the expressed genes had low or median abundance.

Transgenesis induced smaller transcriptome variation than MAB breeding

A screening of differentially expressed genes (DEGs) revealed 984 DEGs (739 up-regulated and 245 down-regulated) in DXT vs. D62B, 1413 DEGs (938 up- and 475 down-regulated) in DXB vs. D62B, and 2599 DEGs (2109 up- and 490 down-regulated) in MH86 vs. D62B

Table 1 Major agronomic traits of four rice plants studied

Sample	Lesion length (cm)	Plant height (cm)[a]	Tillers	Panicle length (cm) [b]	Seed ratio (%)	1000-grains weight (g)
D62B	10.0±2.7	88.4±4.3	11±3	23.9±1.2	94±3.0	25.8±2.8
DXT	0.4±0.3#	86.3±2.0	10±1	24.2±0.9	93±4.7	25.8±2.2
DXB	1.2±0.4#	85.5±3.4	12±2	23.8±1.1	93±2.9	26.0±1.7
MH86	11.3±1.9	113.1±2.8*	10±2	27.0±1.7*	80±5.8*	27.1±1.0

Data included are averages of at least 5 individual plants from which 3 infected leaves (a) or panicles (b) were scored. * represents a significant levels of $p < 0.01$ beween D62B and MH86 (t-test). # represents the significant levels of $p < 0.01$ in DXT and DXB with D62B as the control.D62B: the recipient control; DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; MH86: another rice variety.

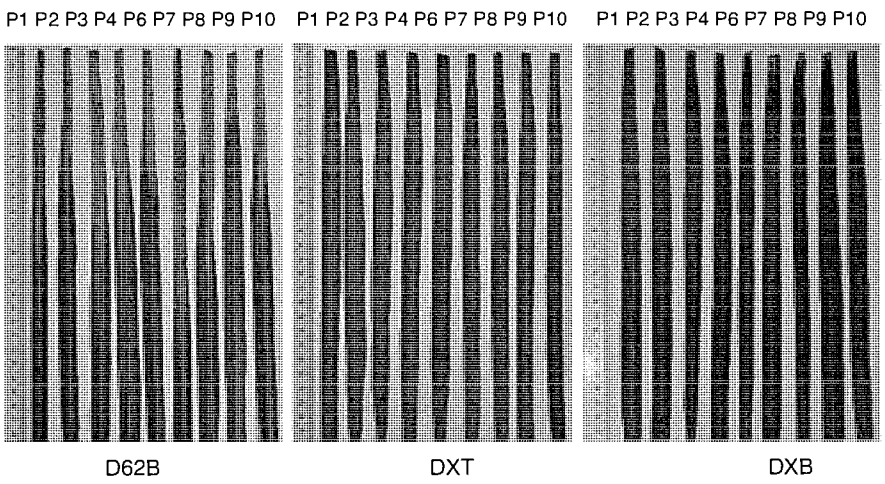

Figure 1 Bacterial blight resistance spectrum analysis of D62B, DXT and DXB. Nine *Xoo* strains from Philippine (P1-P10) were used to innoculate the rice leaves in the high tillering stage. The shorter the yellow portion on a leaf is, the higher plant resistance to *Xoo* infection. DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; D62B: the common parental line of DXT and DXB.

(Additional file 1: Table S1). Eight up-regulated and two down-regulated genes in DXT were further analyzed by qRT-PCR (Additional file 2: Figure S1). Eight of these ten genes profiled exhibited expression consistent with the sequencing data, confirming the results from Next-Gen sequencing; seven of the eight up-regulated genes were indeed significantly up-regulated and one of the two down-regulated gene was significantly down-regulated in DXT (Additional file 2: Figure S1). The discrepancy between the results of deep sequencing and qRT-PCR on two genes assayed may be due to the technical difference between the two techniques [24,25].

Remarkably, the transcriptome variations between the two rice varieties D62B and MH86, measured by the number of DEGs between the two, was the largest, while the transcriptome variations between DXT and DXB was the smallest among the comparisons. More importantly, transgenesis of *Xa21* induced 43.6% less transcriptome variation than MAB breeding. The differences among such transcriptome variations were displayed in Figures 2A to 2C. Overall, the results showed that the transcriptome change due to the introduction of *Xa21* was significantly smaller than that by MAB breeding and the variation between the transcriptomes of two rice varieties D62B and MH86.

Furthermore, a clustering analysis of DEGs (see Methods) showed that the rice plants with close genetic background tended to group together (Figure 2D). As expected, DXT and DXB were closer to their parental line D62B than they were to MH86. Despite the difference in the two breeding strategies, the transgenic line DXT was more closely related to D62B than the MAB line DXB (Figure 2D). These results revealed that transgenesis and MAB breeding did not alter the transcriptome more significantly than another natural rice variety (i.e., MH86). As far as transcriptome variation is concerned, these results suggest that the transgenic rice is closer to its parental line than the MAB rice is to the same parental line.

Transgenesis had less impact on molecular and cellular functions than MAB breeding

In order to appreciate the possible consequences of introducing *Xa21* into D62B by the two breeding strategies, GO functional analysis was performed on the DEGs between DXT and D62B and between DXB and D62B (Figure 3). In total, 31 and 35 functional categories were enriched among the DEGs between DXT and D62B and between DXB and D62B, respectively. Surprisingly, 30 of these enriched functional categories were common (Figure 3B),

Table 2 Statistics of RNA sequencing data, generated by Illumina sequencing, on four rice plants

	D62B	DXT	DXB	MH86
Raw reads	5,801,661	6,161,959	6,101,257	6,104,443
Qualified reads	5,677,504	6,026,127	5,962,165	5,954,422
Unambiguous tag mapping to Gene	2,330,333	2,724,696	2,549,799	2,665,554
Unambiguous tag-mapped Genes	12,048	12,686	12,526	13,349
Unambiguous tag/gene	193.4	214.8	203.6	199.7

D62B: the recipient control; DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; MH86: another rice variety.

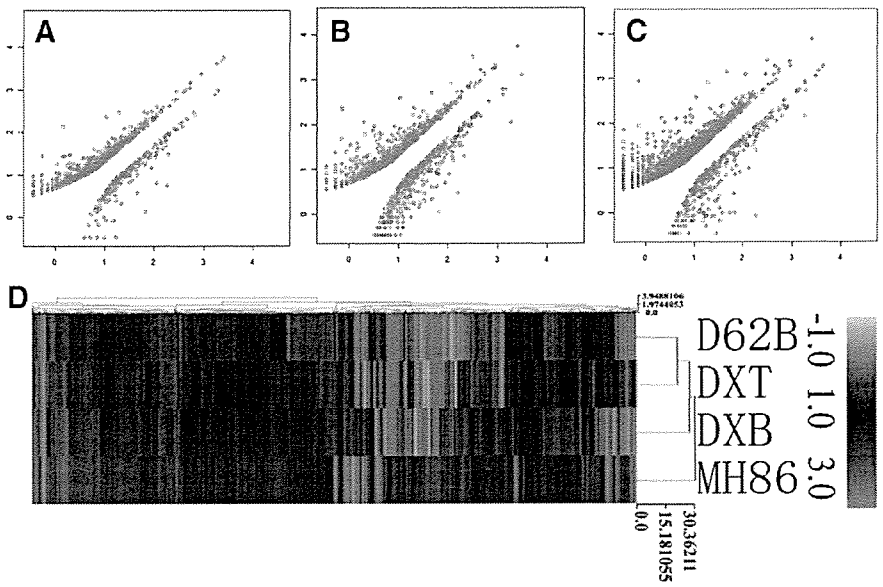

Figure 2 Transcriptome variations and expression relationships among four rice plants studied. Scatter plotes of transcriptome comparison of **(A)** DXT vs. D62B, **(B)** DXB vs. D62B, and **(C)** MH86 vs. D62B, where the horizontal and vertial axes represent the digital gene expression abundance after log10 transformation, and dots in red represent differentially expressed genes. **(D)** Clustering results on the differentially expressed genes of DXT, DXB and MH86 with respect to D62B. Each column in the figure refers to a gene. Digital expression abundance, after log10 transformation, is used in the plot. DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; D62B: the common parental line of DXT and DXB; MH86 :another rice variety.

indicating that most of the DEGs in DXT and DXB have the same or similar functions. The single functional category specific to the DEGs in DXT is the "extracellular region part", and the four categories unique to the DEGs in DXB include "antioxidant", "enzyme regulator", "molecular transducer" and "death" (Figure 3B). Although it is unclear what phenotypic consequence, if any, that these DEGs may lead to, it is evident that the transcriptome variation caused by the DEGs in DXT imposed less cellular perturbation than that by the DEGs in DXB. In other words, the transgenesis of *Xa21* induced less disturbance to the molecular and cellular machineries than the MAB breeding did.

To further understand the effects of the two different breeding strategies, we analyzed the genes expressed exclusively in DXT and exclusively in DXB, along with the ones expressed exclusively in D62B. We identified 758, 821 and 404 genes that were exclusively expressed in DXT, DXB and D62B, respectively (Figure 4A). The DXT-, DXB- and D62B-specific genes were enriched in 21, 33 and 26 GO functional categories, respectively (Figure 4B). Interestingly, the 21 categories enriched in the DXT-specific genes were also enriched in the DXB- and D62B-specific genes, and the 26 categories enriched in the D62B-specific genes were enriched in the DXB-specific genes as well (Figure 4B). This result indicated that although there were a large number of genes expressed exclusively in these plants, they carried the same or similar molecular and/or cellular functions, reflecting the functional elasticity and robustness of these genes. On the other hand, comparing with the genes expressed in the parental line, the genes expressed exclusively in DXT lost functions in 5 categories, while the genes expressed exclusively in DXB gained additional functions in 7 categories. It is possible that these lost and gained functions may be compensated for by the genes commonly expressed in these three rice plants. Nevertheless, this plant-specific gene function analysis also indicated that the perturbation to the molecular and cellular functions induced by transgenesis has a smaller or compatible scale than by MAB breeding.

Genes exclusively expressed in transgenic rice functioned in pathogen defense

Among the DXT-specific genes, 52 encoded transposons and retrotransposons, supporting the notion that mobile elements are typically activated during genetic transformation [26]. In addition, 40 DXT-specific genes encoded protein kinases, receptor-like protein kinases (RLKs), or OsWAK receptor-like protein kinases that have been implicated in stress/defense signaling-signal perception and signal transduction, which are consistent

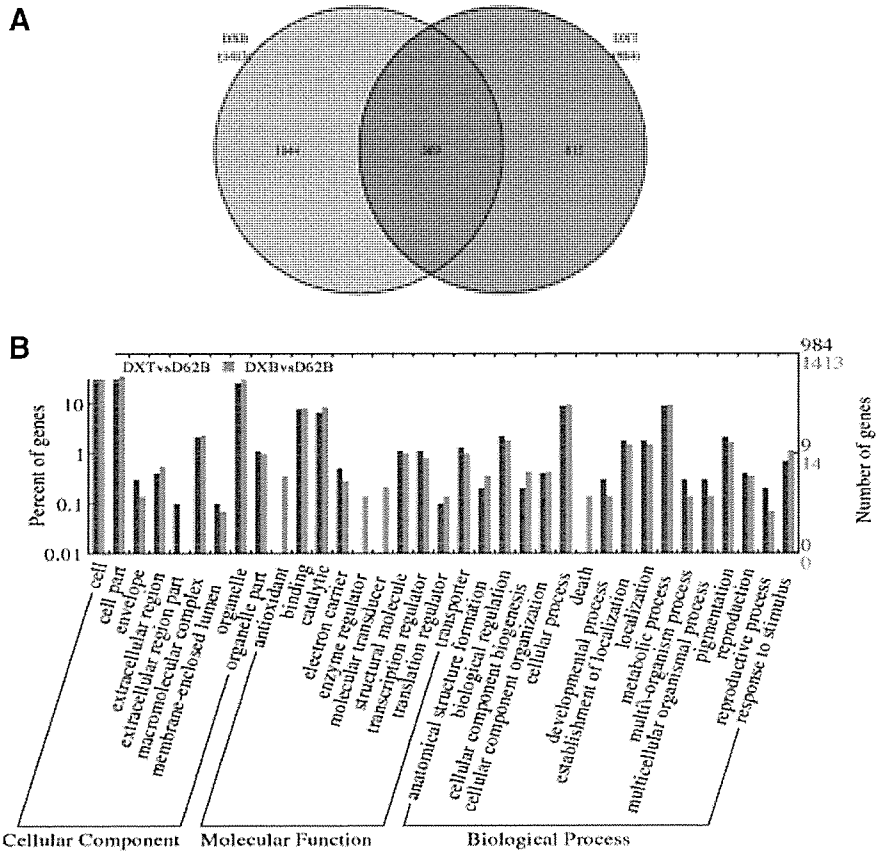

Figure 3 Comparative analysis of the DEGs in DXT and DXB with respect to D62B. (A) Venn diagram for the numbers of DEGs in DXT and DXB. **(B)** GO functional categories of the DEGs in DXT and DXB. The y-axis on the right indicates the number of genes in a category, and the y-axis on the left is the percentage of genes to be analyzed in a category. DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; D62B: the common parental line of DXT and DXB.

with the existing results that show that protein phosphorylation and dephosphorylation play an important role in *Xa21* induced gene regulation in response to pathogen invasion [27-32]. The remaining DXT-specific genes included 14 genes encoding transporter (including potassium, sulfate, metal cation, ABC, and ctr copper transportor), 16 genes encoding putative transcription factors (including 6 AP2 domain containing proteins, 1 ethylene-responsive transcription factor (ERF), and 9 MYB family transcription factors), 8 DXT-specific expressed genes encoding resistance or resistance-like proteins (including 5 NBS-LRR type of proteins such as LR10 [33], RPM1 [34] and I2GA-SH194-2 [35]) and 2 genes encoding DNA methylation related proteins. DNA methylation has been reported to affect rice resistance response [36-38]. The AP2/ERF family is a large family of plant specific transcription factors that share a well-conserved DNA-binding domain that has been reported to activate the expression of abiotic stress-responsive genes via specific binding to the dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element in their promoters [39]. The MYB family has key transcription factors for controlling plant development and response to biotic and abiotic stresses [40]. Taken together, these results strongly suggested that transgenesis of *Xa21* affected mainly those genes that were involved in pathways related to stress response.

Compatible observations were also made on the genes expressed exclusively in DXB, i.e., these genes had the same or similar functions as those exclusively expressed in DXT. Among the DXB-specific genes, 58 encoded transposons and retrotransposons and 24 encoded protein kinases, receptor-like protein kinases (RLKs) or OsWAK receptor-like protein kinases. The remaining DXB-specific genes included 10 genes that encode transporters, 10 that encode putative transcription factors (including 3 MYB domain containing proteins), 13 that encode resistance or resistance-like proteins (including 7 NBS-LRR type of proteins), and 3 that encode DNA methylation-related proteins.

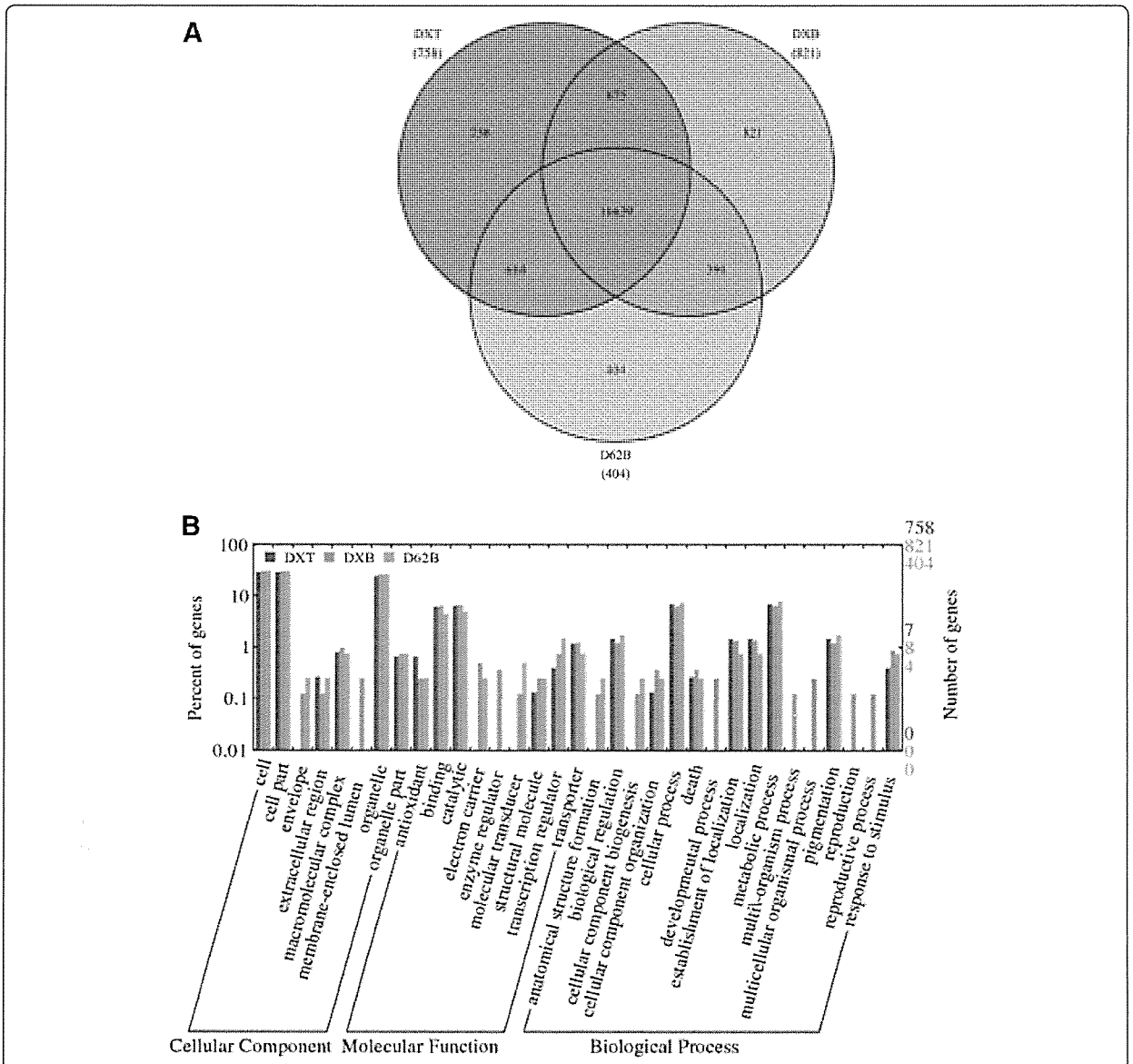

Figure 4 Comparative analysis of the genes exclusively expressed in transgenic line DXT, MAB breeding line DXB and their parental line D62B. (A) Venn diagram for the numbers of genes exclusively expressed in DXT, DXB and D62B. **(B)** GO functional categories of the genes in DXT and DXB. The *y*-axis on the right indicates the number of genes in a category, and the *y*-axis on the left is the percentage of genes to be analyzed in a category. DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; D62B: the common parental line of DXT and DXB.

Most pathways perturbed by transgenesis were also disturbed by MAB breeding

Our final step of the comparative study of transcriptome variation was a pathways analysis on the DEGs in DXT and the DEGs in DXB. To this end, the DEGs in these two plants were mapped to the pathways in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database and searched for significantly enriched KEGG pathways that were potentially affected by gene expression variation [41]. Among the DEGs in DXT and DXB, 476 and 700 were annotated on KEGG pathways, respectively. In comparison, more DEGs, i.e., 1331, between MH86 and D62B were mapped to KEGG pathways. With a statistical significance of $p \leq 0.05$; 11, 16, and 20 signaling pathways were abundant among the DEGs of DXT, DXB and MH86, respectively (Table 3, Additional file 1: Table S2). Seven of the 11 significantly enriched pathways in the transgenic rice were also significantly affected in the MAB rice. The rest four pathways (including Carotenoid biosynthesis, Regulation of autophagy, Arachidonic acid metabolism and Anthocyanin biosynthesis) were also detected in the MAB rice but at a less significant level above the cutoff

Table 3 Pathways enriched in differentially expressed genes in the transgenic line DXT, MAB breeding line DXB and a rice variety MH86 with respect to recipient line D62B

Pathways	DXT	DXB	MH86
Ribosome	√	√	√
Flavonoid biosynthesis	√	√	√
Vitamin B6 metabolism	√	√	√
Biosynthesis of phenylpropanoids	√	√	
Benzoxazinoid biosynthesis	√	√	
Flavone and flavonol biosynthesis	√	√	
Oxidative phosphorylation	√	√	
Carotenoid biosynthesis	√		
Regulation of autophagy	√		
Arachidonic acid metabolism	√		
Anthocyanin biosynthesis	√		
Zeatin biosynthesis		√	√
ABC transporters		√	√
Inositol phosphate metabolism		√	√
Phenylalanine metabolism		√	
Glycine, serine and threonine metabolism		√	
Phagosome		√	
Biosynthesis of secondary metabolites		√	
Circadian rhythm - plant		√	
Sulfur metabolism		√	
Metabolism of xenobiotics by cytochrome P450			√
Spliceosome			√
Endocytosis			√
Biosynthesis of plant hormones			√
Biosynthesis of unsaturated fatty acids			√
Glutathione metabolism			√
Linoleic acid metabolism			√
Biosynthesis of terpenoids and steroids			√
alpha-Linolenic acid metabolism			√
Phosphatidylinositol signaling system			√
Limonene and pinene degradation			√
Non-homologous end-joining			√
Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid			√
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis			√

Listed are significantly enriched KEGG terms with cutoff of $p \leq 0.05$. "√" means significantly affected pathway. Note that the four pathways enriched in DXT but not listed under DXB in the table were also enriched in DXB at a less significant level (with p-values from 0.06 to 0.88).

threshold (Additional file 1: Table S3). Specifically, "Carotenoid biosynthesis", "Regulation of autophagy", "Arachidonic acid metabolism", and "Anthocyanin biosynthesis" had p-values of 0.22, 0.88, 0.06, and 0.67 respectively in the MAB rice.

Discussion

MAB breeding is a well accepted breeding technology for production of safe crops. Comparing crops produced from transgenesis and MAB breeding thus offers a viable approach to assessing the potential impact that transgenic crops might have on human health. As a general principle, substantial equivalence [11] can be followed to assess the safety of transgenic crops. Using this principle as a guideline and yardstick, various aspects of substantial equivalence, e.g., molecular, biological, chemical and nutritional equivalence, can be examined. Taking a systems-biology perspective, we focused on molecular and biological equivalences between transgenic crops and plants from MAB breeding in the current study. This was done by contrasting transcriptome variations of a transgenic rice and a rice by MAB breeding. As a caveat, any results from such a study should not be interpreted to directly address the safety of transgenic plants. But rather, in addition to gaining insights, at a genome scale, into the biological processes and pathways that might be perturbed by transgenesis, such results can be used to pave the way for further study of chemical and nutritional equivalence.

Adopting the above view point and taking *Xa21*, the most widely used resistance gene to rice bacterial leaf blight, as the target gene, we studied the phenotype and transcriptome differences between the transgenic and MAB rice carrying *Xa21*. Included in our expriment system were a transgenic rice (i.e., DXT) and a MAB rice (i.e., DXB) that were developed from the same parental line D62B. DXT was identical to D62B except the key *Xa21* gene. The genetic makeup of DXB, through careful selection over six generations of backcrossing to D62B, was almost identical to that of D62B. Therefore, with exception of the specific breeding techniques used, DXT and DXB carried nearly identical genetic materials, making them ideal for the comparison of the two breeding techniques. A critical component of our experiments and analysis was another natural rice variety, MH86, which does not carry *Xa21*. Here, MH86 played the role of a"control", when compared with D62B, to reveal the natural range of transcriptome variation between the two natural rice plants.

The comparison of transgenic and MAB rice was done at two levels. At the physiological level, we confirmed through extensive, although laborious, in-field screening of various morphological properties to ensure that DXT and DXB were morphologically equivalent or similar.

Due to genetic diversity, the morphological difference between natural varieties are typically greater than that of transgenesis, as we saw in our comparison between MH86 vs. D62B and DXT vs. D62B. At the molecular and cellular level, we carefully studied transcriptome variations as well as function and pathway alterations in DXT and DXB. The scale of transcriptome variations caused by transgenesis was smaller than that by MAB breeding. More importantly, the molecular and cellular functions that may be affected by transgenesis were also affected by MAB breeding, and the majority of pathways perturbed in the transgenic rice were also distorted in the MAB rice. In stark contrast, transcriptome and function variations between MH86 and D62B, which can be regarded as natural variations, were substantially larger than that between DXT and D62B. In short, the variations caused by transgenesis were smaller than that of MAB breeding and were within the natural range of variations.

The results from the current study were in agreement with that in the literature on transgenic wheat and maize. For example, Gregersen *et al.* compare the gene expression profiles of wildtype and transgenic wheat expressing an *aspergillus fumigatus* phytase [42]. The results show that the expression profiles of the two plants are not significantly different. A similar study of transgenic wheat, which carries additional High Molecular Weight subunit genes, reaches the same conclusion that transgenesis has less impact on the transcriptome of wheat grain than conventional breeding [10]. Further, Coll *et al.* uses microarrays to compare the expression profiles of commercial maize variety MON810 and near-isogenic varieties in leaves *in vitro* and also field cultured plants of AristisBt/Aristis and PR33P67/PR33P66 [43,44]. The target gene is *Bt*, a gene non-native to maize encoding insecticidal crystal protein of the soil *bacterium Bacillus thuringiensis* (*B.t.*). The results show that gene expression profiles of MON810 and comparable non-GM maize varieties are more similar to that of conventional lines and natural variation.

On the other hand, the experiment designs of previous studies are far from perfect. In [10], comparative analysis of the transcriptomes of three wheat lines, including a parental line, a conventional breeding line and a transgenic line, reaches the conclusion that transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. However, several additional genes or sequences, including the marker gene (*Bar* gene), the reporter gene (*uidA* gene), and sequences derived from the bacterial plasmid, are present in the transgenic line. The transgenic line, referred to as a "clean fragment" line, still contains the *bar* gene. In fact, selective markers, such as *bar*, are the main concern of biosafety of transgenic crops which induce specific pleiotropic effects [45]. In the studies reported in [42-44], foreign transgenes are used. The current study overcame these issues. First, the target gene *Xa21* was cloned from *Oryza longistaminata*, which is native to rice. Second, other than *Xa21* sequence, DXT did not carry any additional sequences such as that for marker genes and reporter genes. Third, unlike the previous studies where a target gene is under the control of a foreign promoter [42,46], in the current study *Xa21* in DXT carried its own promotor. As a result of these factors, the results from our analysis were specific to the functions of *Xa21* rather than some artifacts of genes foreign to rice.

More importantly, our analysis expanded beyond transcriptome profiling to include analyses of gene functions and signaling pathways that might be altered by the introduction of *Xa21*. Our results showed that most of the molecular and cellular functions affected by transgenesis were influenced by MAB and functional categories that affected by MAB were more than those that affected by transgenesis. Analyzing the pathways perturbed by transgenesis and MAB showed that majority pathways altered in transgenic rice were also distorted in MAB rice. The bigger difference between the transcriptome variations of DXB and D62B could be attributed to two factors. Firstly, the transgenic rice analyzed in our study was carefully selected to have favorable properties for the purpose of the study. In particular, DXT was a single-copy, marker-free, and homozygous *Xa21* transgenic line and also had consistent agronomic traits similar to that of the parental line D62B. The genetic makeup of DXT was identical to D62B with the exception of the *Xa21* gene. Secondly, although six backcrosses have been used to eliminate linkage drag, a few unwanted donor chromosomal segments could still have been retained in DXB when *Xa21* was introgressed into D62B. Therefore, the transcriptome of DXT was closer to that of D62B than DXB to D62B.

Thanks to Next-Gen sequencing, the gene profiling experiments of our study were genome wide and provided a high resolution [47]. In contrast, the previous studies focus on a limited number of genes using microarray profiling which is less accurate and restricted to annotated genes [10,42-44,46,48]. Such a large scale functional analysis performed in the current study has never been attempted before and the functional analyses provided deep insight into the functional equivalence between the transgenesis and MAB breeding.

Conclusions

MAB breeding and transgenesis are two most popular breeding techniques for producing plants of favorable traits. As a newer biotechnology, transgenesis has made visible contributions to increase yield of staple crops. While plants produced by MAB breeding have already been widely accepted, transgenic plants are facing the challenge regarding safety on human health. In the

current study, these two distinct breeding techniques was closely compared. The analysis focused on transcriptome variations in rice plants generated from these two breeding techniques and on that of two natural rice plants as a baseline of the comparison. The study combined careful assessment of agronomic traits, transcriptome profiling by Next-Gen sequencing, and functional and pathway analyses. Two important conclusions can be drawn from the results. First, transcriptome variation caused by transgenesis is significantly smaller than that by MAB breeding and is within the range of natural variation. Second, the functional categories of differentially expressed genes due to these two breeding techniques and the pathways perturbed by these techniques are not substantially different. These results suggest the transgenic rice and rice from MAB breeding that were compared in the current study are substantially equivalent at the molecular and biological levels. The data and results can be used to study chemical and nutritional equivalence of rice generated by transgenesis and MAB breeding.

Methods

Resistance analysis of rice varieties

Both D62B and MH86 are *indica* rice and widely used as parental lines of hybrid rice in China. The transgenic line DXT with *Xa21* was developed from D62B in our previous study [22]. The MAB line DXB with *Xa21* was developed by backcrossing to D62B in the current study. When the rice grew to the high tillering stage, five to seven fully expanded leaves were inoculated with *Xoo* using the leaf-clipping method [49]. The cultures were grown on PSA (Potato-Sugar-Agar) medium (potato, 300 g/L; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g/L; $Na_2HPO_4 \cdot 12H_2O$, 2.0 g/L; sugar, 15 g/L; agar 15 g/L) at 28°C for 3 days. Inoculums were prepared by suspending the bacterial cells in sterile water and adjusting the concentration to about 10^9 cells per milliliter. Phenotype scoring was carried out at 15 days post innoculation (dpi).

Molecular analysis of transgenic and MAB breeding plants

Genomic DNA was isolated from fresh rice leaf tissue using the cetyltrimethylammonium bromide protocol. Transgenic plants were validated by polymerase chain reaction (PCR) and Southern blotting. The primers which were used for molecular analysis of transgenic plants were described detailedly in Gao et al. (2011). MAB breeding plants were validated by PCR using the same primers as transgenic plants.

Quantitative PCR

DNaseI-treated RNA was used for fist strand cDNA synthesis using M-MLV reverse transcriptase (Promega) and oligo$(dT)_{15}$ according to the manufacture's protocols. Specific pairs of primers for SYBR-green detection and quantification of selected DEGs were designed using Primer Express software follwing the primer design guidance. The primer sequences are listed in Additional file 1: Table S5. The endogenous actin gene was run in parallel as control PCR reaction and the untransfromed receptor line was used as the calibrator to normlize the relative expression levels of the target DEG. Triplicate samples for each tested line were prepared for real-time PCR assays. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes of gene expression [50]. PCR reactions were performed using the TranStart Green qPCR SupMix reagent (TransGen Biotech, Inc.) on BioRad CFX96 PCR system.

Illumina sequencing

Sequencing and preliminary data acquisition were finished by Beijing Genomics Institute. The experimental process includes sample preparation and sequencing. The main reagents and supplies used included Illumina Gene Expression Sample Prep Kit and Solexa Sequencing Chip (flowcell), and the main instruments used included Illumina Cluster Station and Illumina HiSeq™ 2000 Systerm. Ten leaves randomly selected from ten individual plants of each tested line were harvested and pooled together for RNA extraction. Total RNA was isolated using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacture's protocols and purified using an RNeasy Plant Kit (Qiagen, Hilden, Germany). The integrity and purity of RNA samples were determined by gel electrophoresis and OD 260/280 nm absorption ratios and the RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE, USA).

Processing of sequencing data

Raw sequencing data in image were transformed by base calling into raw reads. Raw reads were then transformed into clean reads after removal of such reads as 3' adaptor sequences, empty reads, low quality reads, reads which were too long or too short, and reads with only one copy (probably due to sequencing error). Clean reads that were mapped to reference sequences from multiple genes were removed. The remaining clean reads were designated as unambiguous clean or qualified reads.

Gene expression and differentially expressed genes

The number of qualified reads for each gene was tallied and normalized to TPM (number of transcripts per million qualified reads), which was then used as the digital gene expression abundance of the gene. Genes that were differentially expressed, referred to as DEGs, across two plants were identified using the criterion of at least 2-fold change to digital expression abundance and with a cut-off of False Discovery Rate (FDR) no greater than 0.001.

Cluster analysis

All DEGs in DXT, DXB and MH86, with respect to D62B, were aggregated. The $\log_{10}$-transformed TPM values of the genes in the combined set of DEGs were used to cluster the DEGs by hierarchical clustering with euclidean distance and average linkage. The clustering was done and the result was plotted using Multi Experiment Viewer (MeV) [51].

Gene ontology and KEGG pathway analyses

GO functional category analysis was performed separately on DEGs and genes exclusively expressed in a specific plant, e.g., DXT or DXB. This analysis was done by mapping the set of genes of interest (DEGs or plant-specific genes) to the terms in plant GOSlim Ontologies from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/annotation_pseudo_goslim.shtml). WEGO (Web Gene Ontology Annotation Plot [52]) tool was used to plot GO annotation results. For pathway enrichment analysis, all DEGs involved in a comparison were mapped to the terms in the KEGG database to identify significantly enriched KEGG terms. The Kobast 2.0 [53] tool for pathway enrichment analysis was used with a cutoff of p value no greater than 0.05.

Availability of supporting data

The gene expression profiling data on the four rice plants from Illumina HiSeq™ 2000 has been deposited into NCBI/GEO database under the accession number SRA061839.

Additional files

Additional file 1: Table S1. Differentially expressed genes in transgenic rice line DXT, MAB breeding line DXB and a rice variety MH86 with respect to the expression in D62B. **Table S2.** Pathways enriched in the differentially expressed genes in transgenic rice line DXT with respect to the expression in the parental line D62B. **Table S3.** Pathways enriched in the differentially expressed genes in MAB breeding line DXB with respect to the expression in the parental line D62B. **Table S4.** Pathways enriched in the differentially expressed genes in rice variety MH86 with respect to the expression in D62B. **Table S5.** Primers used in qRT-PCR analysis of ten differentially expressed genes.

Additional file 2: Figure S1. Real-time PCR analysis of 10 differentially expressed genes. Expression patterns of LOC_Os10g41838, LOC_Os08g20500, LOC_Os11g39320, LOC_Os09g26560, LOC_Os11g39190, LOC_Os10g30970, LOC_Os09g36420 and LOC_Os09g19229 are consistent with digital gene expression abundance from deep sequencing. Triplicate samples for each tested lines were used for real-time PCR assays. Actin gene was used as internal controls and the recipient D62B was used as the calibrator. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression. The vertical axes represent the relative expression levels of target genes with respect to the control of actin gene. DXT: transgenic line with *Xa21*; DXB: MAB line with *Xa21*; D62B: the common parental line of DXT and DXB.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LG carried out the molecular analysis and field score of rice varieties, analyzed the results and contributed to the drafting of the manuscript; YC and GL participated in data analysis; ZX performed qPCR experiments; GJ analyzed the leaf blight resistance of transgenic and MAB rice; WZhang conceived the research topic, analyzed the results and wrote the paper; WZhai conceived the study, designed the experiments and coordinated the research. All authors read and approved the final manuscript.

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ISAAA
INTERNATIONAL SERVICE FOR THE ACQUISITION OF AGRI-BIOTECH APPLICATIONS

EXECUTIVE SUMMARY

BRIEF 49

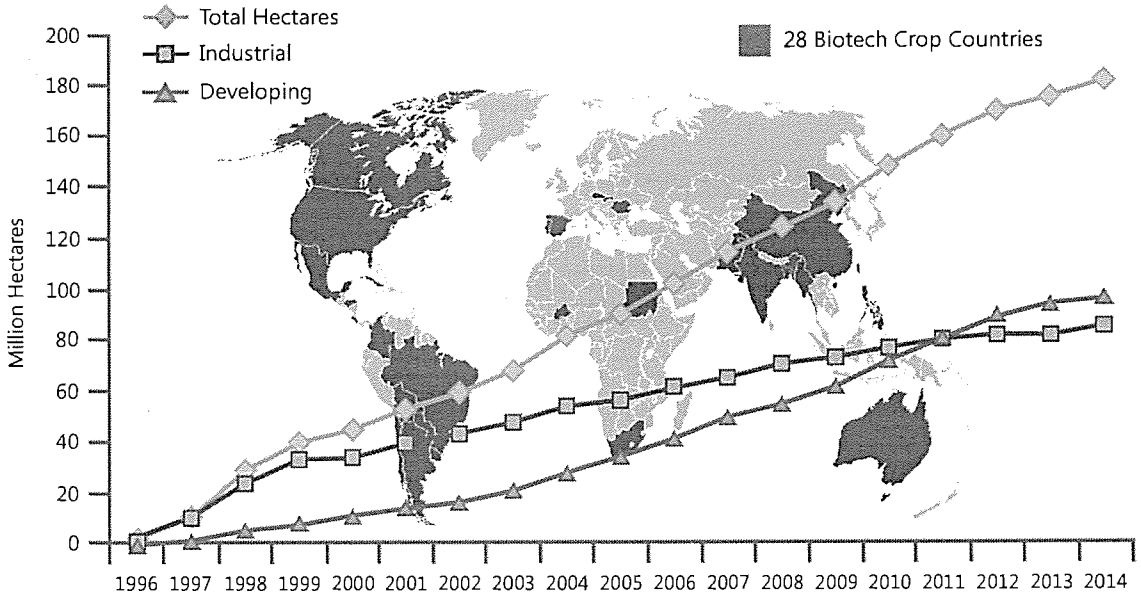
Global Status of Commercialized Biotech/GM Crops: 2014

By

Clive James
Founder and Emeritus Chair of ISAAA

Dedicated to the late Nobel Peace Laureate, Norman Borlaug,
founding patron of ISAAA, on the centenary of his birth, 25 March 2014

GLOBAL AREA OF BIOTECH CROPS
Million Hectares (1996-2014)



A record 18 million farmers, in 28 countries, planted 181.5 million hectares (448 million acres) in 2014, a sustained increase of 3 to 4% or 6.3 million hectares (~16 million acres) over 2013.

Source: Clive James, 2014.

No. 49 - 2014

AUTHOR'S NOTE:

Global totals of millions of hectares planted with biotech crops have been rounded off to the nearest million and similarly, subtotals to the nearest 100,000 hectares, using both < and > characters; hence in some cases this leads to insignificant approximations, and there may be minor variances in some figures, totals, and percentage estimates that do not always add up exactly to 100% because of rounding off. It is also important to note that countries in the Southern Hemisphere plant their crops in the last quarter of the calendar year. The biotech crop areas reported in this publication are planted, not necessarily harvested hectarage in the year stated. Thus, for example, the 2014 information for Argentina, Brazil, Australia, South Africa, and Uruguay is hectares usually planted in the last quarter of 2014 and harvested in the first quarter of 2015 with some countries like the Philippines having more than one season per year. Thus, for countries of the Southern hemisphere, such as Brazil, Argentina and South Africa the estimates are projections, and thus are always subject to change due to weather, which may increase or decrease actual planted hectares before the end of the planting season when this Brief has to go to press. For Brazil, the winter maize crop (safrinha) planted in the last week of December 2014 and more intensively through January and February 2015 is classified as a 2014 crop in this Brief consistent with a policy which uses the first date of planting to determine the crop year. ISAAA is a not-for-profit organization, sponsored by public and private sector organizations. All biotech crops hectare estimates reported in all ISAAA publications are only counted once, irrespective of how many traits are incorporated in the crops. Importantly, all reported biotech crop hectares are for officially approved and planted products, and do not include unofficial plantings of any biotech crops. At the time when this Brief went to press, estimates of economic benefits, productivity, landsaving, and carbon data were provisional for the period 1996-2013 (Brookes and Barfoot, 2015, Forthcoming); and pesticide data is for 1996-2012 (Brookes and Barfoot, 2014). Details of the references listed in the Executive Summary are found in the full Brief 49.

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ISAAA prepares this Brief and supports its free distribution to developing countries. The objective is to provide information and knowledge to the scientific community and society on biotech/GM crops to facilitate a more informed and transparent discussion regarding their potential role in contributing to global food, feed, fiber and fuel security, and a more sustainable agriculture. The author takes full responsibility for the views expressed in this publication and for any errors of omission or misinterpretation.

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EXECUTIVE SUMMARY

Global Status of Commercialized Biotech/GM Crops: 2014

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EXECUTIVE SUMMARY

Global Status of Commercialized Biotech/GM Crops: 2014

By

Clive James, Founder and Emeritus Chair of ISAAA

Dedicated to the late Nobel Peace Laureate, Norman Borlaug, founding patron of ISAAA, on the centenary of his birth, 25 March 2014

Remarkably, in 2014 global biotech crop hectarage continued to grow for the 19th consecutive year of commercialization; 18 million farmers in 28 countries planted more than 181 million hectares in 2014, up from 175 million in 27 countries in 2013. Notably, Bangladesh, a small poor country approved Bt brinjal/eggplant for the first time on 30 October 2013, and in record time – less than 100 days after approval – small farmers commercialized Bt brinjal on 22 January 2014. Innate™ potato, another food crop, was approved in the US in November 2014. It has lower levels of acrylamide, a potential carcinogen in humans, and suffers less wastage from bruising; potato is the fourth most important food staple in the world. A safer product and decreased wastage in a vegetatively propagated and perishable crop, can contribute to higher productivity and food security. Also in November 2014, a new biotech alfalfa (event KK179) with up to 22% less lignin, which leads to higher digestibility and productivity, was approved for planting in the US. The first biotech drought tolerant maize, planted in the US in 2013 on 50,000 hectares increased over 5 fold to 275,000 hectares in 2014 reflecting high acceptance by US farmers. Importantly, a new 2014 comprehensive global meta-analysis, on 147 published biotech crop studies over the last 20 years worldwide confirmed the significant and multiple benefits that biotech crops have generated over the past 20 years, 1995 to 2014; on average GM technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. These findings corroborate earlier and consistent results from other annual global studies which estimated increases in crop productivity valued at US$133.3 billion for the period 1996-2013.

Introduction

This Executive Summary focuses on the highlights of ISAAA Brief 49, details of which are presented and discussed in the full Brief, "Global Status of Commercialized Biotech/GM Crops: 2014".

Biotech crop hectarage increases yet again in 2014, in their 19th consecutive year of commercialization.

A record 181.5 million hectares of biotech crops were grown globally in 2014, at an annual growth rate of between 3 and 4%, up 6.3 million hectares from 175.2 million hectares in 2013. This year, 2014, was the 19th year of commercialization, 1996-2014, when growth continued after a remarkable 18 consecutive years of increases every single year; notably 12 of the 18 years were double-digit growth rates.

Biotech crops are the fastest adopted crop technology in the world.

The global hectarage of biotech crops has increased more than 100-fold from 1.7 million hectares in 1996 to181.5 million hectares in 2014 – this makes biotech crops the fastest adopted crop technology in recent times. This impressive adoption rate speaks for itself, in terms of its sustainability, resilience and the significant benefits it delivers to both small and large farmers as well as consumers.

A new and rigorous 2014 comprehensive global meta-analysis of 147 published biotech crop studies over the last 20 years, confirmed the significant and multiple benefits that biotech crops have generated over the past 20 years (1995 to 2014).

The meta-analysis was performed by Klumper and Qaim (2014) on 147 published biotech crop studies conducted during the last 20 years, using primary data from farm surveys or field trials world-wide and reporting impacts of GM soybean, maize, or cotton on crop yields, pesticide use, and/or farmer profits. The meta-analysis concluded that ***"on average GM technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. Yield gains and pesticide reductions are larger for insect-resistant crops than for herbicide-tolerant crops. Yield and profit gains are higher in developing countries than in developed countries."*** The authors concluded that ***"this meta-analysis confirms that in spite of impact heterogeneity, the average agronomic and economic benefits of GM crops are large and significant. Impacts vary especially by modified crop trait and geographic region. Yield gains and pesticide reductions are larger for IR crops than for HT crops. Yield and farmer profit gains are higher in developing countries than in developed countries. Recent impact studies used better data and methods than earlier studies, but these improvements in study design did not reduce the estimates of GM crop advantages. Rather, NGO reports and other publications without scientific peer review seem to bias the impact estimates downward. But even with such biased estimates included, mean effects remain sizeable."*** The authors of the meta-analysis note that it reveals ***"robust evidence of GM crop benefits for farmers in developed and developing countries."*** It is noteworthy that the findings of this meta-analysis corroborates results from previous peer reviewed studies including the annual global impact study on biotech crops conducted by Brookes and Barfoot of PG Economics and regularly referenced in the Annual ISAAA Briefs.

Millions of risk-averse farmers, both large and small, world-wide, have concluded that the returns from planting biotech crops are high, hence repeat planting is virtually 100%; good returns on their investment is the critical test applied by demanding farmers when judging the performance of any technology.

In the 19 year period 1996 to 2014, millions of farmers in almost 30 countries worldwide, adopted biotech crops at unprecedented rates. The most compelling and credible testimony for biotech crops is that during the 19 year period 1996 to 2014, millions of farmers in ~30 countries worldwide, elected to make more than 100 million independent decisions to plant and replant an accumulated hectarage of more than 1.8 billion hectares exceeding 4 billion acres for the first time in 2014. This is an area equivalent to >180% the size of the total land mass of the US or China which is an enormous area. There is one principal and overwhelming reason that underpins the trust and confidence of risk-averse farmers in biotechnology – biotech crops deliver substantial, and sustainable, socio-economic and environmental benefits. Comprehensive analytical studies by many organizations including a 2011 EU

study have confirmed that biotech crops are safe and deliver substantial agronomic and environmental benefits, and result in significant reductions in pesticide usage.

28 countries, up one from 27 in 2013, grew biotech crops in 2014.

Of the 28 countries which planted biotech crops in 2014 (Table 1 and Figure 1), 20 were developing (including the new biotech crop country Bangladesh) and only 8 were industrial countries. Each of the top 10 countries, of which 8 were developing, grew more than 1 million hectares providing a broad-based worldwide foundation for continued and diversified growth in the future. More than half the world's population, ~60% or ~4 billion people, live in the 28 countries planting biotech crops.

Bangladesh, one of the smaller and poorest countries in the world, approved and commercialized Bt brinjal in record time in 2014. Vietnam and Indonesia moved towards planting their first biotech crops in 2015, for a total of 9 biotech countries in Asia.

Bangladesh approved a biotech crop (Bt brinjal/eggplant) for planting for the first time on 30 October 2013, and in record time – less than 100 days after approval – commercialization was initiated on 22 January 2014 when 20 very small farmers planted their first crop of Bt brinjal; a total of 120 farmers planted 12 hectares of Bt brinjal in 2014. This feat, which is an excellent working model for other small poor countries, could not have been achieved without strong political will and support from the government, particularly from the Minister of Agriculture Matia Chowdhury. This approval by Bangladesh is important in that it serves as an exemplary model for other small poor countries. Also, very importantly, Bangladesh has broken the impasse experienced in trying to gain approval for commercialization of Bt brinjal in both India and the Philippines.

It is noteworthy that in Asia, two other developing countries, Vietnam and Indonesia also approved cultivation of biotech crops in 2014 for commercialization in 2015 (these hectarages are not included in the data base for this Brief). Vietnam approved biotech maize and Indonesia approved a drought tolerant sugarcane for food, whilst approval for feed is pending; 50 hectares of biotech seed setts of sugarcane were planted in 2014 for planned commercialization in 2015. With the addition of Vietnam and Indonesia this would bring the total number of countries in Asia commercializing biotech crops to nine.

Increased adoption of biotech drought tolerant maize in the US

The estimated hectares of DroughtGard™ maize with event MON 87460 planted for the first time in the US in 2013 was 50,000 hectares and in 2014 was of the order of 275,000 hectares. This is equivalent to a large 5.5-fold year-to-year increase in planted hectares between 2013 and 2014, and reflects strong US farmer acceptance of the first biotech-derived drought-tolerant maize technology to be deployed globally. It is noteworthy that Event MON 87460 was donated by Monsanto to the Water Efficient Maize for Africa (WEMA), a public-private partnership (PPP) designed to deliver the first biotech drought tolerant maize to selected African countries starting 2017.

A selection of "new" biotech crops was recently approved and planned for commercialization in 2015 and beyond; they include two new food staples, potato and the vegetable brinjal (eggplant).

In 2014, the US approved the following two new biotech crops for cultivation starting in 2015; Innate™

Table 1. Global Area of Biotech Crops in 2014: by Country (Million Hectares)**

Rank	Country	Area (million hectares)	Biotech Crops
1	USA*	73.1	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
2	Brazil*	42.2	Soybean, maize, cotton
3	Argentina*	24.3	Soybean, maize, cotton
4	India*	11.6	Cotton
5	Canada*	11.6	Canola, maize, soybean, sugar beet
6	China*	3.9	Cotton, papaya, poplar, tomato, sweet pepper
7	Paraguay*	3.9	Soybean, maize cotton
8	Pakistan*	2.9	Cotton
9	South Africa*	2.7	Maize, soybean, cotton
10	Uruguay*	1.6	Soybean, maize
11	Bolivia*	1.0	Soybean
12	Philippines*	0.8	Maize
13	Australia*	0.5	Cotton, canola
14	Burkina Faso*	0.5	Cotton
15	Myanmar*	0.3	Cotton
16	Mexico*	0.2	Cotton, soybean
17	Spain*	0.1	Maize
18	Colombia*	0.1	Cotton, maize
19	Sudan*	0.1	Cotton
20	Honduras	<0.1	Maize
21	Chile	<0.1	Maize, soybean, canola
22	Portugal	<0.1	Maize
23	Cuba	<0.1	Maize
24	Czech Republic	<0.1	Maize
25	Romania	<0.1	Maize
26	Slovakia	<0.1	Maize
27	Costa Rica	<0.1	Cotton, soybean
28	Bangladesh	<0.1	Brinjal/Eggplant
	Total	**181.5**	

* 19 biotech mega-countries growing 50,000 hectares, or more, of biotech crops
** Rounded off to the nearest hundred thousand

Source: Clive James, 2014.

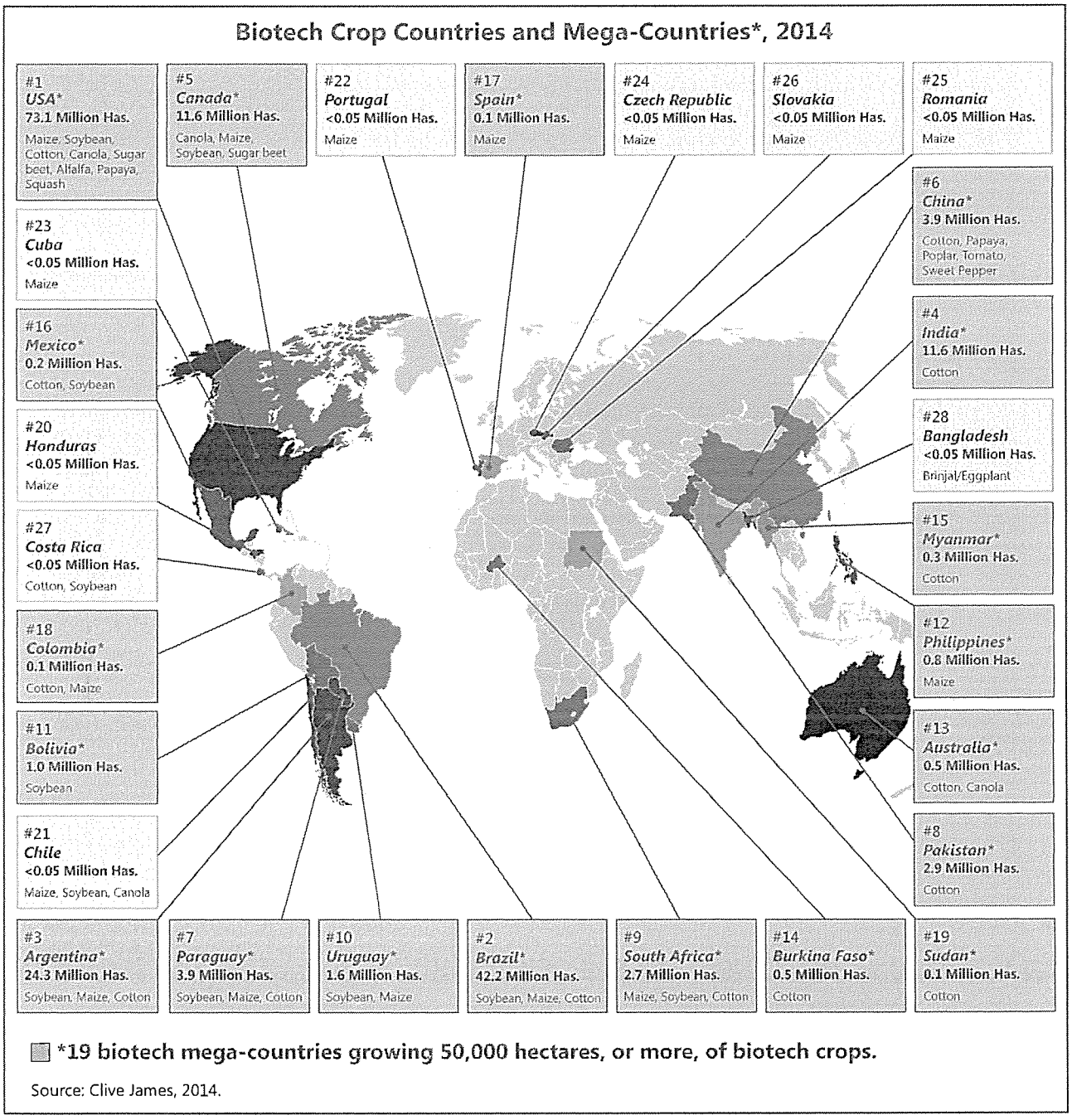

Figure 1. Global Map of Biotech Crop Countries and Mega-Countries in 2014

potato, a food staple with lower levels of acrylamide, a potential carcinogen, and less wastage due to bruising; and reduced lignin alfalfa with event KK179, to be marketed as HarvXtra™ with higher digestibility and higher yield. Another product Enlist™ Duo is a representative example of the second generation of HT products featuring dual-action/weed management systems for dealing with herbicide resistant weeds. Others in the same class include a dicamba/glyphosate soybean product, and event SYHTOH2 soybean tolerant to glufosinate, isoxaflutole and mesotrione. Enlist™ Duo has herbicide tolerance to both glyphosate and 2,4-D in soybean and maize. Indonesia has approved drought tolerant sugarcane with plans to plant in 2015 and Brazil has two products – Cultivance™, an HT soybean, and a home-grown virus resistant bean for commercialization in 2016. Finally, Vietnam has approved biotech maize (HT and IR) for the first time with commercialization planned for 2015. In summary, in addition to the current biotech food crops which directly benefit consumers (white maize in South Africa, sugar beet and sweet corn in the US and Canada, and papaya and squash in the US) new biotech food crops include the queen of the vegetables (brinjal) in Bangladesh and potato, the fourth most important food staple in the world.

- **Innate™ potato** developed by the private company, Simplot, in the US, was approved for commercialization in the US by APHIS/USDA in November 2014. Innate™ has 50 to 75% lower levels of acrylamide, a potential carcinogen in humans, produced when potatoes are cooked at high temperatures. Innate™ potato is also less susceptible to bruising. Given that potato is a perishable food product, quality can be significantly and negatively impacted by damage to the tubers during harvest, handling and processing. Innate™potatoes are an excellent example of how biotech crops can enhance food safety, quality and provide benefits for all stakeholders, growers, processors and consumers. It is noteworthy that Innate™ potato was developed by transferring genes from one potato variety to another. Simplot claims that Innate™ potato is a safe and superior product that will confer the following benefits to farmers, processors and consumers: lower levels of asparagine, which in turn lowers the potential for production of undesirable acrylamide, a potential carcinogen, when potatoes are cooked at high temperatures; will not discolor when peeled; fewer spots due to bruising; they store better; reduce wastage and thus contribute to food security. Consumer surveys by Simplot indicate that 91% of those surveyed were comfortable with the Innate™ breeding method. RNA interference technology was used to silence four genes that lowered enzyme levels that in turn led to lower acrylamide level. The company plans to initiate commercialization on a modest hectarage in 2015, prioritizing the fresh potato market and the potato chip market whilst keeping Innate™ production separated from conventional potatoes for the export market. Simplot is planning on submitting applications to the major markets, Canada, Mexico and Japan.

Approval of Innate™ could open new windows of opportunity for biotech potatoes globally. Potato is the fourth most important food staple in the world after rice, wheat, and maize. Plant protection constraints are important in potato production because the potato is a vegetatively propagated crop, where the tubers and not the "true seed" are used to propagate the crop commercially. Thus, unlike crops propagated through the seed, potatoes do not benefit from the natural barrier provided by the seed for blocking transmission of many plant pathogens. Hence, like other tuber crops, the prevalence and importance of diseases is high in potatoes, compared with seed propagated crops. Global yield loss in potatoes due to fungal and bacterial pathogens is estimated at 22%, plus 8% for viruses for a total of 30% for all diseases. These disease losses are in addition to the estimated losses of 18% for insect pests and 23% for weeds. Without crop protection, up to 70% of attainable potato production could potentially be lost to pests such as Colorado beetle and virus vectors (aphids and leafhoppers),

diseases caused by fungi, bacteria and a complex of viruses, including potato virus Y (PVY) and potato leaf roll virus (PLRV) as well as nematodes, which cause devastating losses in localized areas. Seed certification programs, for field tubers grown for propagation, and plant tissue cultural systems, both requiring infrastructure and recurrent use of resources to produce clean potato stock annually, are used in industrial countries to provide effective control of some diseases particularly insect vectored viruses including PVY and PLRV. Certification is not very effective against the spread of destructive late blight and certification requires adequate infrastructure which is often not available in developing countries. Thus, potato suffers very high losses from pests and diseases, which biotech can effectively control.

Of the many pests that attack potatoes, late-blight (caused by the fungus *Phytophthora infestans*) is the single most important disease, accounting for up to 15% of potato yield losses due to plant pathogens – the disease that caused the Irish Famine of 1845. More than 150 years after the famine, conventional technology has still failed to confer resistance and late-blight is still the most important disease of potatoes world wide responsible for economic losses estimated at US$7.5 billion annually. Potato is widely grown in many developing countries like Bangladesh, India, and Indonesia, where field trials are already underway for assessing biotech resistance to late-blight disease of potatoes. The approval of Innate™ potato in the US could have important implications globally particularly for developing countries, because it opens up new opportunities to apply biotech to a "new" crop by stacking several important traits already developed (late-blight resistance), approved (Innate™), or already commercialized (PVY, PLRV and Bt in the US in the late 1990s). It is noteworthy that recently, Simplot has pioneered this strategy by licensing biotech late blight resistant potato from the John Innes Institute in the UK and developed an enhanced Innate™ with late blight resistance potato, low acrylamide potential, reduced black spot bruising and lowered reducing sugars. The company has submitted an application for non regulated status of the enhanced Innate™ product to APHIS, which has already invited public comments on the application.

- **Reduced lignin alfalfa event KK179** (to be marketed as HarvXtra™) was recently deregulated by APHIS for cultivation in the US. Alfalfa is a perennial and the fourth largest crop by hectarage in the US after maize, soybean, and wheat, occupying up to 8 to 9 million hectares. It is the major forage crop in the US and globally, where it occupies approximately 30 million hectares. Biotech herbicide tolerant RR® alfalfa has already been grown since 2005 in the US. In November 2014, the US approved the planting of biotech alfalfa, event KK179, to be marketed as HarvXtra™, as a stack with RR® alfalfa with up to 22% reduction in lignin when compared to conventional alfalfa at the same stage of growth. This results in a reduced overall accumulation of total lignin in alfalfa forage. The amounts of lignin in event KK179 forage are generally similar to those found in conventional forage harvested several days earlier under similar production conditions. The reduced lignin alfalfa increases forage quality compared to conventional forage of the same age, maximizes forage yield by delaying harvest for several days, and gives farmers more flexibility in forage harvest timing. Thus, event KK179 maximizes forage quality with lower lignin levels; optimizes forage yields by allowing farmers to delay harvest for several days during which more forage biomass is accumulated; and allows more flexible harvest schedules to deal with adverse weather and varying labor schedules.

- **Enlist™ Duo** is a representative example of a second generation of herbicide tolerant products featuring dual-action/weed management systems for dealing with herbicide resistant weeds – others in the same class includes a dicamba/glyphosate soybean product and event SYHTOH2 soybean tolerant to the herbicides glufosinate, isoxaflutole and mesotrione. **Enlist™ Duo** products contain two pyramided

genes to confer tolerance to herbicide glyphosate and 2,4-D choline. The product was deregulated in the US to manage broad spectrum of weeds including hard-to-control and resistant weeds such as glyphosate-resistant Palmer amaranth, waterhemp and giant ragweed. Maize and soybean farmers can use the Enlist™ Duo seeds as a component in their stewardship of rotating various herbicide tolerant seeds and products on their farms – an important strategy to retain the value, effectiveness and durability of herbicide tolerant crops. A full launch of Enlist products is pending import approval in China which approved the last product in June 2013; asynchronous approval for cultivating and import of new products is a major challenge which needs urgent attention by all stakeholders.

18 million farmers benefit from biotech crops – 90% were small resource-poor farmers.

In 2014, approximately 18 million farmers, the same as 2013, grew biotech crops – remarkably, about 90%, or 16.5 million, were risk-averse small, poor farmers in developing countries. In China, 7.1 million small farmers benefited from biotech cotton and in India there were 7.7 million beneficiary farmers cultivating a total of more than 15 million hectares of Bt cotton. The latest provisional economic data available for the period 1996 to 2013 indicates that farmers in China gained US$16.2 billion and in India US$16.7 billion. In addition to economic gains, farmers benefited enormously from at least a 50% reduction in the number of insecticide applications, thereby reducing farmer exposure to insecticides, and importantly contributed to a more sustainable environment and better quality of life.

For the third consecutive year in 2014, developing countries planted more biotech crops than industrial countries.

Latin American, Asian and African farmers collectively grew 96 million hectares or 53% of the global 181 million biotech hectares (versus 54% in 2013) compared with industrial countries at 85 million hectares or 47% (versus 46% in 2013), equivalent to a gap of 11 million hectares in favor of developing countries. In the long term, this trend is expected to continue despite the fact that in 2014 the US had the highest increase (3.0 million hectares) whereas Brazil (with an increase of 1.9 million hectares in 2014) had the highest year-to-year increase for the last five years. The higher hectarage in developing countries is contrary to the prediction of critics who, prior to the commercialization of the technology in 1996, prematurely declared that biotech crops were only for industrial countries and would never be accepted and adopted by developing countries, particularly small poor farmers.

During the period 1996-2013 cumulative provisional economic benefits in industrial countries were at US$65.2 billion compared to US$68.1 billion generated by developing countries. In 2013, developing countries had 49.5% equivalent to US$10.1 billion of the total US$20.4 billion gain, with industrial countries at US$10.3 billion (Brookes and Barfoot, 2015, Forthcoming).

Stacked traits occupied 28% of the global 181 million hectares.

Stacked traits continued to be an important and growing feature of biotech crops – 13 countries planted biotech crops with two or more traits in 2014, of which 10 were developing countries. About 51 million hectares equivalent to 28% of over 181 million hectares were stacked in 2014, up from 47 million hectares or 27% of the 175 million hectares in 2013; this steady and growing trend of more stacked traits is expected to continue. In 2014, 5.8 million hectares of HT/Bt soybean were grown in Brazil, Argentina, Paraguay and Uruguay in Latin America.

The 5 lead biotech developing countries in the three continents of the South: Brazil and Argentina in Latin America, India and China in Asia, and South Africa on the continent of Africa, grew 47% of global biotech crops and represent ~41% of world population.

The five lead developing countries in biotech crops in the three continents of the South are China and India in Asia, Brazil and Argentina in Latin America, and South Africa on the continent of Africa. They collectively grew 84.7 million hectares (47% of global) and together represent ~41% of the global population of 7 billion, which could reach 10.9 billion, or more, by the turn of the century in 2100. Remarkably, the population in Sub Saharan Africa alone could escalate from ~1 billion today (~13% of global) to a possible high of 3.8 billion (~38% of global) by the end of this century in 2100. Global food security, exacerbated by high and unaffordable food prices, is a formidable challenge to which biotech crops can contribute but are not a panacea.

USA maintains leadership role, and in 2014 its increase in year-to-year hectarage was higher than Brazil, which has recorded the highest increase of any country for the last five years.

The US continued to be the lead producer of biotech crops globally with 73.1 million hectares (40% of global), with an average adoption rate of over ~90% across its principal biotech crops; year-to-year growth in the US in 2014 was 4%. It is noteworthy that in 2014, the US increase in hectarage (3.0 million hectares) was higher than any country in the world including Brazil (1.9 million hectares) which had recorded the highest increase of all countries in the world for the last five years. The higher increase in the US in 2014 was principally due to an 11% increase in total planted hectarage to a record 34.3 million hectares of soybean planted. Despite very high levels of adoption in 2013, adoption in 2014 increased in all three principal crops – soybean adoption increased from 93% to 94%, maize from 90% to 93% and cotton from 90% to 96%.

Brazil continues to be second only to the US in biotech crop hectarage.

In 2014, Brazil ranked second only to the USA in biotech crop hectarage in the world with 42.2 million hectares (up from 40.3 million in 2013); the increase in 2014 was 1.9 million hectares equivalent to a growth rate of 5%. For the last five years, Brazil was the engine of growth globally. In 2013, it increased its hectarage by 3.7 million hectares of biotech crops, more than any other country in the world, however, in 2014, the highest year-over-year increase was in the US at 3.0 million hectares. In 2014, Brazil grew 23% (same as 2013) of the global hectarage of 181 million hectares. In the future, Brazil is expected to close the gap with the US. An efficient and science-based approval system in Brazil facilitates fast adoption. In 2014, Brazil commercially planted, for the second year, the stacked soybean with insect resistance and herbicide tolerance on 5.2 million hectares, up substantially from 2.2 million hectares in 2013. Notably, EMBRAPA, Brazil's agricultural R&D organization, with an annual budget of US$1 billion, has gained approval to commercialize its home-grown biotech virus resistant bean, planned for 2016 and a herbicide tolerant soybean which it developed in a public-private partnership with BASF, which is waiting for an EU import approval prior to a planned commercialization in 2016.

Canada increases hectarage of biotech crops whereas area in Australia decreases because of continuing severe drought.

Canada grew 11.6 million hectares of biotech crops in 2014, up from 10.8 million hectares in 2013,

as farmers planted more biotech canola and soybean. Canada planted 8 million hectares of biotech canola (95% adoption) and over 2 million hectares of biotech soybean. Australia posted a decrease of ~200,000 hectares of biotech cotton (99% adoption) due to a severe drought. The decrease in cotton plantings was offset by an increase of ~50% for herbicide tolerant canola to 342,000 hectares.

India continues to benefit enormously from Bt cotton.

India cultivated a record 11.6 million hectares of Bt cotton planted by 7.7 million small farmers with an adoption rate of 95%, up from 11.0 million hectares in 2013. Notably, the increase from 50,000 hectares of Bt cotton in 2002 (when Bt cotton was first commercialized) to 11.6 million hectares in 2014, represents an unprecedented 230-fold increase in thirteen years. Brookes and Barfoot's latest provisional estimate indicated that India had enhanced farm income from Bt cotton by US$16.7 billion in the twelve year period 2002 to 2013 and US$2.1 billion in 2013 alone, similar to 2012.

Status of biotech crops in China

In 2014, 7.1 million small farmers (0.5 to 0.6 hectare/farm) successfully planted 3.9 million hectares of biotech cotton at an adoption rate of 93% of its 4.2 million total cotton hectarage. In addition ~8,500 hectares of virus resistant papaya were planted in Guangdong, Hunan Island and this year's new province of Guangxi; plus ~543 hectares of Bt poplar, the same as last year. Despite China's decreased total cotton hectarage from 4.6 million hectares in 2013 to 4.2 million hectares in 2014 (mainly due to low prices and high stockpiles of cotton in China), biotech cotton adoption has increased from 90% in 2013 to 93% in 2014. Impressively, virus resistant papaya plantings increased by ~50% from 5,800 hectares in 2013 to 8,475 hectares in 2014. In addition to the 7.1 million farmers benefiting directly from biotech cotton, there maybe an additional 10 million secondary beneficiary farmers cultivating 22 million hectares of crops which are alternate hosts of cotton bollworm that benefit from decreased pest infestation due to the extensive planting of Bt cotton. Thus, the actual total number of beneficiary farmers of biotech Bt cotton in China alone may well substantially exceed 7.1 million farmers. Latest provisional data shows that economic gains at the farmer level from Bt cotton for the period 1997 to 2013 was US$16.2 billion and US$1.6 billion for 2013 alone.

In the shorter term, biotech maize, and for the longer term Bt rice, offer significant benefits and have momentous implications for China, Asia, and the rest of the world, in the near, mid and long term; this is due to the fact that rice is the most important food staple, and maize the most important feed crop in the world. China's research and commercialization of Bt maize, herbicide tolerant maize and phytase maize as well as biotech rice, can make very important potential contributions to global food and feed needs as well as that of China. Whereas President Xi Jinping has endorsed the technology that is used in imported biotech soybean and maize in very large quantities by China (63 million tons of soybean and 3.3 million tons of maize in 2013), domestic production of staple food crops has not been implemented to-date, although biotech papaya, consumed as a fresh fruit/food is widely accepted with hectarage increasing by ~50% in 2014 to over 8,000 hectares. President Xi JinPing stated at the Communist Party Conference in December 2013 that, because the technology is new it's reasonable that society should hold controversial views and doubts. Importantly, now China, through the Ministry of Agriculture, has launched a large national public information media campaign to increase the awareness of the public regarding biotech crops including the benefits they offer China. Continuing high priority to R&D support for biotech crops in China (US$4 billion for the period 2008 to 2020)

reflect the country's long term commitment to biotech crops. China imports increasing quantities of maize (~90% of which is biotech) and consumes one-third of global soybean production; China imports 65% of global soybean exports of which over 90% is biotech.

Status in Africa

Africa continued to make progress in 2014 with Sudan increasing its Bt cotton hectarage substantially to 90,000 hectares by ~46%, with South Africa and Burkina Faso marginally lower mainly because of uncertainty of planting conditions. Encouragingly, an additional seven African countries (listed alphabetically): Cameroon, Egypt, Ghana, Kenya, Malawi, Nigeria, and Uganda have conducted field trials on the following broad range of staple and orphan crops: rice, maize, wheat, sorghum, bananas, cassava, and sweet potato. The WEMA project is expected to deliver its first biotech stacked drought tolerant maize with insect control (Bt) in South Africa as early as 2017, followed by Kenya and Uganda, and then by Mozambique and Tanzania, subject to regulatory approval.

Five EU countries planted 143,016 hectares of biotech Bt maize. Spain was by far the largest adopter, planting 92% of the total Bt maize hectarage in the EU.

Five EU countries, same as last year, planted 143,016 hectares of Bt maize, down marginally by 3% from 2013, mainly due to lower total plantings of maize, particularly in Spain which reported a record adoption rate of 31.6% and grew 92% of all the Bt maize in the EU. Modest increases were reported in three countries: Portugal, Romania and Slovakia and marginal decreases in two countries: Spain and Czechia. Spain led the EU with 131,538 hectares of Bt maize, down 3% from 136,962 in 2014. Generally in the EU countries, there is a disincentive for farmers to plant Bt maize because of the negative effect of onerous and over-demanding EU farmer reporting procedures.

Status of approved events for biotech crops

As of the end of October 2014, a total of 38 countries (37 + EU - 28) have granted regulatory approvals to biotech crops for use as food, feed or for environmental release since 1994. From these countries, 3,083 regulatory approvals have been issued by competent authorities across 27 GM crops and 357 GM events. 1,458 are for food use (direct use or for processing), 958 for feed use (direct use or for processing) and 667 for environmental release or planting. Japan has the most number of approved events (201), followed by the U.S.A. (171 not including stacked events), Canada (155), Mexico (144), South Korea (121), Australia (100), New Zealand (88), Taiwan (79), Philippines (75), European Union (73 including approvals that have expired or under renewal), Colombia (73), South Africa (57) and China (55). Maize still has the most number of events (136 events in 29 countries), followed by cotton (52 events in 21 countries), canola (32 events in 13 countries), potato (31 events in 10 countries), and soybean (30 events in 28 countries).

Among the GM events, the herbicide-tolerant soybean event GTS-40-3-2 has the most number of approvals (52 approvals in 26 countries + EU-28). It is followed by the herbicide-tolerant maize event NK603 (52 approvals in 25 countries + EU-28), insect-resistant maize MON810 (50 approvals in 25 countries + EU-28), insect resistant maize Bt11 (50 approvals in 24 countries + EU-28), insect-resistant maize TC1507 (47 approvals in 22 countries + EU-28), herbicide-tolerant maize GA21 (41 approvals in 20 countries + EU-28), insect-resistant cotton MON531 (39 approvals in 19 countries + EU-28),

insect-resistant maize MON89034 (39 approvals in 22 countries + EU-28), herbicide-tolerant soybean A2704-12 (39 approvals in 22 countries + EU-28), insect-resistant maize MON88017 (37 approvals in 20 countries + EU-28), herbicide-tolerant maize T25 (37 approvals in 18 countries + EU-28) and insect-resistant cotton MON 1445 (37 approvals in 17 countries + EU-28).

Global value of biotech seed alone was ~US$15.7 billion in 2014

Global value of biotech seed alone was ~US$15.7 billion in 2014. A 2011 study estimated that the cost of discovery, development and authorization of a new biotech crop/trait is ~US$135 million. In 2014, the global market value of biotech crops, estimated by Cropnosis, was US$15.7 billion, (up slightly from US$15.6 billion in 2013); this represents 22% of the US$72.3 billion global crop protection market in 2013, and 35% of the ~US$45 billion commercial seed market. The estimated global farm-gate revenues of the harvested commercial "end product" (the biotech grain and other harvested products) are more than ten times greater than the value of the biotech seed alone.

FUTURE PROSPECTS

Feeding the World of 2050

Feeding over 9 billion people in 2050 is one of, if not THE most daunting challenges facing mankind during the remaining years of this century. The fact that the majority of the world's population is not even aware of the magnitude of the challenge makes the task even more difficult. The following paragraphs chronicle some of the salient and critical facts in relation to the dimensions of feeding the world of 2050 and beyond.

- Global population, which was only 1.7 billion at the turn of the century in 1900, is now 7.2 billion, expected to climb to 9.6 billion by 2050, and will be close to 11 billion at the end of this century in 2100. Globally, 870 million people are currently chronically hungry and 2 billion are malnourished.
- Coincidentally, a change is occurring in favor of a less efficient higher protein diet, including significantly more meat in more prosperous developing countries led by China and India.
- Need to increase crop productivity, by at least 60% or more by 2050 and do so in an improved and sustainable use of less resources – less land, water, fertilizer and less pesticides.
- Increased demand for crop biomass to produce biofuels in response to more energy required for a more demanding and affluent growing world population.
- Respond to the additional new challenges associated with climate change, with more frequent and severe droughts with implications for availability and use of water – agriculture uses 70% of the fresh water in the world, a rate that is not sustainable by 2050 with 2 billion more people.

Rates of growth in crop productivity have declined subsequent to the significant contribution of the green revolutions of wheat and rice. It is now evident that conventional crop technology alone will not allow us to feed over 9 billion in 2050 and neither is biotechnology a panacea. An option being proposed by the global scientific community is a balanced, safe and sustainable approach, using the best of conventional crop technology (well adapted germplasm) and the best of biotechnology (appropriate GM and/non-GM traits) to achieve **sustainable intensification** of crop productivity on

the 1.5 billion hectares of cropland globally. The returns on investments in agriculture are high and furthermore they directly impact on poverty alleviation, particularly small resource-poor farmers and the rural landless dependent on agriculture, representing the majority of the world's poorest people.

Biotech crops contribution to Food Security, Sustainability and Climate Change

Provisional data for 1996 to 2013 showed that biotech crops contributed to Food Security, Sustainability and Climate Change by: increasing crop production valued at US$133.3 billion; providing a better environment, by saving ~500 million kg a.i. of pesticides in 1996-2012; in 2013 alone reducing CO_2 emissions by 28 billion kg, equivalent to taking 12.4 million cars off the road for one year; conserving biodiversity in the period 1996-2013 by saving 132 million hectares of land; and helped alleviate poverty by helping 16.5 million small farmers, and their families totaling >65 million people, who are some of the poorest people in the world. Biotech crops can contribute to a **"sustainable intensification"** strategy favored by many science academies worldwide, which allows productivity/production to be increased only on the current 1.5 billion hectares of global crop land, thereby saving forests and biodiversity. Biotech crops are essential but are not a panacea and adherence to good farming practices, such as rotations and resistance management, are a must for biotech crops as they are for conventional crops.

Contribution of biotech crops to Sustainability

Biotech crops are contributing to sustainability in the following five ways:

- **Contributing to food, feed and fiber security and self sufficiency, including more affordable food, by increasing productivity and economic benefits sustainably at the farmer level**

  Economic gains at the farm level of ~US$133.3 billion were generated globally by biotech crops during the eighteen year period 1996 to 2013, of which 30% were due to reduced production costs (less ploughing, fewer pesticide sprays and less labor) and 70% due to substantial yield gains of 441.4 million tons. The corresponding figure for 2013 alone was 88% of the total US$20.4 billion gain due to increased yield (equivalent to 64 million tons), and 12% due to lower cost of production (Brookes and Barfoot, 2015, Forthcoming).

- **Conserving biodiversity, biotech crops are a land saving technology**

  Biotech crops are a land-saving technology, capable of higher productivity on the current 1.5 billion hectares of arable land, and thereby can help preclude deforestation and protect biodiversity in forests and in other in-situ biodiversity sanctuaries – a sustainable intensification strategy. Approximately 13 million hectares of biodiversity – rich tropical forests, are lost in developing countries annually. If the 441.4 million tons of additional food, feed and fiber produced by biotech crops during the period 1996 to 2013 had not been produced by biotech crops, an additional 132 million hectares (Brookes and Barfoot, 2015, Forthcoming) of conventional crops would have been required to produce the same tonnage. Some of the additional 132 million hectares would probably have required fragile marginal lands, not suitable for crop production, to be ploughed, and for tropical forest, rich in biodiversity, to be felled to make way for slash and burn agriculture in developing countries, thereby destroying biodiversity.

- **Contributing to the alleviation of poverty and hunger**

  To-date, biotech cotton in developing countries such as China, India, Pakistan, Myanmar, Burkina Faso and South Africa have already made a significant contribution to the income of 16.5 million small resource-poor farmers in 2014. This can be enhanced in the remaining years of this decade 2011 to 2020 principally with biotech cotton and maize.

- **Reducing agriculture's environmental footprint**

  Conventional agriculture has impacted significantly on the environment, and biotechnology can be used to reduce the environmental footprint of agriculture. Progress to-date includes: a significant reduction in pesticides; saving on fossil fuels; decreasing CO_2 emissions through no/less ploughing; and conserving soil and moisture by optimizing the practice of no till through application of herbicide tolerance. The accumulative reduction in pesticides, based on the latest information for the period 1996 to 2012, was estimated at ~500 million kilograms (kgs) of active ingredient (a.i.), a saving of 8.7% in pesticides, which is equivalent to an 18.5% reduction in the associated environmental impact of pesticide use on these crops, as measured by the Environmental Impact Quotient (EIQ). EIQ is a composite measure based on the various factors contributing to the net environmental impact of an individual active ingredient. The corresponding data for 2012 alone was a reduction of 36 million kgs a.i. (equivalent to a saving of 8% in pesticides) and a reduction of 23.6% in EIQ (Brookes and Barfoot, 2014).

  Increasing efficiency of water usage will have a major impact on conservation and availability of water globally. Seventy percent of fresh water is currently used by agriculture globally, and this is obviously not sustainable in the future as the population increases by almost 30% to over 9.6 billion by 2050. The first biotech maize hybrids with a degree of drought tolerance were commercialized in 2013 in the USA, and the first tropical biotech drought tolerant maize is expected by ~2017 in sub-Saharan Africa. Drought tolerance is expected to have a major impact on more sustainable cropping systems worldwide, particularly in developing countries, where drought will likely be more prevalent and severe than industrial countries.

- **Helping mitigate climate change and reducing greenhouse gases**

  The important and urgent concerns about the environment have implications for biotech crops, which contribute to a reduction of greenhouse gases and help mitigate climate change in two principal ways. First, permanent savings in carbon dioxide (CO_2) emissions through reduced use of fossil-based fuels, associated with fewer insecticide and herbicide sprays. Provisionally in 2013 alone, this was an estimated saving of 2.1 billion kg of CO_2, equivalent to reducing the number of cars on the roads by 0.93 million. Secondly, additional savings from conservation tillage (need for less or no ploughing facilitated by herbicide tolerant biotech crops) for biotech food, feed and fiber crops, led to an additional soil carbon sequestration equivalent in 2013 to 25.9 billion kg of CO_2, or removing 11.5 million cars off the road for one year. Thus in 2013, the combined permanent and additional savings through sequestration was equivalent to a saving of 28 billion kg of CO_2 or removing 12.4 million cars from the road up from 11.8 million in 2012 (Brookes and Barfoot, 2015, Forthcoming).

Droughts, floods, and temperature changes are predicted to become more prevalent and more severe as we face the new challenges associated with climate change, and hence, there will be a need for faster crop improvement programs to develop varieties and hybrids that are well adapted to more rapid changes in climatic conditions. Several biotech crop tools and techniques, including tissue culture, diagnostics, genomics, molecular marker-assisted selection (MAS) zinc fingers, and TALENS, and biotech crops can be used collectively for 'speeding the breeding' and help mitigate the effects of climate change. Biotech crops are already contributing to reducing CO_2 emissions by precluding the need for ploughing a significant portion of cropped land, conserving soil, particularly moisture, and reducing pesticide spraying as well as sequestering CO_2.

In summary, collectively the above five thrusts have already demonstrated the capacity of biotech crops to contribute to sustainability in a significant manner and for mitigating the formidable challenges associated with climate change – global warming, and the potential for the future is enormous. Biotech crops can increase productivity and income significantly, and hence, can serve as an engine of rural economic growth that can contribute to the alleviation of poverty for the world's small and resource-poor farmers.

Stewardship and Resistance Management of Biotech Crops

The two major biotech crop traits of insect resistance (IR) and herbicide tolerance (HT) have made an enormous contribution to global food, feed and fiber production since they were first approved for commercial cultivation in 1996, almost 20 years ago. In 2014, insect resistance and herbicide tolerance traits, were deployed singly or stacked in the four principal biotech crops of maize, soybean, cotton and canola, and were planted globally on 181 million hectares in 28 countries. Moreover, in the 19 year period, 1996 to 2014 the IR/HT biotech crops have gained the trust of millions of farmers world-wide and as a result have achieved a near-optimal adoption of 90% or more in virtually all the principal countries growing biotech crops. The IR/HT biotech crops have provided a successful complementary and alternative system to the conventional pesticide-based crop production systems and they are judged by farmers to be efficient, convenient and environment-friendly. These same two trait(s) have also been successfully incorporated in a range of other commercialized biotech crops including alfalfa, brinjal (eggplant), sugar beet and poplar; the two traits have also been successfully incorporated in the other two major staples of rice and wheat for future deployment as new commercial biotech crops.

Irrespective of whether the technology is conventional or biotech, the wide-spread adoption of insect resistance and herbicide tolerance leads, over time, to insect pest resistance and resistant weeds, thereby diminishing their benefits to farmers. **The issues of resistance management of IR/HT traits were anticipated and discussed by the scientific community, regulators and policy makers prior to the introduction of biotech crops in 1996.** Policy approaches were considered to manage development of resistance in IR/HT crops including the deployment of refugia, integration of IRM into general insect pest management (IPM) schemes using insect resistant management (IRM) strategies, and post release monitoring of biotech crops for early detection of resistance. Coincidentally, new scientific methods evolved around gene pyramiding, and stacking of traits to enable more effective management and stewardship of resistance in the new biotech crops. Thus, resistance management including IRM and stewardship, and good farming practices including rotation have played a significant role in the successful large scale adoption and acceptance of IR/HT biotech crops from the very beginning in 1996. These approaches are credited with prolonging the life of biotech crops, and making them more

durable than conventional technology thereby extending the benefits to farmers from planting IR/HT biotech crops season-after-season.

As anticipated, studies have confirmed that the first generation IR and HT traits are becoming susceptible to resistant targeted insect pests and weeds respectively. Single or stacked IR/HT GM crops involving single and multiple gene(s) in maize in the USA have led to field-evolved resistance of insect pests. Hence, approaches for managing Bt resistance must be assigned a high priority, particularly as more crops feature Bt genes (simple and stacked) and in 2014 already occupied 55 million hectares. Similarly, several studies indicate that a considerable number of weeds have shown resistance to the application of herbicides including the widely used glyphosate, thereby potentially limiting the future use of the product in its current form. Thus, the management of insect resistance and stewardship of IR/HT biotech crops have assumed greater importance and deserves priority and appropriate attention and implementation at the field level.

The two decades of experience and the trend in technological development suggest that the following 12 elements be considered to achieve effective and strict implementation of resistance management and stewardship:

- Planting of refugia and innovative methods for deploying them in simple but creative schemes such as refuge in the bag (RIB)
- Integration of IRM in integrated pest management (IPM) systems
- Stricter implementation of package of recommended practices
- Post release monitoring and timely reporting of detection of resistance
- Ensuring seed purity and adequate expression of traits
- Assurance of supply of high quality IR/HT seeds
- Gene pyramiding and stacking of insect resistance and herbicide tolerance traits
- Integrating multiple modes-of-action for IR/HT traits
- Development of innovative and more resilient new technologies capable of reversing resistance
- Timely replacement of current IR/HT products with improved versions
- Education, training and outreach to the farming community in managing IR/HT biotech crops and,
- Strengthen compliance of regulatory requirements

Early as possible approvals of the second generation of IR/HT crops such as Bollgard-III™ and Enlist™ products with dual and triple modes-of-action for insect and weed tolerant traits is important, and helps overcome the current challenges of managing the insect and weed resistance to IR/HT crops. The wide scale use of the refuge-in-bag (RIB) strategy and regulatory compliance needs to be strictly implemented. **Importantly, all stakeholders including the scientific community, farmers, policy makers and the private sector must be made aware of their collective responsibility and the fact that the overall system of managing resistance will NOT work if any single stakeholder is delinquent in its implementation.**

Status of Golden Rice

Women and children are the most vulnerable to vitamin A deficiency (VAD), the leading cause of childhood blindness and inability of the immune systems to combat disease. **WHO reports in 2009**

and 2012 that 190 to 250 million preschool children worldwide are still affected by VAD annually. Studies showed that vitamin A supplementation could reduce all mortality in children younger than 5 years by 24-30%. This means that vitamin A availability for 8 million late infancy and pre-eschool age children in undernourished settings could prevent 1.3 to 2.5 million child deaths annually. Golden Rice (GR) is being developed by the Philippine Rice Research Institute (PhilRice) and the International Rice Research Institute (IRRI). IRRI reports that as of March 2014, the research, analysis and testing of beta carotene-enriched Golden Rice continues, in partnership with collaborating national research agencies in the Philippines, Indonesia, and Bangladesh. The Golden Rice event R (GR2-R) was introgressed into selected mega varieties, field tested for three seasons to evaluate the agronomic and product performance under Philippine field conditions.

Preliminary results of the conducted multi locational trials show that while the target level of beta-carotene in the grain was attained, yield was on an average lower than yields from comparable local varieties already preferred by farmers. Hence, the new objective of increasing yield became the focus of the current research to include other versions of GR2 such as GR2-E and others. At IRRI, the Golden Rice trait is being bred into mega varieties to get suitable advance lines, and once attained the series of confined field trials will resume. IRRI and its many research partners remain committed to developing a high-performing Golden Rice variety that benefits farmers and consumers. The important mission of the Golden Rice project – to contribute to improving the health of millions of people suffering from micronutrient deficiency – demands that every step and aspect of the scientific study of Golden Rice be carefully planned. IRRI and all participating organizations will continue to rigorously follow all biosafety and other regulatory protocols in continuing the research to develop and disseminate Golden Rice.

Once released, Golden Rice has the potential to provide beta carotene fortified carbohydrate staple, totaling an estimated 2,006,869 calories per day in the major countries of the South suffering from VAD. The following is the breakdown by region per day: people living in South Asia (1,130,648 calories), Southeast Asia (660,979), Africa (125,124), Latin America (75,238), and Central Asia (14,880) for a total of 2,006,869 calories per day – these are the regions where most VAD occurs (HarvestPlus, Personal Communications).

Potential New Biotech Crops in the next 5 to 10 years

One of the concerns often voiced by critics of biotech crops is the narrow focus on four principal crops (soybean, maize, cotton, and canola) and two traits, (herbicide tolerance and insect resistance). However, in the last five years there have been a significant broadening of the number of commercialized biotech crops to include a significant hectarage of sugar beet, and alfalfa along with continued small hectarages of squash, papaya, eggplant and poplar, for a total of 10 commercial biotech crops in 2014.

Global Information on biotech crops undergoing field trials is of interest to many but it is not always easy to access the information. Appendix 7 in the full Brief provides an incomplete listing of 71 selected new biotech crop/trait(s) that have, at a minimum, been field tested at the equivalent of contained field trials (CFT). The list provides the reader with a general global overview of the possible future scope of new biotech crops that may become available (subject to regulatory approval) during the next 5 to 10 years. The data base simply lists biotech crops by crop, trait(s), technology developer/ facilitator, and countries where field tests have been conducted. Whereas the list of 71 entries is not exhaustive, in reviewing the data base of 71 entries, the following are some of the general features that maybe of interest:

- About half of the 71 entries involve products field tested in developing countries and the other half are in industrial countries; the general drift in favor of developing countries is both timely and appropriate given the greater need for food, feed, and fiber in the countries of the South, in Africa, Asia and Latin America.
- About one quarter is "new" crops that substantially diversify the current portfolio of 10 commercial biotech crops and they are by and large pro-poor orphan crops that can make an important contribution to food security for poor people. The new biotech crops include apple, banana, camelina, cassava, citrus, chickpea, cowpea, groundnut, mustard, pigeon pea, potato, rice, safflower, sugarcane and wheat.
- The range of traits include those for improved drought and salinity tolerance, yield enhancement, efficient nitrogen utilization, increased nutrition and food quality, resistance to pests and diseases, including resistance to viruses.
- About half of the listed entries represent technologies developed by public sector organizations or are crop biotech transfer projects involving public-private sector partnerships. This, combined with the fact that about half of the trials are being conducted in developing countries, with an increasing number in Africa which presents the greatest challenges, is encouraging news for the development community globally.

Non-transgenic Biotech Products

Up until now transgenic modification has been achieved using Agrobacterium or the gene gun. New advanced biotech applications such as **zinc finger nucleases (ZFN) technology, clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease systems and transcription activator-like effector nucleases (TALENs)**, are being used to increase the efficiency and precision of the transformation process. These new techniques allow the cutting of the DNA at a pre-determined location and the precise insertion of the mutation, or single nucleotide changes at an optimal location in the genome for maximum expression. These techniques are well advanced – ZFN has already been used to successfully introduce herbicide tolerance and TALENs has been used to delete or "snip out" the gene in rice that confers susceptibility to the important bacterial blight disease of rice. **However, experts in the field believe that potentially the "real power" of these new technologies is their ability to "edit" and modify multiple native plant genes (non GM), coding for important traits such as drought and, generating improved crops that are not transgenic.** Regulators in the US have initially opined that changes not involving transgenics will be treated differently; this could have a very significant impact on the efficiency and timing of the current resource-intensive regulation/ approval process and the acceptance of the products by the public.

Powdery mildew-resistant wheat was developed by researchers from the Chinese Academy of Sciences through advanced gene editing methods. Researchers deleted genes encoding for proteins that repress defenses against the mildew using TALENs and CRISPR genome editing tools. Wheat is a hexaploid and thus required deletion of multiple copies of the genes. This also represents a significant achievement in modifying food crops without inserting foreign genes, hence considering it as a non GM technique.

Another class of new applications, still at the early stages of development, are **plant membrane transporters** that are being researched to overcome a range of crop constraints from abiotic and biotic stresses to enhancement of micronutrients. It is noteworthy that of the current 7 billion global population, almost one billion is undernourished but another one billion is malnourished, **lacking**

critical micro nutrients: iron (anemia), zinc and vitamin A. Adequate supply of nutritious foods with enhanced levels of important micronutrients is critical for human health. Recent advances show that specialized plant membrane transporters can be used to enhance yields of staple crops, increase micronutrient content and increase resistance to key stresses, including salinity, pathogens and aluminum toxicity, which in turn could expand available arable land. Acid soils are estimated to occupy 30% of land globally.

CLOSING COMMENTS

The Way Forward – The Role of Public-Private Partnerships (PPP)

In reviewing crop biotech transfer projects over the last decade the progress and promise of public-private sector partnerships (PPP) is striking. The first PPP biotech crop transfer project was facilitated by ISAAA in the early 1990s. The tripartite project involved three partners: the developing country partner was Mexico (more specifically the biotech lab CINVESTAV) which in conjunction with Ministry of Agriculture had identified resistance to virus diseases in potatoes, grown by small farmers as a top priority because conventional technology did not offer a solution; the private sector partner was Monsanto which agreed to donate the coat protein events that confer virus resistance to PVX and PVY in potatoes. Importantly, Monsanto also agreed to train scientists from CINVESTAV in the use of the new technology. The third partner was the Rockefeller Foundation which provided the entire funding for the 3 year project, because of its innovative nature and was consistent with the Foundation's program in crop biotechnology.

Following the implementation of the Mexican project, ISAAA further explored the possibility of building a biotech transfer project in which more than one country would share the same donated technology, thus providing a multiplier effect for technology transfer. The project that evolved featured the donation of an event for conferring resistance to the lethal papaya ringspot virus (PRSV) of papaya. The developing county partners were five countries in South East Asia all of whom had identified PRSV as a common need and top priority because conventional technology did not offer a solution. The five developing country partners in South East Asia (where lead public sector laboratories in crop biotechnology were involved) were, in alphabetic order: Indonesia, Malaysia, Philippines, Thailand and Vietnam. The private sector partner was Monsanto which agreed to donate the event(s) for virus resistance to PRSV in papaya for use by small farmers in the five respective partner countries. As in the Mexican project, Monsanto also agreed to train scientists from the five countries in South East Asia in the use of the new technology; the funding was provided by different donor agencies for a three year period. Subsequent to the establishment of the PRSV project, ISAAA facilitated a network of the five countries to share experiences and expedite progress with the technology. The network also provided an appropriate cost-effective mechanism for exchange of information and reciprocal training of project scientists among the five labs. Following interaction of the countries in the network, the five countries collectively identified a second papaya trait deemed important by all parties – delayed ripening. It is an important trait for a perishable fruit such as papaya which suffers significant post-harvest losses in the tropics – the technology for delayed ripening was donated by Zeneca.

In the last decade or so, several aid agencies and foundations have established projects to facilitate donation and transfer of biotech crop applications from both the private and public sector for the

benefit of developing countries particularly for small resource-poor farmers. Examples include, AATF based in Nairobi serving the needs of African countries, and Agricultural Biotechnology Support Project (ABSPII) which is a United States Agency for International Development (USAID) bilateral program, with global activities and operated by Cornell University.

A preliminary review of the initiatives involved in biotech crop transfer projects from both the public and private sector, suggests that public-private partnerships (PPP) projects have been encouragingly successful and offer advantages that increases the probability of delivering an approved biotech crop product at the farmer level within a reasonable time frame. Four PPP case studies have been selected to review and illustrate the diversity in characteristics of the four model projects: Bt brinjal in Bangladesh, herbicide tolerant soybean in Brazil, drought tolerant sugarcane in Indonesia, and the WEMA project for drought tolerance in maize in selected countries in Africa. For the convenience of readers, short descriptions of each of the four case studies, with more specific details are summarized in four boxes at the end of this closing chapter.

Norman Borlaug's Legacy and Advocacy of Biotech Crops

It is fitting to close this ISAAA Brief for 2014, by chronicling the counsel of the late 1970 Nobel Peace Laureate, Norman Borlaug, on biotech/GM crops, whose birth centenary was honored on 25 March 2014. Norman Borlaug, who saved a billion people from hunger, was awarded the Nobel Peace Prize for the impact of his semi-dwarf wheat technology on the alleviation of hunger. Norman Borlaug was the founding patron of ISAAA, and also the greatest advocate for biotechnology and biotech/GM crops worldwide, because he knew, better than anyone else their critical and paramount importance in feeding the world of tomorrow.

The following are two memorable and historical self-explanatory quotes from the man who knew more than anyone about feeding the world of tomorrow, because he had achieved it in the green revolution and understood the profundity of the proverb – **reading is learning, seeing is believing, but doing is knowing – knowledge**. This Brief seeks to share knowledge about biotech crops whilst respecting the rights of readers to make their own decisions about biotech/GM crops.

Borlaug Quotes:

"Over the past decade, we have been witnessing the success of plant biotechnology. This technology is helping farmers throughout the world produce higher yield, while reducing pesticide use and soil erosion. The benefits and safety of biotechnology has been proven over the past decade in countries with more than half of the world's population."

"What we need is courage by the leaders of those countries where farmers still have no choice but to use older and less effective methods. The Green Revolution and now plant biotechnology are helping meet the growing demand for food production, while preserving our environment for future generations" (ISAAA, 2009).

Case Study 1 – Insect Resistant (IR) Bt Brinjal in Bangladesh

Brief Description: The Bt brinjal project in Bangladesh may lay claim to be the first crop biotechnology transfer project to deliver a product that has already been commercialized by farmers. Bt brinjal was developed as an international public private partnership, between an Indian seed company Mahyco generously donating technology to the Bangladesh public sector R&D institute Bangladesh Agricultural Research Institute (BARI) facilitated by the Cornell University led project, ABSP-II, and funded by USAID. Bangladesh approved Bt brinjal for commercial cultivation on 30 Oct 2013 and in record time – less than 100 days – on 22 January 2014 a group of small farmers planted the first commercial product in their own fields. In 2014, a total of 12 hectares of Bt brinjal were planted by 120 farmers and the area is expected to increase substantially in 2015. This feat would not have been possible without strong support for the project from the Government of Bangladesh and in particular the political will and support of the Minister of Agriculture, the Honorable Matia Chowdhury. Bt brinjal drastically reduces pesticide application, increases marketable yield and improves fruit quality. Farmers have successfully sold Bt brinjal fruits in the open market labelled as *"BARI Bt Begun #, no pesticide used"*. More specific details are provided below.

Country: Bangladesh
Crop: Brinjal/Eggplant
Area: ~50,000 hectares farmed by ~150,000 smallholder farmers (0.3 ha average farm size
Importance: The poor man's vegetable crop, known as "the queen of the vegetables"
Gene: *cry1Ac* gene from *Bacillus thuringiensis* (Bt)
Trait(s): Insect resistance (IR); imparts protection against the lethal insect pest fruit and shoot borer" (*Leucinodes orbonalis*) which often requires small farmers to apply a polluting insecticide spray every other day and even then adequate control is not possible
Event: Elite Event EE-1
Technology Donor: The private sector company Mahyco, from India
Technology Recipient: Bangladesh Agricultural Research Institute (BARI)
Donor Funding Agency: USAID
Facilitator: Agricultural Biotechnology Support Program II (ABSPII) managed by Cornell University
Status of Approval: Approved for food, feed and environmental release on 30 Oct 2013 and commercialized in less than 100 days later on 22 January 2014
Varieties Approved: Brinjal-1 (Uttara), Bt Brinjal-2 (Kajla), Bt Brinjal-3 (Nayantara) and Bt Brinjal-4 (Iswardi/ISD 006)
Commercialization: 120 farmers planted Bt brinjal on 12 hectares in 2014
Number of Potential Beneficiary Farmers: 150,000 of the poorest and smallest farmers in Bangladesh which has a per capita of less than US$1,000 per annum
Socio-Economic Impact: Increases marketable yield by at least 30% and reduces the number of insecticide applications by 70-90%, resulting in a net economic benefit of US$1,868 per hectare; this is equivalent to a gain of up to US$200 million per annum nationally

Case Study 2 – Herbicide Tolerant (HT) Soybean in Brazil

Brief Description: In 2010, the Brazilian regulator authority CTNBio approved the commercial cultivation of a new herbicide tolerant soybean variety developed through a public-private partnership jointly executed by the private sector company BASF Germany and the public sector R&D institute EMBRAPA, the Brazilian Agricultural Research Cooperation. In this collaborative project, BASF provided EMBRAPA with *csr1-2* gene which confers tolerance to the herbicide imidazolinone, whilst the Brazilian institution also provided an additional gene and was responsible for the insertion of the trait into well adapted soybean germplasm. EMBRAPA and BASF share the patent for the new varieties, which represent the first home-grown biotech crop developed through PPP and approved in Brazil. Commercialization in Brazil is waiting on final import approval from the EU. It is expected that the new HT varieties will be commercialized in Brazil by 2016, increasing the choice of weed management options for Brazilian growers. More specific information is provided below.

Country: Brazil
Crop: Soybean
Area: ~31 million hectares
Importance: Most important export crop of Brazil
Gene: *csr1-2* from *Arabidopsis thaliana* conferring tolerance to imidazolinone herbicides
Trait(s): Herbicide tolerance
Event: BPS-CV127-9
Technology Provider: BASF, Germany/EMBRAPA, Brazil (there are 2 main patents supporting the product development, one gene from BASF and another from EMBRAPA, 4 soy gene transfers
Technology Recipient: BASF, Germany/EMBRAPA, Brazil
Donor Funding Agency: BASF, Germany/EMBRAPA, Brazil
Facilitator/Collaborator: BASF, Germany/EMBRAPA, Brazil
Status of Approval: Approved for commercial cultivation in 2009 (December), but pending EU final Import approval
Variety Approved: Varieties to be sold under the brand name Cultivance™
Commercialization: Expected planting as commercial crop in 2016
Potential Beneficiaries: Include farmers, seed growers and consumers
Socio-Economic Impact: Cultivance™ expected to reach up to 20% of market share on 31 million hectares of soybean with an export value of US$17 billion

Case Study 3 – Drought Tolerant (DT) Sugarcane in Indonesia

Brief Description: In May 2013, Indonesia – the second largest (2.4 million tonnes, valued at US$1.6 billion) raw sugar importing country in the world, issued food and environmental safety certificates for the country's first home-grown genetically modified drought tolerant sugarcane. The biotech sugarcane variety "Cane PRG Drought Tolerant NX1-4T" was developed under a public-private partnership between the Indonesian State-owned sugar company, PT. Perkebunan Nusantara XI (PTPN-11) and Ajinomoto Company, Japan in collaboration with Jember University in East Java, Indonesia. The drought tolerant sugarcane varieties can withstand water stress up to 36 days and under drought stress can yield substantially higher than the control variety BL-19; yield increases from 2 to 75% in the first planting, 14 to 57% in the first ratoon, and from 11 to 44% in the second ratoon. It is expected that the first home-grown drought tolerant sugarcane will be officially planted in Indonesia in 2015, pending approval of the product for feed. More specific information is provided below.

Country: Indonesia
Crop: Sugarcane
Area: 450,000 hectares
Importance: Indonesia is the second largest sugar importing country in the world
Gene: *betA* from *Rhizobium meliloti*
Trait(s): Drought tolerance
Event: NX1-4T
Technology Provider: Ajinomoto, Japan
Technology Recipient: PT. Perkebunan Nusantara XI (PTPN-11), Indonesia
Donor Agency: Govt of Indonesia
Facilitator/Collaborator: Jember University, East Java, Indonesia
Status of Approval: Approved for food and environmental release in 2013, pending feed approval
Variety Approved: Cane PRT Drought Tolerant NX1-4T
Commercialization: Expected first commercial planting in 2015

Case Study 4 – Drought Tolerant (DT) Maize for Africa WEMA (South Africa, Kenya, Uganda, Mozambique, and Tanzania)

Brief Description: Monsanto donated the biotech drought tolerant (DT) maize technology (MON 87460), DroughtGard™ to the public sector agriculture R&D institutions in five countries in Sub Saharan Africa including South Africa, Kenya, Uganda, Mozambique, and Tanzania through a public-private partnership project entitled "Water Efficient Maize for Africa (WEMA)". WEMA is coordinated by the African Agricultural Technology Foundation (AATF) based in Nairobi in collaboration with Monsanto and CIMMYT for further technology development. The project is funded jointly by the Gates Foundation, the Howard G. Buffett Foundation and USAID. The first stacked biotech insect resistant/drought tolerant (Bt/DT) maize hybrids are expected to be available to farmers (subject to regulatory approval) as early as 2017. South Africa is expected to be the first country to deploy the technology in 2017, followed by Kenya and Uganda which are expected to conduct confined field trials (CFT) in 2015. The three countries have conducted CFTs with the DT maize for at least 5 seasons (Uganda 5th, Kenya 6th, and South Africa 7th season) with very encouraging results. Kenya is in its 3rd season CFT for Bt maize (MON 810 also donated by Monsanto subsequent to the initiation of the project) and Uganda is in the 2nd season of field testing. In Mozambique, a revised Biosafety decree and implementing regulations received approval by the Council of Ministers in October 2014, and the country is due to initiate WEMA CFTs in 2015. Tanzania has made substantive progress towards amendment of the 2009 Biosafety regulations for the CFTs. It is projected that the WEMA stacked DT/Bt maize hybrids may yield up to 20 to 35% more grain than other commercial hybrids under moderate drought, resulting in an additional 2 to 5 million metric tons of maize to feed about 14 to 21 million people in Africa. More specific information is provided below.

Countries: South Africa, Kenya, Uganda, Tanzania and Mozambique
Crop: Maize
Area: ~8 million hectares in the five countries
Importance: Africa grows 90% of its maize under rainfed conditions and up to 25% of the area suffers from frequent droughts
Gene: Cold shock protein gene (*CspB*) from *Bacillus subtilis*
Trait(s): Drought tolerance
Event: Event MON87460, to be deployed as a stacked hybrid maize, also featuring a Bt gene (MON 810) for insect control also donated by Monsanto subsequent to the initiation of the project. The DT event is the same as that deployed in the 50,000 hectares of biotech drought tolerant maize in the US in 2013, which increased 5.5-fold to 275,000 hectares in the US in 2014.
Technology Donor: Monsanto, USA
Technology Recipients: South Africa, Kenya, Uganda, Mozambique and Tanzania
Donor Agencies: The Gates Foundation, the Howard G. Buffet Foundation and USAID
Facilitator Agencies: African Agricultural Technology Foundation (AATF), NARIs in the 5 WEMA countries, CIMMYT
Status of Approval: First deployment of stacked DT/Bt expected in South Africa in 2017, followed by Kenya and Uganda who are expected to conduct confined field trials (CFT) of the stacked product next year, 2015. Revised Biosafety decree and implementing regulations endorsed in Mozambique which paves the way for CFTs to be conducted in 2015, and positive discussion on amendment of biosafety regulations proceeding in Tanzania.
Commercialization: To begin (subject to regulatory approval) in South Africa in 2017
Socio-Economic Impact: Could increase maize production by up to 2 to 5 million metric tons under moderate drought, to feed about 14 to 21 million people in Africa.

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PLOS ONE

A Meta-Analysis of the Impacts of Genetically Modified Crops

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Abstract

Background: Despite the rapid adoption of genetically modified (GM) crops by farmers in many countries, controversies about this technology continue. Uncertainty about GM crop impacts is one reason for widespread public suspicion.

Objective: We carry out a meta-analysis of the agronomic and economic impacts of GM crops to consolidate the evidence.

Data Sources: Original studies for inclusion were identified through keyword searches in ISI Web of Knowledge, Google Scholar, EconLit, and AgEcon Search.

Study Eligibility Criteria: Studies were included when they build on primary data from farm surveys or field trials anywhere in the world, and when they report impacts of GM soybean, maize, or cotton on crop yields, pesticide use, and/or farmer profits. In total, 147 original studies were included.

Synthesis Methods: Analysis of mean impacts and meta-regressions to examine factors that influence outcomes.

Results: On average, GM technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. Yield gains and pesticide reductions are larger for insect-resistant crops than for herbicide-tolerant crops. Yield and profit gains are higher in developing countries than in developed countries.

Limitations: Several of the original studies did not report sample sizes and measures of variance.

Conclusion: The meta-analysis reveals robust evidence of GM crop benefits for farmers in developed and developing countries. Such evidence may help to gradually increase public trust in this technology.

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Introduction

Despite the rapid adoption of genetically modified (GM) crops by farmers in many countries, public controversies about the risks and benefits continue [1–4]. Numerous independent science academies and regulatory bodies have reviewed the evidence about risks, concluding that commercialized GM crops are safe for human consumption and the environment [5–7]. There are also plenty of studies showing that GM crops cause benefits in terms of higher yields and cost savings in agricultural production [8–12], and welfare gains among adopting farm households [13–15]. However, some argue that the evidence about impacts is mixed and that studies showing large benefits may have problems with the data and methods used [16–18]. Uncertainty about GM crop impacts is one reason for the widespread public suspicion towards this technology. We have carried out a meta-analysis that may help to consolidate the evidence.

While earlier reviews of GM crop impacts exist [19–22], our approach adds to the knowledge in two important ways. First, we include more recent studies into the meta-analysis. In the emerging literature on GM crop impacts, new studies are published continuously, broadening the geographical area covered, the methods used, and the type of outcome variables considered. For instance, in addition to other impacts we analyze effects of GM crop adoption on pesticide quantity, which previous meta-analyses could not because of the limited number of observations for this particular outcome variable. Second, we go beyond average impacts and use meta-regressions to explain impact heterogeneity and test for possible biases.

Our meta-analysis concentrates on the most important GM crops, including herbicide-tolerant (HT) soybean, maize, and cotton, as well as insect-resistant (IR) maize and cotton. For these crops, a sufficiently large number of original impact studies have

been published to estimate meaningful average effect sizes. We estimate mean impacts of GM crop adoption on crop yield, pesticide quantity, pesticide cost, total production cost, and farmer profit. Furthermore, we analyze several factors that may influence outcomes, such as geographic location, modified crop trait, and type of data and methods used in the original studies.

Materials and Methods

Literature search

Original studies for inclusion in this meta-analysis were identified through keyword searches in relevant literature databanks. Studies were searched in the ISI Web of Knowledge, Google Scholar, EconLit, and AgEcon Search. We searched for studies in the English language that were published after 1995. We did not extend the review to earlier years, because the commercial adoption of GM crops started only in the mid-1990s [23]. The search was performed for combinations of keywords related to GM technology and related to the outcome of interest. Concrete keywords used related to GM technology were (an asterisk is a replacement for any ending of the respective term; quotation marks indicate that the term was used as a whole, not each word alone): GM*, "genetically engineered", "genetically modified", transgenic, "agricultural biotechnology", HT, "herbicide tolerant", Roundup, Bt, "insect resistant". Concrete keywords used related to outcome variables were: impact*, effect*, benefit*, yield*, economic*, income*, cost*, soci*, pesticide*, herbicide*, insecticide*, productivity*, margin*, profit*. The search was completed in March 2014.

Most of the publications in the ISI Web of Knowledge are articles in academic journals, while Google Scholar, EconLit, and AgEcon Search also comprise book chapters and grey literature such as conference papers, working papers, and reports in institutional series. Articles published in academic journals have usually passed a rigorous peer-review process. Most papers presented at academic conferences have also passed a peer-review process, which is often less strict than that of good journals though. Some of the other publications are peer reviewed, while many are not. Some of the working papers and reports are published by research institutes or government organizations, while others are NGO publications. Unlike previous reviews of GM crop impacts, we did not limit the sample to peer-reviewed studies but included all publications for two reasons. First, a clear-cut distinction between studies with and without peer review is not always possible, especially when dealing with papers that were not published in a journal or presented at an academic conference [24]. Second, studies without peer review also influence the public and policy debate on GM crops; ignoring them completely would be short-sighted.

Of the studies identified through the keyword searches, not all reported original impact results. We classified studies by screening titles, abstracts, and full texts. Studies had to fulfill the following criteria to be included:

- The study is an empirical investigation of the agronomic and/or economic impacts of GM soybean, GM maize, or GM cotton using micro-level data from individual plots and/or farms. Other GM crops such as GM rapeseed, GM sugarbeet, and GM papaya were commercialized in selected countries [23], but the number of impact studies available for these other crops is very small.
- The study reports GM crop impacts in terms of one or more of the following outcome variables: yield, pesticide quantity (especially insecticides and herbicides), pesticide costs, total variable costs, gross margins, farmer profits. If only the number of pesticide sprays was reported, this was used as a proxy for pesticide quantity.
- The study analyzes the performance of GM crops by either reporting mean outcomes for GM and non-GM, absolute or percentage differences, or estimated coefficients of regression models that can be used to calculate percentage differences between GM and non-GM crops.
- The study contains original results and is not only a review of previous studies.

In some cases, the same results were reported in different publications; in these cases, only one of the publications was included to avoid double counting. On the other hand, several publications involve more than one impact observation, even for a single outcome variable, for instance when reporting results for different geographical regions or derived with different methods (e.g., comparison of mean outcomes of GM and non-GM crops plus regression model estimates). In those cases, all observations were included. Moreover, the same primary dataset was sometimes used for different publications without reporting identical results (e.g., analysis of different outcome variables, different waves of panel data, use of different methods). Hence, the number of impact observations in our sample is larger than the number of publications and primary datasets (Data S1). The number of studies selected at various stages is shown in the flow diagram in Figure 1. The number of publications finally included in the meta-analysis is 147 (Table S1).

Effect sizes and influencing factors

Effect sizes are measures of outcome variables. We chose the percentage difference between GM and non-GM crops for five different outcome variables, namely yield, pesticide quantity, pesticide cost, total production cost, and farmer profits per unit area. Most studies that analyze production costs focus on variable costs, which are the costs primarily affected through GM technology adoption. Accordingly, profits are calculated as revenues minus variable production costs (profits calculated in this way are also referred to as gross margins). These production costs also take into account the higher prices charged by private companies for GM seeds. Hence, the percentage differences in profits considered here are net economic benefits for farmers using GM technology. Percentage differences, when not reported in the original studies, were calculated from mean value comparisons between GM and non-GM or from estimated regression coefficients.

Since we look at different types of GM technologies (different modified traits) that are used in different countries and regions, we do not expect that effect sizes are homogenous across studies. Hence, our approach of combining effect sizes corresponds to a random-effects model in meta-analysis [25]. To explain impact heterogeneity and test for possible biases, we also compiled data on a number of study descriptors that may influence the reported effect sizes. These influencing factors include information on the type of GM technology (modified trait), the region studied, the type of data and method used, the source of funding, and the type of publication. All influencing factors are defined as dummy variables. The exact definition of these dummy variables is given in Table 1. Variable distributions of the study descriptors are shown in Table S2.

Statistical analysis

In a first step, we estimate average effect sizes for each outcome variable. To test whether these mean impacts are significantly

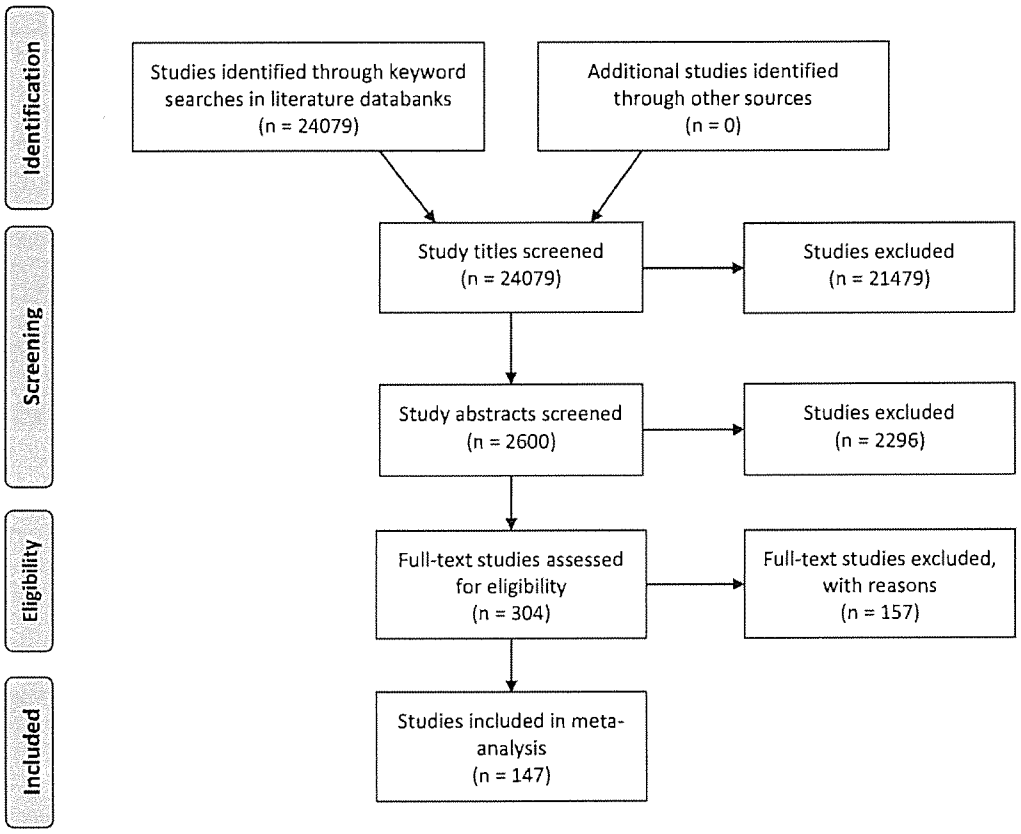

Figure 1. Selection of studies for inclusion in the meta-analysis.
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different from zero, we regress each outcome variable on a constant with cluster correction of standard errors by primary dataset. Thus, the test for significance is valid also when observations from the same dataset are correlated. We estimate average effect sizes for all GM crops combined. However, we expect that the results may differ by modified trait, so that we also analyze mean effects for HT crops and IR crops separately.

Meta-analyses often weight impact estimates by their variances; estimates with low variance are considered more reliable and receive a higher weight [26]. In our case, several of the original studies do not report measures of variance, so that weighting by variance is not possible. Alternatively, weighting by sample size is common, but sample sizes are also not reported in all studies considered, especially not in some of the grey literature publications. To test the robustness of the results, we employ a

Table 1. Variables used to analyze influencing factors of GM crop impacts.

Variable name	Variable definition
Insect resistance (IR)	Dummy that takes a value of one for all observations referring to insect-resistant GM crops with genes from *Bacillus thuringiensis* (Bt), and zero for all herbicide-tolerant (HT) GM crops.
Developing country	Dummy that takes a value of one for all GM crop applications in a developing country according to the World Bank classification of countries, and zero for all applications in a developed country.
Field-trial data	Dummy that takes a value of one for all observations building on field-trial data (on-station and on-farm experiments), and zero for all observations building on farm survey data.
Industry-funded study	Dummy that takes a value of one for all studies that mention industry (private sector companies) as source of funding, and zero otherwise.
Regression model result	Dummy that takes a value of one for all impact observations that are derived from regression model estimates, and zero for observations derived from mean value comparisons between GM and non-GM.
Journal publication	Dummy that takes a value of one for all studies published in a peer-reviewed journal, and zero otherwise.
Journal/academic conference	Dummy that takes a value of one for all studies published in a peer-reviewed journal or presented at an academic conference, and zero otherwise.

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different weighting procedure, using the inverse of the number of impact observations per dataset as weights. This procedure avoids that individual datasets that were used in several publications dominate the calculation of average effect sizes.

In a second step, we use meta-regressions to explain impact heterogeneity and test for possible biases. Linear regression models are estimated separately for all of the five outcome variables:

$$\%\Delta Y_{hij} = \alpha_h + \mathbf{X}_{hij}\boldsymbol{\beta}_h + \varepsilon_{hij}$$

$\%\Delta Y_{hij}$ is the effect size (percentage difference between GM and non-GM) of each outcome variable h for observation i in publication j, and $\mathbf{X}_{hij}$ is a vector of influencing factors. α_h is a coefficient and $\boldsymbol{\beta}_h$ a vector of coefficients to be estimated; ε_{hij} is a random error term. Influencing factors used in the regressions are defined in Table 1.

Results and Discussion

Average effect sizes

Distributions of all five outcome variables are shown in Figure S1. Table 2 presents unweighted mean impacts. As a robustness check, we weighted by the inverse of the number of impact observations per dataset. Comparing unweighted results (Table 2) with weighted results (Table S3) we find only very small differences. This comparison suggests that the unweighted results are robust.

On average, GM technology has increased crop yields by 21% (Figure 2). These yield increases are not due to higher genetic yield potential, but to more effective pest control and thus lower crop damage [27]. At the same time, GM crops have reduced pesticide quantity by 37% and pesticide cost by 39%. The effect on the cost of production is not significant. GM seeds are more expensive than non-GM seeds, but the additional seed costs are compensated through savings in chemical and mechanical pest control. Average profit gains for GM-adopting farmers are 69%.

Results of Cochran's test [25], which are reported in Figure S1, confirm that there is significant heterogeneity across study observations for all five outcome variables. Hence it is useful to further disaggregate the results. Table 2 shows a breakdown by modified crop trait. While significant reductions in pesticide costs are observed for both HT and IR crops, only IR crops cause a consistent reduction in pesticide quantity. Such disparities are expected, because the two technologies are quite different. IR crops protect themselves against certain insect pests, so that spraying can be reduced. HT crops, on the other hand, are not protected against pests but against a broad-spectrum chemical herbicide (mostly glyphosate), use of which facilitates weed control. While HT crops have reduced herbicide quantity in some situations, they have contributed to increases in the use of broad-spectrum herbicides elsewhere [2,11,19]. The savings in pesticide costs for HT crops in spite of higher quantities can be explained by the fact that broad-spectrum herbicides are often much cheaper than the selective herbicides that were used before. The average farmer profit effect for HT crops is large and positive, but not statistically significant because of considerable variation and a relatively small number of observations for this outcome variable.

Impact heterogeneity and possible biases

Table 3 shows the estimation results from the meta-regressions that explain how different factors influence impact heterogeneity. Controlling for other factors, yield gains of IR crops are almost 7 percentage points higher than those of HT crops (column 1). Furthermore, yield gains of GM crops are 14 percentage points higher in developing countries than in developed countries. Especially smallholder farmers in the tropics and subtropics suffer from considerable pest damage that can be reduced through GM crop adoption [27].

Most original studies in this meta-analysis build on farm surveys, although some are based on field-trial data. Field-trial results are often criticized to overestimate impacts, because farmers may not be able to replicate experimental conditions. However, results in Table 3 (column 1) show that field-trial data do not overestimate the yield effects of GM crops. Reported yield gains from field trials are even lower than those from farm surveys. This is plausible, because pest damage in non-GM crops is often more severe in farmers' fields than on well-managed experimental plots.

Table 2. Impacts of GM crop adoption by modified trait.

Outcome variable	All GM crops	Insect resistance	Herbicide tolerance
Yield	21.57*** (15.65; 27.48)	24.85*** (18.49; 31.22)	9.29** (1.78; 16.80)
n/m	451/100	353/83	94/25
Pesticide quantity	−36.93*** (−48.01; −25.86)	−41.67*** (−51.99; −31.36)	2.43 (−20.26; 25.12)
n/m	121/37	108/31	13/7
Pesticide cost	−39.15*** (−46.96; −31.33)	−43.43*** (−51.64; −35.22)	−25.29*** (−33.84; −16.74)
n/m	193/57	145/45	48/15
Total production cost	3.25 (−1.76; 8.25)	5.24** (0.25; 10.73)	−6.83 (−16.43; 2.77)
n/m	115/46	96/38	19/10
Farmer profit	68.21*** (46.31; 90.12)	68.78*** (46.45; 91.11)	64.29 (−24.73; 153.31)
n/m	136/42	119/36	17/9

Average percentage differences between GM and non-GM crops are shown with 95% confidence intervals in parentheses. *, **, *** indicate statistical significance at the 10%, 5%, and 1% level, respectively. *n* is the number of observations, *m* the number of different primary datasets from which these observations are derived.
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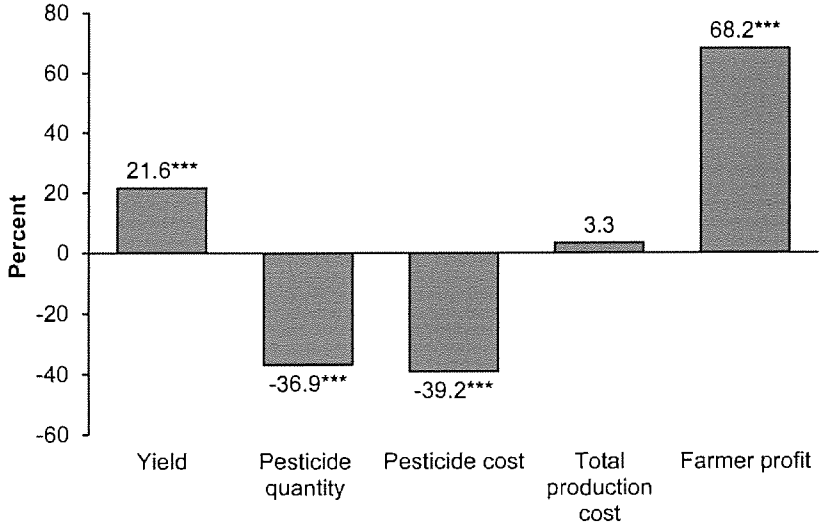

Figure 2. Impacts of GM crop adoption. Average percentage differences between GM and non-GM crops are shown. Results refer to all GM crops, including herbicide-tolerant and insect-resistant traits. The number of observations varies by outcome variable; yield: 451; pesticide quantity: 121; pesticide cost: 193; total production cost: 115; farmer profit: 136. *** indicates statistical significance at the 1% level.
doi:10.1371/journal.pone.0111629.g002

Another concern often voiced in the public debate is that studies funded by industry money might report inflated benefits. Our results show that the source of funding does not significantly influence the impact estimates. We also analyzed whether the statistical method plays a role. Many of the earlier studies just compared yields of GM and non-GM crops without considering possible differences in other inputs and conditions that may also affect the outcome. Net impacts of GM technology can be estimated with regression-based production function models that control for other factors. Interestingly, results derived from regression analysis report higher average yield effects.

Finally, we examined whether the type of publication matters. Controlling for other factors, the regression coefficient for journal publications in column (1) of Table 3 implies that studies published in peer-reviewed journals show 12 percentage points higher yield gains than studies published elsewhere. Indeed, when only including observations from studies that were published in journals, the mean effect size is larger than if all observations are included (Figure S2). On first sight, one might suspect publication bias, meaning that only studies that report substantial effects are accepted for publication in a journal. A common way to assess possible publication bias in meta-analysis is through funnel plots [25], which we show in Figure S3. However, in our case these funnel plots should not be over-interpreted. First, only studies that report variance measures can be included in the funnel plots, which holds true only for a subset of the original studies used here. Second, even if there were publication bias, our mean results would be estimated correctly, because we do include studies that were not published in peer-reviewed journals.

Further analysis suggests that the journal review process does not systematically filter out studies with small effect sizes. The journal articles in the sample report a wide range of yield effects, even including negative estimates in some cases. Moreover, when combining journal articles with papers presented at academic conferences, average yield gains are even higher (Table 3, column 2). Studies that were neither published in a journal nor presented at an academic conference encompass a diverse set of papers, including reports by NGOs and outspoken biotechnology critics. These reports show lower GM yield effects on average, but not all meet common scientific standards. Hence, rather than indicating publication bias, the positive and significant journal coefficient may be the result of a negative NGO bias in some of the grey literature.

Concerning other outcome variables, IR crops have much stronger reducing effects on pesticide quantity than HT crops (Table 3, column 3), as already discussed above. In terms of pesticide costs, the difference between IR and HT is less pronounced and not statistically significant (column 4). The profit gains of GM crops are 60 percentage points higher in developing countries than in developed countries (column 6). This large difference is due to higher GM yield gains and stronger pesticide cost savings in developing countries. Moreover, most GM crops are not patented in developing countries, so that GM seed prices are lower [19]. Like for yields, studies published in peer-reviewed journals report higher profit gains than studies published elsewhere, but again we do not find evidence of publication bias (column 7).

Conclusion

This meta-analysis confirms that – in spite of impact heterogeneity – the average agronomic and economic benefits of GM crops are large and significant. Impacts vary especially by modified crop trait and geographic region. Yield gains and pesticide reductions are larger for IR crops than for HT crops. Yield and farmer profit gains are higher in developing countries than in developed countries. Recent impact studies used better data and methods than earlier studies, but these improvements in study design did not reduce the estimates of GM crop advantages. Rather, NGO reports and other publications without scientific peer review seem to bias the impact estimates downward. But even with such biased estimates included, mean effects remain sizeable.

One limitation is that not all of the original studies included in this meta-analysis reported sample sizes and measures of variance. This is not untypical for analyses in the social sciences, especially when studies from the grey literature are also included. Future

Table 3. Factors influencing results on GM crop impacts (%).

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Variables	**Yield**	**Yield**	**Pesticide quantity**	**Pesticide cost**	**Total cost**	**Farmer profit**	**Farmer profit**
Insect resistance (IR)	6.58** (2.85)	5.25* (2.82)	−37.38*** (11.81)	−7.28 (5.44)	5.63 (5.60)	−22.33 (21.62)	−33.41 (21.94)
Developing country	14.17*** (2.72)	13.32*** (2.65)	−10.23 (8.99)	−19.16*** (5.35)	3.43 (4.78)	59.52*** (18.02)	60.58*** (17.67)
Field-trial data	−7.14** (3.19)	−7.81** (3.08)	–#	−17.56 (11.45)	−10.69* (5.79)	–#	–#
Industry-funded study	1.68 (5.30)	1.05 (5.21)	37.04 (23.08)	−7.77 (10.22)	–#	–#	–#
Regression model result	7.38* (3.90)	7.29* (3.83)	9.67 (10.40)	–#	–#	−11.44 (24.33)	−9.85 (24.03)
Journal publication	12.00*** (2.52)	–	9.95 (6.79)	−3.71 (4.09)	−3.08 (3.30)	48.27*** (15.48)	–
Journal/academic conference	–	16.48*** (2.64)	–	–	–	–	65.29*** (17.75)
Constant	−0.22 (2.84)	−2.64 (2.86)	−4.44 (10.33)	−16.13 (4.88)	−1.02 (4.86)	8.57 (24.33)	−1.19 (24.53)
Observations	451	451	121	193	115	136	136
R^2	0.23	0.25	0.20	0.14	0.12	0.12	0.14

Coefficient estimates from linear regression models are shown with standard errors in parentheses. Dependent variables are GM crop impacts measured as percentage differences between GM and non-GM. All explanatory variables are 0/1 dummies (for variable definitions see Table 1). The yield models in columns (1) and (2) and the farmer profit models in columns (6) and (7) have the same dependent variables, but they differ in terms of the explanatory variables, as shown. *, **, *** indicate statistical significance at the 10%, 5%, and 1% level, respectively. # indicates that the variable was dropped because the number of observations with a value of one was smaller than 5.

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impact studies with primary data should follow more standardized reporting procedures. Nevertheless, our findings reveal that there is robust evidence of GM crop benefits. Such evidence may help to gradually increase public trust in this promising technology.

Supporting Information

Figure S1 Histograms of effect sizes for the five outcome variables.
(PDF)

Figure S2 Impacts of GM crop adoption including only studies published in journals.
(PDF)

Figure S3 Funnel plots for the five outcome variables.
(PDF)

Table S1 List of publications included in the meta-analysis.
(PDF)

Table S2 Distribution of study descriptor dummy variables for different outcomes.
(PDF)

Table S3 Weighted mean impacts of GM crop adoption.
(PDF)

Data S1 Data used for the meta-analysis.
(PDF)

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Author Contributions

Conceived and designed the research: WK MQ. Analyzed the data: WK MQ. Contributed to the writing of the manuscript: WK MQ. Compiled the data: WK.

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Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variances

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The aim of the present study was to assess possible adverse effects of transgene expression in leaves of field-grown barley relative to the influence of genetic background and the effect of plant interaction with arbuscular mycorrhizal fungi. We conducted transcript profiling, metabolome profiling, and metabolic fingerprinting of wild-type accessions and barley transgenics with seed-specific expression of (1,3-1, 4)-β-glucanase (GluB) in Baronesse (B) as well as of transgenics in Golden Promise (GP) background with ubiquitous expression of codon-optimized *Trichoderma harzianum* endochitinase (ChGP). We found more than 1,600 differential transcripts between varieties GP and B, with defense genes being strongly overrepresented in B, indicating a divergent response to subclinical pathogen challenge in the field. In contrast, no statistically significant differences between ChGP and GP could be detected based on transcriptome or metabolome analysis, although 22 genes and 4 metabolites were differentially abundant when comparing GluB and B, leading to the distinction of these two genotypes in principle component analysis. The coregulation of most of these genes in GluB and GP, as well as simple sequence repeat-marker analysis, suggests that the distinctive alleles in GluB are inherited from GP. Thus, the effect of the two investigated transgenes on the global transcript profile is substantially lower than the effect of a minor number of alleles that differ as a consequence of crop breeding. Exposing roots to the spores of the mycorrhizal *Glomus* sp. had little effect on the leaf transcriptome, but central leaf metabolism was consistently altered in all genotypes.

food safety | glucanase | chitinase | sustainability

Breeding for improved grain weight, higher grain yield, disease resistance, and climatic adaptation by selection of spontaneous mutations shaped the modern barley (*Hordeum vulgare* L.) crop plant beginning as early as 10,000 years ago. With the technical advance to generate transgenic crops with improved agronomic performance, it has become necessary to assess the substantial equivalence of transgenic crop plants; that is, validate that no undesired side effect of the genetic modification has occurred relative to their parental lines (see ref. 1 for review). The availability of the "omics" techniques opens the possibility to probe substantial equivalence in nontargeted global analyses, providing unbiased results.

We have recently developed a 44-K barley microarray based on the assembly of 444,652 barley ESTs into 28,001 contigs and 22,937 singletons, of which 13,265 are represented on the array (2). In contrast, a comprehensive analysis of the metabolome (i.e., all metabolites in a specimen) is not possible because of the immense diversity of primary and secondary plant metabolites (3, 4). Thus, investigating the metabolome requires the prioritization of metabolite subsets as defined by their physicochemical properties or abundance. Although approaches to metabolite profiling are fueled by a multitude of individual targeted metabolite assays of high specificity and accuracy, metabolite fingerprinting aims at obtaining global metabolite patterns by NMR- or MS-based applications, only allowing for suboptimal recovery of individual metabolites (3).

When applied to pathway-engineered transgenic plants, global transcriptome and metabolome analyses could not reveal substantial differences between genetically modified (GM) and non-GM plants. No significant alterations in transcriptome were exhibited in wheat plants expressing *Aspergillus fumigatus* phytase compared with the corresponding non-GM variety, except for changes associated with seed development (5). Similarly, GC-MS-analyzed fructan-producing transgenic potato tubers did not exhibit significant changes, except for metabolites directly connected to the introduced pathway (6), and *Arabidopsis* expressing up to three *Sorghum bicolor* genes involved in the biosynthesis of the cyanogenic glucoside dhurrin did not exhibit any robust transcriptional changes compared to the parental lines (7).

Assessing the influence of natural genotypic variation and environmental factors on multiparallel datasets is of paramount importance to better evaluate the impact of transgene expression. To avoid unnecessary bias, the regarded transgene should not directly influence metabolic pathways in the target plant. An NMR comparison of wheat-flour metabolome derived from field-grown transgenic wheat expressing high molecular weight glutenin and the corresponding parental line revealed that, despite some differences in central free amino acid and sugar metabolism between GM and non-GM varieties, year and field site had a stronger effect on the dataset than expression of the transgene (8). Metabolome analysis of *Bt*-maize by NMR also revealed significant differences in free amino acid contents of the parental line; however, other likely-influential factors were not assessed in this study (9). Comparative transcript profiling of different maize cultivars harboring an identical *Bt* transgene insertion event revealed that the variability between cultivars was much greater than the influence of the transgene (10, 11). Independently, comparison of the potato tuber proteome of 21

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The authors declare no conflict of interest.

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Data deposition footnote: Microarray data obtained in this study can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19296.

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tetraploid cultivars with eight potato landraces and five transgenic potato lines led to the same conclusion (12).

In the present study, we investigated two transgenic barley cultivars. The first, hereafter termed ChGP, was designed for ubiquitous expression of a secreted, codon-optimized 42-kDa endochitinase *cThEn(GC)* from *Trichoderma harzianum* (13) in the variety Golden Promise (GP). *Trichoderma* chitinases can degrade rigid fungal cell walls of mature hyphae, conidia, chlamydospores, and sclerotia, in addition to the soft structure of hyphal tips (14–16). Recombinant *cThEn(GC)* conferred growth inhibition to the necrotrophic fungal root pathogens *Rhizoctonia oryzae* and *Rhizoctonia solani* AG8 in vitro (13). Overexpression of *cThEn(GC)* in tobacco and potato yielded high levels of transgene expression, and the transgenics displayed medium-level to complete-resistance phenotypes toward the necrotrophic fungal pathogens *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, and *R. solani* (17).

The second transgenic line employed in the study was pJH271 Beta-Glu-307 (271.06 × Baronesse), hereafter termed GluB, that exhibits hordein-D-promoter-driven, endosperm-specific expression of the chimeric heat-stable (1,3-1,4)-β-glucanase from *Bacillus amyloliquefaciens* and *Bacillus macerans* (18). The GluB transgenics were generated in the GP background, outcrossed to the elite cultivar Baronesse (B), and selected for high yield and good field performance by the single-seed descent method. GluB plants accumulate the recombinant enzyme in storage protein vacuoles and lack β-glucan in endosperm cell walls (19, 18). Expression of (1,3-1,4)-β-glucanase in the endosperm improves the nutritional value of barley for poultry (20, 21).

Making use of comparative, parallel transcriptome profiling, targeted metabolome profiling, and nontargeted metabolite fingerprinting, the present study assesses substantial equivalence in leaves of field-grown transgenic barley relative to the variation between cultivars and to the effects caused by the interaction with mycorrhizal fungi.

Results

Generation and Analysis of Transgenic Barley Plants Expressing Recombinant *Trichoderma* Endochitinase. We constitutively expressed the codon-optimized recombinant *T. harzianum* endochitinase ThEn42(GC) (13) in barley *cv.* Golden Promise either (*i*) fused to the barley chitinase 26 (HvChi26) secretion signal peptide driven by the Cauliflower mosaic virus 35S (CaMV 35S) promoter (Fig. S1*A*) or (*ii*) fused to the chitinase 33 (HvChi33) secretion signal peptide and driven by the maize ubiquitin promoter (Fig. S1*B*). After identification of primary transformants with expression of recombinant endochitinase by immunological detection and subsequent selection for homozygous T_1 transformants (*SI Materials and Methods*), we chose for further study two Ubi::ChGP (Ubi::ChGP-9 and -19) transgenic lines and one 35S::ChGP transgenic line that exhibited the strongest endochitinase expression. We assayed tissue specificity of chitinase activity (Fig. 1*A* and *SI Materials and Methods*, with a quantitative method using the fluorogenic substrate methylumbelliferyl-chitotrioside (Fig. S2*A*). Chitinase activity was highest in coleoptiles, reaching up to 320 $\mu g \cdot g^{-1}$ FW in both Ubi::ChGP lines and 280 $\mu g \cdot g^{-1}$ FW in 35S::ChGP-36 (Fig. 1*A*). Although CaMV 35S-driven chitinase expression in roots was close to that in leaves, ubiquitin-promoter-driven chitinase expression was 6- and 3-fold lower in roots compared with primary leaves or coleoptiles, respectively. Both Ubi::ChGP lines yielded very similar results.

To assess, whether chitinase expression confers antifungal activity, we checked if resistance to *R. solani* AG8 was increased in the transgenics, being quantified as number of wilted leaves per plant (Fig. 1*B* and *SI Materials and Methods*). Compared with the GP wild-type, only 35S::ChGP plants exhibited significantly milder disease symptoms after 1 week of cocultivation with *R. solani* AG8. This finding suggests that, despite the proof of concept obtained from the 35S::ChGP plants, endochitinase amounts might be too low in roots of the two Ubi::ChGP lines to diminish susceptibility toward the highly virulent *R. solani* AG8. Nevertheless, we selected line Ubi::ChGP-9 for further experiments to minimize effects of chitinase expression on the growth of challenging fungi in the field, which could influence both the transcriptome and the metabolome.

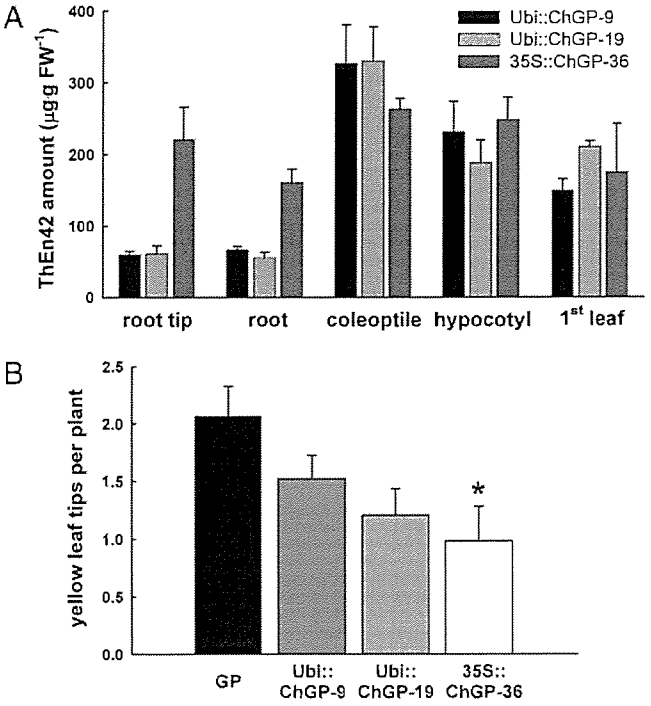

Fig. 1. Characterization of ChGP transformants tissue-specific accumulation of endochitinase ThEn42 in ChGP transformants and its effect on root infections by *R. solani* AG8. (*A*) Amounts of endochitinase in tissues of ChGP transformants. Seedlings of the transgenic barley lines Ubi::ChGP-9 (black bars), Ubi::ChGP-19 (light gray bars), and 35S::ChGP-36 (dark gray bars). Endochitinase content in root tips, upper parts of the roots, coleoptiles, hypocotyls, and first leaves (*Left* to *Right*) were determined with a fluorometric assay (Fig. S2*A* and *SI Materials and Methods*). Data are the mean of five replicate samples ± SEM (*B*) Reduced disease symptoms on ChGP transformants after root inoculation with *R. solani* AG8 (*SI Materials and Methods*). Significant differences to GP with $P < 0.05$ are indicated by an asterisk above the bars and were calculated with a Welch's modified *t* test (29).

Metabolome Analysis of Field-Grown Barley Leaves. We conducted both a targeted metabolite profiling and a metabolite fingerprinting approach with field-grown plants of the four barley genotypes GP, ChGP, B, and GluB that were cultivated in the field at the Giessen Experimental Station (Giessen, Germany; *SI Materials and Methods*). The plants were grown with and without amendment of soil in the plots with Amykor (Amykor Wurzel-Vital), a mixture of the mycorrhizal fungi *Glomus mosseae* and *Glomus intraradices*. Targeted analysis of 72 metabolites, including major carbohydrates, free amino acids, carboxylates, phosphorylated intermediates, major antioxidants (such as ascorbate, glutathione, and tocopherol), as well as carotenoids (for complete dataset, see Table S1), revealed only three differences associated with endochitinase expression in the GP background. In contrast, the contents of sucrose, starch, the amino acids Gln, Ala, and Leu, as well as of the carboxylate oxoglutarate were significantly reduced in GluB compared to B (Table S1). Comparisons of the two unmodified varieties B and GP revealed more consistent differences (e.g., UDPGlc and the amino acids, Tyr, Phe, Ala, Leu, and Cys) (Fig. 2*A*). Furthermore, several consistent changes in central leaf metabolism

in response to Amykor treatment were revealed (Fig. 2*B*): although the amounts of free hexoses and central phosphorylated intermediates (3PGA, PEP, RuBP, Glc1P), free inorganic phosphate and the carboxylates isocitrate and malate increased upon treatment, the contents of sucrose, the two major amino acids Glu and Asp, as well as chlorophyll, lutein, and glutathione all decreased in response to mycorrhizal inoculation (Fig. 2*B*).

The elevated pool sizes of several phosphorylated intermediates indicate improved phosphate availability as a possible consequence of successful mycorrhizal root colonization. Thus, it was instructive to determine the extent of mycorrhizal root colonization in plots with and without treatment with Amykor. Quantification of fungal genomic DNA by qPCR based on the *G. mossae* ITS sequence relative to host ubiquitin revealed increased fungal abundance in roots from treated compared with untreated plots (Fig. S3), despite considerable amounts of fungal DNA in plants (e.g., in GP) from untreated plots. Furthermore, microscopic analysis of plants grown in plots treated with Amykor confirmed arbuscle formation in all specimens (*SI Materials and Methods*), demonstrating that mycorrhiza were intact and functional. To validate that the characteristic changes in the leaf metabolome were brought about by improved mycorrhizal colonization of plants in the Amykor-treated plots, we determined the same targeted metabolome profile of ChGP and GP plants grown under controlled conditions in the greenhouse with soil that was either devoid of mycorrhizal inoculum or fortified with the same dosage of Amykor as in the field experiment. The contents of phosphorylated intermediates and hexoses were altered in a similar fashion between mycorrhizal and non-mycorrhizal plants of both examined genotypes in the field and in the greenhouse experiment (Table S2), providing strong indication for successful symbiotic interactions in all genotypes in the field experiment (*SI Materials and Methods*).

In a principal component analysis (PCA), the targeted metabolome data were able to distinguish both the effect of mycorrhizal infection and cultivar-specific differences by principal components 1 (PC1) and PC2, respectively (Fig. 3*A*; see Fig. S4*A* for the corresponding hierarchical cluster analysis). Interestingly, the metabolite profile in GluB transgenics was less affected by mycorrhizal infections compared to the other genotypes, and GluB was more distant to non-GM B plants than the ChGP transgenics was from their wild-type counterpart. As indicated by individual metabolite contents (mentioned in the previous paragraph), sugars, major amino acids, and phosphorylated intermediates strongly loaded on PC1 in response to mycorrhizal infections. Likewise, sugar and free amino acid contents contributed strongly to PC2, distinguishing cultivar-specific differences. Nearly identical results were obtained when data from the 307 most significant mass signals of a metabolite fingerprinting approach were fed into the PC analysis (Fig. 3*B*), except that GP and ChGP were more distant to each other in the Amykor treatment compared to untreated samples.

Transcriptome Analysis of Field-Grown Barley Leaves. Our next goal was to compare the discriminatory power of the metabolome analysis to that of the corresponding transcriptome dataset obtained from identical sample pools (*SI Materials and Methods*). PCA resulted in a similar discrimination of genotypes as reported for the metabolome analyses, with GluB again being distant from B (Fig. 3*C*; see Fig. S4*B* for the corresponding hierarchical cluster analysis). In contrast to the metabolome analysis, treatment with Amykor could not be clearly resolved in the PC analysis; indeed, no statistically significant differentially transcribed genes were detected in three of the four examined genotypes in response to the Amykor treatment. Only in GP, 4 out of 31,198 features detected on the array were differentially expressed in response to the mycorrhizal fungi (Table S3). However, 1,660 genes (697 up

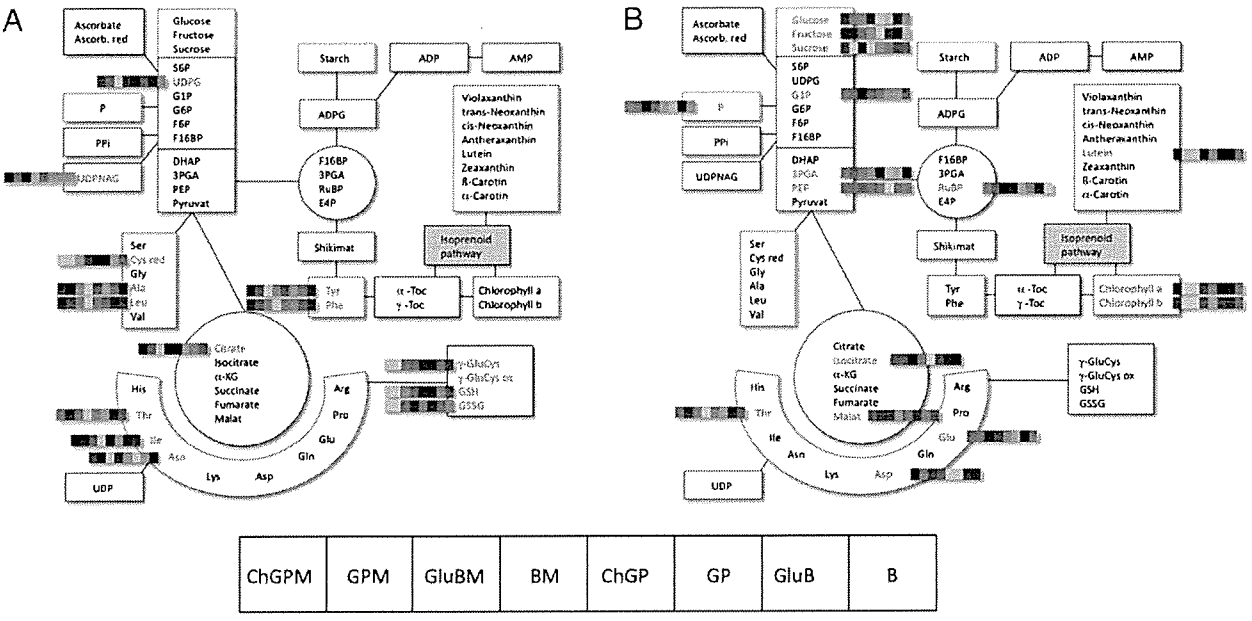

Fig. 2. Differentially abundant metabolites in barley leaves. Overview of differentially abundant metabolites from the targeted profiling approach with leaf material from 4-month-old, field-grown barley plants representing the treatments (*A*) cultivar or (*B*) Amykor. The schematic metabolic diagrams in (*A*) and (*B*) represent a map of all analyzed metabolites. The heat map strips next to the metabolite names were taken from the hierarchical cluster analysis (Fig. S4*A*) conducted with the program Cluster v2.11 (30), with red signals denoting an increased metabolite content relative to average and green signals indicating decreased metabolite contents relative to average. The consistent sample order in these strips is indicated at the bottom of the figure using the genotype and treatment abbreviations used throughout the text and as explained below. The entire dataset, including the results of the significance tests, are given in Table S1. Please note that the color pattern has no implications on statistically significant differences in pairwise comparisons, which were calculated with a Welch-Satterthwaite test embedded in the VANTED software v1.7 (31) and are displayed in Table S1. GP, Golden Promise; B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; M, Amykor treatment.

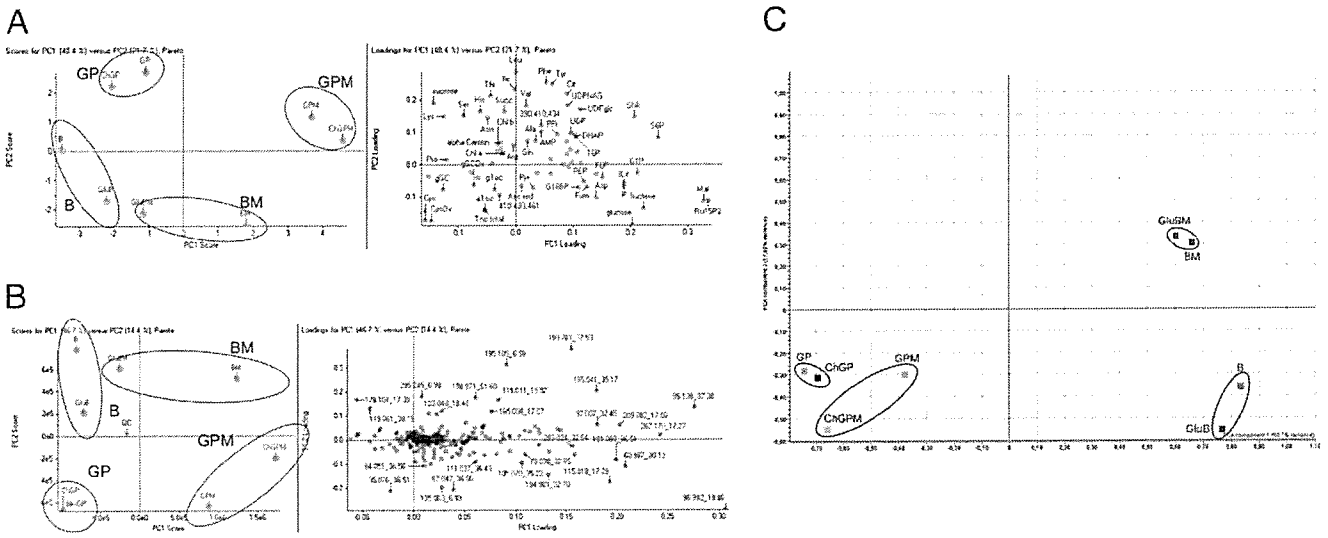

Fig. 3. Principal component analysis (PCA) of multiparallel datasets obtained from field-grown barley leaves. (*A*) PCA based on 72 metabolites that were analyzed in a targeted fashion (complete dataset displayed in Table S1): For PCA, mean values of four replicate samples per genotype and treatment were taken and the resulting data points labeled as described below. (*Left*) PCA plot of principal component 1 (PC1) versus principal component 2 (PC2). Circles are drawn around spots derived from the genotype of identical cultivar or treatment and are labeled by letters as indicated below. (*Right*) loadings plot for PC1 and PC2. The 72 metabolites are individually labeled. (*B*) PCA of metabolite fingerprinting data. The analysis was computed for the 307 most significant mass signals obtained by metabolite fingerprinting and is based on mean values from four replicate samples (see *Materials and Methods*). Compounds are labeled according to the quantified transition. Data arrangement and labeling are as described in *A*. (*C*) PCA of transcriptome data. PCA was performed based on data from two replicate hybridizations per genotype and treatment. RNA was extracted from aliquots of pooled sample material also used for metabolome analysis. From the 1,660 genes differentially expressed between cultivars B and GP (Table S3), five of the most significant ones were confirmed by qRT-PCR analysis of independent sample aliquots (Fig. S2*B*). GP, Golden Promise; B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; M, Amykor treatment.

and 863 down) were differentially transcribed between the cultivars GP and B (Tables S3 and Dataset S1), indicating strong cultivar-specific expression patterns. The result of the microarray data analysis was confirmed in independent sample pools by qRT-PCR, picking five genes with cultivar-specific transcript abundance (Fig. S2*B*). Along with genes involved in carbohydrate metabolism and genes coding for storage proteins, defense-associated genes were strongly overrepresented among the differentially regulated genes in the GP to B comparison (Fig. S2*C*). This result likely reflects a greater level of disease resistance obtained deliberately or fortuitously over years of breeding and selection for ever better-adapted and higher-yielding modern barley varieties. Of particular interest, 22 differentially transcribed genes were found between GluB and its non-GM counterpart B (Table S3), corresponding with the distance of these two genotypes in the PCA. Sixteen of these 22 differential genes, (i.e., approx. 73%) were also discriminated in the GP to B comparison. Although surprising at first glance, this finding can be explained by the pedigree of GluB. GluB was produced by transformation of GP with glucanase transgene, which was later introduced into the cultivar B by outcrossing and selection of single-seed descendants. Thus, differential gene expression between GluB and B could be caused by retention of a few GP alleles in the GluB genotype.

To obtain data in support of this hypothesis, we attempted to refine the chromosomal location of the 16 genes that were differentially transcribed in both the GluB to B and the GP to B comparisons on the current genetic map of barley (http://www.harvest-web.org/hweb/bin/gmap.wc?wsize=1263 × 854). Although 14 of the unigenes had no assigned map position, 2 could be located between 142 cM and 167 cM on the lower arm of chromosome 7H. Analysis of two simple sequence repeat (SSR) markers located in the region of interest revealed that both carried the GP allele (Fig. 4 and Fig. S5).

Discussion

The comprehensive dataset generated in the present study provides a comparison of the alterations in leaf transcriptome and metabolome caused by (*i*) the presence of transgenes, (*ii*) cultivar, and (*iii*) biotic interactions in the root. This dataset leads us to four major conclusions.

First, the effect of recombinant *Trichoderma* chitinase on both the metabolome and transcriptome was negligible compared to the differences between the wild-type cultivars GP and B.

Second, the metabolome analysis has proven to be as sensitive as the survey of the corresponding transcriptome as a means to detect minor differences between B and the out-bred transgenic GluB. In addition, both targeted and untargeted metabolome analyses discerned an influence of mycorrhizal infection on leaf

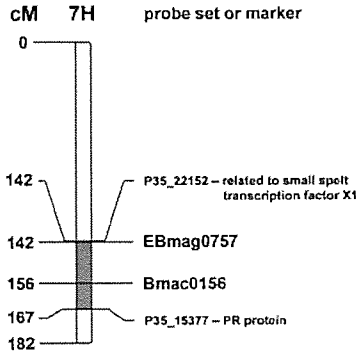

Fig. 4. Inheritance of GP alleles in GluB on the lower arm of barley chromosome 7H. Two of the 16 unigenes differentially expressed in both the B vs. GP and the B vs. GluB comparisons could be mapped to the current physical barley map (www.harvest-web.org) and were located at 142 cM and 167 cM on chromosome 7H, respectively. Analysis of the two polymorphic SSR markers EBmag0757 (142 cM) and Bmac0156 (156 cM) revealed retention of the respective GP alleles in GluB that had been generated by introgressing the *GluB* transgene from GP into B. Locations of the employed markers and the two unigenes of interest on the genetic map are given on the left, their names and annotations are to the right to the chromosomal sketch.

metabolism that was not achieved by transcriptome analysis alone. In targeted experiments, on the other hand, differential display was used successfully to identify transcripts of five differentially expressed genes from leaves of mycorrhiza-colonized tomato (22). Based on our results, metabolome analysis represents a more immediate probe of physiological status of the plant than the transcriptome. We have found that a subset of phosphorylated intermediates of central metabolism was more abundant in leaves of Amykor-treated than in control plants, reflecting improved phosphate availability in treated plants.

Third, comparisons of the metabolome and transcriptome between the transgenic GluB and wild-type B revealed four differentially abundant metabolites and 22 deregulated transcripts, suggesting a more distant positioning in the PCA between these two lines, as compared to ChGP and GP. Although the reason for the deviation in the metabolome of GluB and B remain elusive, about 73% of the differentially transcribed genes between GluB and B were similarly deregulated between GP and B. The evidence for genetic linkage of 2 out of the 16 coregulated genes between GluB and GP by SSR marker analysis indicates that the differences in the transcript profiles of GluB and B could be attributed to retention of introgressed GP traits in the GluB background. This finding could also hold true for the differences in the GluB and B metabolome profiles. Our finding suggests that introgression of a few alleles can convey a stronger effect on substantial equivalence than the introduction of the two regarded transgenes.

Fourth, compared with the previously addressed slight changes, the data compiled for GP and B revealed 1,660 differentially regulated genes and a considerable, albeit minor, number of steady-state metabolite pools that were substantially different. Targeted qRT-PCR analysis of five genes that strongly differed in expression between GP and B disclosed that, for most of them, transcripts were only specifically abundant in one of the two cultivars. Defense-associated genes such as pathogenesis-related gene-4 (*PR-4*) were overrepresented in the 1,660 genes. Because we did not include a substantial number of defense-related metabolites in our targeted metabolome analysis, the difference in defense priming between GP and B remained obscure in the metabolite dataset. As no symptoms of infection were visible on any genotype at sampling date, our data indicates that subclinical or latent infections at the field site had triggered defense-gene expression. Thus, our results suggest that B, unlike GP, was in an alert state with basal expression of pathogenesis-related genes. The variety GP lacks most resistance genes (23) and exhibits a much weaker defense response compared to bred high-end varieties. We can thus estimate that past breeding of elite lines, such as B for putative disease resistance, represent the strongest effect on global gene expression between cultivars in the field, where plants are subjected to perpetual challenge by microbial pathogens and pests. Such large differences are not expected to be caused by single transgenes, although evidence on pathogen-challenged disease-resistant GM crops is thus far unavailable. Although resistance toward pathogen challenge in the field should be increased in ChGP because of the presence of endochitinase, transcript profiles of ChGP, and because GP did not exhibit significant differences, unlike the B to GP comparison described above. This result means that endochitinase expression did not affect the transcriptome in challenged plants. In comparison, strong differences in transcript profiles of Bt maize compared with non-GM cultivars were to be expected upon corn borer infestation, representing sick and healthy plants, respectively.

Conclusion

In summary, our results substantially extend observations that cultivar-specific differences in transcriptome and metabolome greatly exceed effects caused by transgene expression. Furthermore, we provide evidence that, (*i*) the impact of a low number of alleles on the global transcript and metabolite profile is stronger than transgene expression and that, more specifically, (*ii*) breeding for better adaptation and higher yields has coordinately selected for improved resistance to background levels of root and leaf diseases, and this selection appears to have an extensive effect on substantial equivalence in the field during latent pathogen challenge.

Materials and Methods

Barley Seed. The seed used in this study represented barley lines pYW210-9-(4001-4360), pYW210-19-(4701-6100), pYW300-36-(7121-7187), pJH271-Beta Glu-307 and the cultivars Golden Promise and Baronesse. Line pYW210-9-(4001-4360), termed ChGP, and lines pYW210-19-(4701-6100) and pYW300-36-(7121-7187), which are constitutively expressing endochitinase ThEn42 (GC) from *T. harzianum* (13), were produced for this study (see below). Line pJH271-Beta Glu-307, termed GluB, constitutively expresses a thermostable (1,3-1,4)-β-glucanase and was described earlier (18).

Double-Cassette Vector Construction with the Ubiquitin Promoter and Barley Transformation. For construction of plasmid pYW300 (Fig. S1*A*), the Cauliflower mosaic virus 35S promoter was amplified from plasmid *pBI221* (Clontech Inc.), digested with *Hin*dIII and *Pst*I, and inserted into *Hin*dIII- and *Pst*I-digested plasmid pAM110-cTHEn42(GC) (Fig. S1*C*). The *Hin*dIII-*Not*I fragment of this plasmid was moved into pAM300b (Fig. S1*D*), and the *Hin*dIII-*Eco*RI fragment from this intermediate vector was then inserted into the pJH 260 binary vector as described for the vectors with the ubiquitin promoter (see below). The sequence of the plasmid pYW300 has been assigned GenBank Accession number DQ469639.

Plasmid pYW210 (Fig. S1*B*) was constructed in the binary cloning vector pJH260 derived from pBIN19, as follows: The fragment containing *cThEn42(GC)* provided with the *pUbi 1* promoter and the *SP(HvChi33)* signal sequence was excised from plasmid pAM110-cThEn42(GC) (Fig. S1*C*) with *Hin*dIII and *Not*I. The resulting fragment was inserted into *Hin*dIII-/*Not*I-digested plasmid pAM300b (Fig. S1*D*), yielding plasmid pAM300. A *Hin*dIII / *Eco*RI fragment of the insert was cloned into pJH260 to produce plasmid pYW210 (GenBank Accession Number DQ469636). For barley transformation, see *SI Materials and Methods*.

Metabolome Profiling and Metabolite Fingerprinting. Targeted analysis of free amino acids (24), major leaf carbohydrates (25), ascorbic acid, tocopherols and glutathione (26), carotenoids (2), phosphorylated intermediates and carboxylates (27) was conducted as previously described.

Untargeted metabolome profiling by ESI-MS was carried out on a QTrap3200 mass spectrometer (Applied Biosystems) after metabolite extraction and ion exchange chromatography as described (26). Negative Ions were generated at −4.5 kV and a declustering potential of −20 V. The entrance potential was from −6 to −4 V, and gas pressures were 20 psi (curtain), 30 psi (nebulizer), and 20 psi (turbogas). A mass range of *m/z* 60–610 was recorded with one scan per second over 80 min. Peak alignment was performed after import into Marker View (Applied Biosystems) with a retention time tolerance of 0.75 min and a mass tolerance of 1.0 amu. Maximal number of peaks was set to 500. Retention time corrections and normalization was done with the internal standard pipes (*m/z* 301.1; RT 16.6 min). For PC analysis of fingerprinting data, quality control samples were generated as described (28) by pooling equal-volume amounts from all analyzed samples. Artifact peaks were removed before the analysis was conducted with MarkerView.

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REVIEW ARTICLE

An overview of the last 10 years of genetically engineered crop safety research

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Abstract

The technology to produce genetically engineered (GE) plants is celebrating its 30th anniversary and one of the major achievements has been the development of GE crops. The safety of GE crops is crucial for their adoption and has been the object of intense research work often ignored in the public debate. We have reviewed the scientific literature on GE crop safety during the last 10 years, built a classified and manageable list of scientific papers, and analyzed the distribution and composition of the published literature. We selected original research papers, reviews, relevant opinions and reports addressing all the major issues that emerged in the debate on GE crops, trying to catch the scientific consensus that has matured since GE plants became widely cultivated worldwide. The scientific research conducted so far has not detected any significant hazards directly connected with the use of GE crops; however, the debate is still intense. An improvement in the efficacy of scientific communication could have a significant impact on the future of agricultural GE. Our collection of scientific records is available to researchers, communicators and teachers at all levels to help create an informed, balanced public perception on the important issue of GE use in agriculture.

Keywords

Biodiversity, environment, feed, food, gene flow, -omics, substantial equivalence, traceability

History

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Introduction

Global food production must face several challenges such as climate change, population growth, and competition for arable lands. Healthy foods have to be produced with reduced environmental impact and with less input from non-renewable resources. Genetically engineered (GE) crops could be an important tool in this scenario, but their release into the environment and their use as food and feed has raised concerns, especially in the European Union (EU) that has adopted a more stringent regulatory framework compared to other countries (Jaffe, 2004).

The safety of GE crops is crucial for their adoption and has been the object of intense research work. The literature produced over the years on GE crop safety is large (31 848 records up to 2006; Vain, 2007) and it started to accumulate even before the introduction of the first GE crop in 1996. The dilution of research reports with a large number of commentary papers, their publication in journals with low impact factor and their multidisciplinary nature have been regarded as negative factors affecting the visibility of GE crop safety research (Vain, 2007). The EU recognized that the GE crop safety literature is still often ignored in the public debate even if a specific peer-reviewed journal (http://journals.cambridge.org/action/displayJournal?jid=ebs) and a publicly accessible database (http://bibliosafety.icgeb.org/) were created with the aim of improving visibility (European Commission, 2010).

We built a classified and manageable list of scientific papers on GE crop safety and analyzed the distribution and composition of the literature published from 2002 to October 2012. The online databases PubMed and ISI Web of Science were interrogated to retrieve the pertinent scientific records (Table S1 – Supplementary material). We selected original research papers, reviews, relevant opinions and reports addressing all the major issues that emerged in the debate on GE crops. The 1783 scientific records collected are provided in .xls and .ris file formats accessible through the common worksheet programs or reference manager software (Supplementary materials). They were classified under the scheme given in Table 1, according to the major issues emerging from the literature. Beyond a numerical analysis of the literature, we provide a short explanatory summary of each issue.

General literature (GE gen)

Here we group all the reviews and critical comments offering a broad view of the issues concerning the release of the GE crops into the environment and their use as food and feed, including the regulatory frameworks and risk assessment procedures.

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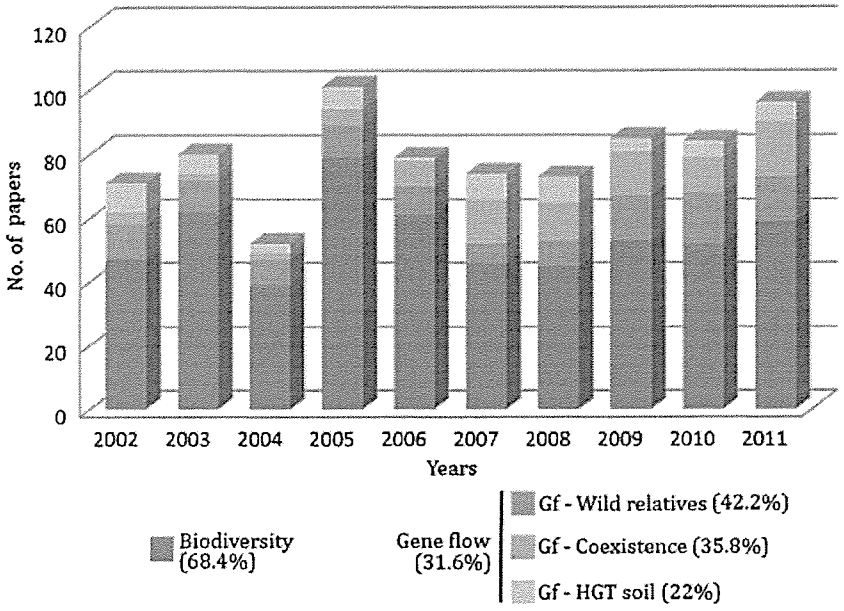
Table 1. Classification of 1783 scientific records on GE crop safety published between 2002 and 2012.

Topic	No. of papers	%*
General literature (GE gen)	166	9.3
Interaction of GE crops with the environment (GE env)	847	47.5
Biodiversity	579	32.5
Gene flow	268	15
Gf – Wild relatives	113	6.3
Gf – Coexistence	96	5.4
Gf – Horizontal gene transfer in soil	59	3.3
Interaction of GE crops with humans and animals (GE food&feed)	770	43.2
Substantial equivalence	46	2.6
Non-targeted approaches to equivalence assessment	107	6
GE food/feed consumption	312	17.5
Traceability	305	17.1

*Percentage of the total number of collected papers.

Figure 1. Main topics of the scientific papers belonging to the GE env group.



The weight of the **GE gen** section, in terms of number or records, is low in our database (9.3% – 166/1783) compared to **GE env** (47.5% – 847/1783) and **GE food&feed** (43.2% – 770/1783) (Table 1). The literature grouped in **GE gen** reflects the difference between the EU and the US regulatory frameworks: the former is based on the evaluation of the process by which the GE crop is obtained and the application of the precautionary principle, the latter is based on the evaluation of the product. The adoption of such different concepts resulted in the need for new legislation and new authorities in the EU, whereas in the US new regulations were integrated into the existing legislation and institutions (Jaffe, 2004).

Other countries have been inspired by these two systems in developing their own regulatory framework (Ramessar et al., 2008). As a result, the regulations on the release of GE crops into the environment and their use as food and feed are not uniform (Gómez-Galera et al., 2012; Jaffe, 2004; McHughen & Smyth, 2008; Ramessar et al., 2008). This lack of harmonization, and the frequent non-scientific disputes in the media that are not balanced by an effective communication from the scientific and academic world, greatly contribute to enhance the concerns on GE crops.

The EU funded more than 50 research programs in 2001–2010, for a total budget of 200 million euros, with the intent to gain new scientific evidence addressing the public concern on the safety of GE crops. A summary report of these programs highlighted that the use of biotechnology and of GE plants *per se* does not imply higher risks than classical breeding methods or production technologies (European Commission, 2010).

Interaction of GE crops with the environment (GEenv)

Biodiversity

Biodiversity preservation is unanimously considered a priority by the scientific community and society at large. This topic is predominant in **GE env** (68.4%) throughout the decade (Table 1; Figure 1). The literature is highly heterogeneous, since the potential impact of GE crops on biodiversity can be investigated at different levels (crop, farm and landscape) and different organisms or microorganisms (target and non-target) can be considered.

The GE crops commercialized so far are herbicide and/or pest resistant. Glyphosate tolerance obtained by

expressing an *Agrobacterium tumefaciens* enolpyruvyl shikimate 3-phosphate synthase (EPSPS), and the production of insecticidal proteins derived from *Bacillus thuringiensis* (Bt), are by far the most widespread GE traits.

The literature considering the effects on biodiversity of non-target species (birds, snakes, non-target arthropods, soil macro and microfauna) is large and shows little or no evidence of the negative effects of GE crops (Carpenter, 2011 and references therein; Raven, 2010; Romeis et al., 2013). Two reviews about pest resistant GE crops published by Lövei et al. (2005, 2009) reported negative impacts on non-target arthropods; however, these reports have been criticized mainly for the statistical methods and the generalizations between crops expressing Bt proteins (commercialized), proteinase inhibitors (only a transgenic cotton line SGK321 present in the Chinese market) and lectins (not commercialized) (Gatehouse, 2011; Shelton et al., 2009). Negative impacts of Bt plants on non-target arthropods and soil microfauna have not been reported in recent papers (e.g. de Castro et al., 2012; Devos et al., 2012; Lu et al., 2012; Verbruggen et al., 2012 Wolfenbarger et al., 2011). Indeed, the positive impacts have been emphasised.

If we consider the effect of GE crops on the target species, weeds or pests, a reduction of biodiversity is obviously expected and necessary for the success of the crop. For instance, cases of area-wide pest suppression due to the adoption of Bt crops (where also the non-adopters of GE crops received beneficial effects), have been reported (Carpenter, 2011 and references therein). This is also the case of the UK Farm Scale Evaluations (FSE), a series of studies which highlighted that the adoption of a management system based on herbicide tolerant GE crops generally resulted in fewer weeds and weed seeds. These results have been used as proof of the negative environmental impact of herbicide tolerant crops, but indeed they demonstrate the effectiveness of such a management system (Carpenter, 2011 and references therein). On the other hand, higher reductions on biodiversity is generally expected with non-GE crops and herbicide/insecticide applications, because the chemicals used are often more toxic and persistent in the environment (Ammann, 2005).

Concerns have been raised about possible outbreak of resistant populations of target species due to the high selection pressures produced by the repetitive sowing of GE herbicide and pest resistant crops. Glyphosate resistant weeds have been reported (Shaner et al., 2012), as well as Bt resistant pests (Baxter et al., 2011; Gassman et al., 2011). Glyphosate tolerance appears more relevant because, while new Bt proteins are available which can be combined in strategies of stacking, or pyramiding, to reduce the risks of insect resistance (Sanahuja et al., 2011), it seems difficult to find herbicides equivalent to glyphosate in terms of efficacy and environmental profile; therefore, proper management of weed control is necessary (Shaner et al., 2012).

Gene flow

In an agricultural context, gene flow can be defined as the movement of genes, gametes, individuals or groups of individuals from one population to another, and occurs both spatially and temporally (Mallory-Smith & Sanchez Olguin, 2011). For instance, GE crop plants may be capable of surviving through seed or asexual propagules for years in the field, or they may be able to fertilize sexually compatible non-GE plants (non-GE crop or wild relative plants). The occurrence of gene flow may lead to the spread and persistence of transgenes into the environment or the market.

We have subdivided this topic into three subgroups: gene flow to wild relatives (Gf – Wild relatives), to other crops (Gf – Coexistence) or to microorganisms (Gf – Horizontal gene transfer in the soil). The literature on *Gene flow* makes up 31.6% of the **GEenv** literature and is clearly a "hot topic" because its share increased considerably after 2006 (Table 1; Figure 1).

Gf – Wild relatives

This topic represents 42.2% of the *Gene flow* literature (Table1; Figure 1). For estimating the gene flow to wild relatives, the knowledge of several factors is necessary: the reproductive biology of the GE crop, the presence or absence of sexually compatible wild relatives within the reach of GE pollen, and the reproductive biology and the fitness of any hybrid.

The formation of hybrids between GE crops and wild relatives is possible and documented (Londo et al., 2010; Mizuguti et al., 2010). Hybrid fitness determines the chance of transgene introgression, that is, permanent incorporation into the wild receiving population, which was reported in some cases (Reichman et al., 2006; Schoenenberger et al., 2006; Warwick et al., 2008). The risk of introgression should be evaluated case-by-case, considering the features of the transgene(s) incorporated into the GE crop.

The presence of spontaneous populations of GE canola with multiple herbicide resistance genes, probably due to multiple events of hybridization, has been reported (Schafer et al., 2011). Zapiola and Mallory-Smith (2012) recently described a new herbicide tolerant intergeneric hybrid of transgenic creeping bentgrass. Other cases have been reviewed (Chandler & Dunwell, 2008). Pest-resistant GE crops (i.e. Bt crops) may pose more risks than herbicide-resistant crops, because the introgression of a pest resistance transgene may confer fitness advantages to wild plants. Pest resistant wild plant populations may in turn exert selective pressure on the pest populations even in the absence of transgenic crops.

Strategies to mitigate the effect of the transgene(s) in pre- and post -hybridization phases have been proposed (e.g. male sterility, delayed flowering, genes that reduce fitness). However, none of them can be considered completely effective for transgene containment and complete segregation of GE crops is not possible. In any case, there is no evidence of negative effects of transgene introgression so far (Kwit et al., 2011).

It should be kept in mind that the gene flow between cultivated and wild species and its impact on biodiversity is an issue that exists independently of GE crops. The literature is rich in examples of natural invasive hybrids, disappearance of local genotypes (genetic swamping) and resistance to herbicides appearing in wild populations due to natural mutation (Kwit et al., 2011).

Gf – Coexistence

Gene flow from a GE to a non-GE crop can lead to an unwanted presence of the transgene in non-GE products. This issue involves not only the movement of pollen, but also the seeds that could remain in the field and give rise to volunteers, and the mechanical admixture of materials occurring during harvest, transportation and storage. The establishment of populations becoming partially wild (ferals) functioning as a natural reservoir of the transgene must also be considered, as well as the survival chances of the GE crops in the wild.

The coexistence issue goes beyond the matter of gene flow and involves several social and economic aspects, such as the manageability of complex agricultural scenarios where different agricultural systems (organic, conventional and biotech) coexist and a full traceability system is in force.

The collected records on coexistence account for 35.8% of the *Gene flow* literature and their number increased significantly after 2006 (Table 1; Figure 1). Even in the US, the coexistence issue is becoming actively discussed (http://www.gmo-compass.org/eng/news/548.docu.html).

Strategies of coexistence have been investigated for several species, such as maize (Devos et al., 2008; Langhof et al., 2010; Rühl et al., 2011), canola (Colbach, 2008; Gruber et al., 2005), soybean (Gryson et al., 2009), flax (Jhala et al., 2011), wheat (Foetzki et al., 2012), potato, cotton and sugar beet (European Commission, 2006). Maize has been the most intensively studied crop, followed by canola and wheat. Isolation distances, harvesting and post-harvesting practices have been proposed in order to avoid unwanted mixing of GE and non-GE-crop.

The feasibility of a coexistence plan is not only evaluated from a scientific point of view but also considering the extra economic costs due to the containment practices; such extra costs must find compensation in extra income from GE crops (Demont & Devos, 2008). In the EU, the scenario on coexistence is very poor currently, considering that only three GE crops are authorized for cultivation (MON 810 and T25 maize and "Amflora" potato), with only MON810 actually commercialized, and Spain accounting for 87% of the entire cultivated surface with GE crops (James, 2011).

Gf – Horizontal gene transfer in soil

Soil microorganisms may uptake the transgene(s) present into the GE crop. In fact, bacteria are naturally capable of acquiring genetic material from other organisms through horizontal gene transfer (HGT). To obtain a GE plant it can be necessary to introduce a gene that makes it possible to select the transgenic cells in tissue culture, by giving them an advantage over the non-transgenic cells. This is frequently achieved with bacterial antibiotic resistance genes that play the role of selectable marker genes (SMGs, recently reviewed by Rosellini, 2012). SMG presence in GE crops is not necessary in the field, and it has raised concerns about the spread of antibiotic resistance genes into the environment and their consumption as food or feed (see below).

The transfer of these genes to bacteria and the possible outbreak of "super pathogenic bacteria" resistant to antibiotics has been a matter of detailed investigation by the scientific community. The number of publications on this topic accounts for 22% of the *Gene flow* literature, with a stable presence in recent years (Table 1; Figure 1).

The results obtained so far clearly indicate that soil bacteria can uptake exogenous DNA at very low frequency (10^{-4} to 10^{-8}) in laboratory experiments (Ceccherini et al., 2003; de Vries et al., 2003), whereas experiments in the field did not show any evidence of HGT (Badosa et al., 2004; Demanèche et al., 2008, 2011; Ma et al., 2011). Moreover, in the unlikely event that soil bacteria acquired the resistance to an antibiotic among those currently used in the laboratory to select GE plants, this would not affect the population of natural antibiotic resistant bacteria already present in the soil (D'Costa, 2006; Forsberg et al., 2012) or imply any additional risk for human and animal health.

The substitution of antibiotic SMGs with plant-derived genes (Rosellini, 2011, 2012), their elimination (Ferradini et al., 2011 and references therein) and in general the elimination of any unwanted DNA sequence in the final GE crop is recommended (EFSA, 2011), as proposed with new approaches to plant genetic engineering such as the so-called intragenic (Nielsen, 2003; Rommens, 2004) or cisgenic (Jacobsen & Schouten, 2007) techniques.

Interaction of GE crops with humans and animals (GE food&feed)

Substantial equivalence

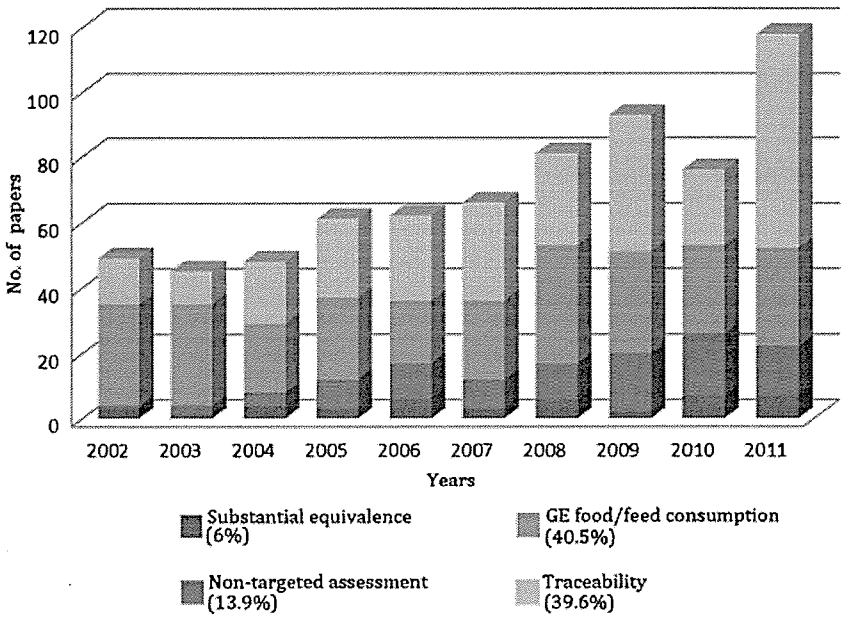
One of the crucial aspects of the risk assessment procedure for a GE crop is to verify if the insertion and/or the expression of the transgene produces alterations in the host organism. The concept of substantial equivalence implies that the GE crop be compared with an isogenic counterpart, that is, the same genotype without the transgene(s).

The demonstration of substantial equivalence is a two-step procedure. First, the GE crop is assessed for agronomic, morphological and chemical characteristics, such as macro- and micro-nutrients, anti-nutrients and toxic molecules. The results of this analysis will provide information on the necessity for further analysis of the nutritive value. Any difference which falls within the range of the normal variability for the crop is considered safe (Colquhoun et al., 2006; EFSA, 2011). This methodology has been agreed internationally (Codex, FAO, OECD, WHO) and involves the quantification of selected molecules, in a so-called "targeted approach" (Kok & Kuiper, 2003). If compositional differences are detected, then they have to be assessed with respect to their safety (Ramessar et al., 2007; EFSA, 2011).

The principle of substantial equivalence has been used for risk assessment of the GE crops commercialized so far (Kier & Petrick, 2008; König et al., 2004) and the results support the fact that these crops are equivalent to their non-transgenic counterparts (Parrot et al., 2010).

Concerns have been expressed about the efficacy of the method for detecting unintended effects. Field comparisons in multiple locations have been recommended in order to minimize the differences due to the environmental effects and large data collections have been created (www.cropcomposition.org).

Figure 2. Main topics of the scientific papers belonging to the GE food&feed group.



It is noteworthy that substantial equivalence represents an important common ground of the process-based and product-based regulatory frameworks. This clearly indicates a large consensus amongst scientists worldwide on GE crop evaluation (Kok et al., 2008). Substantial equivalence accounts for 6% of the scientific records collected in **GE food&feed** (Table 1; Figure 2). The literature is composed mainly by the publications produced by the companies that developed the GM cultivars, as part of the authorization process for commercialization. Public availability of the data on which these studies are based should be guaranteed.

Nontargeted approaches to equivalence assessment

The targeted approach to substantial equivalence assessment has an obvious limitation in the number of compounds that are analyzed. On the contrary, the so-called "–omic" approaches (transcriptomics, proteomics, metabolomics) can analyze a larger number of molecules (Kier & Petrick, 2008). Several GE crops were compared to their isogenic counterparts using –omic approaches and in some cases differences were observed. However, the interpretation of these results is difficult due to the non-homogeneity of the experimental designs. Moreover, the differences emerging from the –omic analyses have to be cleaned up from the environmental effects and their biological relevance weighted in terms of food and feed safety (Rieroch et al., 2011 and references therein).

It appears that the application of the –omics methods as standard procedure in the risk assessment of GE crop does not actually provide manageable information, and needs further development and validation. In this scenario, the substantial equivalence concept remains a robust and safe reference to determine the presence of unintended effects (European Commission, 2010). The weight of the nontargeted assessment topic increased significantly over the years, especially in 2009–2011 leading to a significant number of publications (13.9%) (Table 1; Figure 2).

GE food/feed consumption

The scientific records grouped under this topic are numerous and constitute 40.5% of the **GE food&feed** literature, clearly indicating the importance of the human health issues. The distribution over the year is uniform, but a peak was observed in 2008, probably due to the scientific fervors that followed the publication of experimental studies conducted by the private companies after 2006 (Table 1; Figure 2). According to the literature, the concerns about GE food/feed consumption that emerge from the scientific and social debates can be summarized as follows: safety of the inserted transgenic DNA and the transcribed RNA, safety of the protein(s) encoded by the transgene(s) and safety of the intended and unintended change of crop composition (Dona & Arvanitoyannis, 2009; Parrot et al., 2010).

Safety of the inserted transgenic DNA and the transcribed RNA

DNA. It is estimated that, with a normal diet, humans consume between 0.1 and 1 g of DNA/day from different sources (e.g. meat, vegetables) (Parrot et al., 2010). This DNA is partly digested, but it can also stimulate the immune-system or promote bacterial biofilm formation (Rizzi et al., 2012). The DNA sequences that drive the expression of the transgenes in the plant cell are generally derived from viruses or bacteria. Concerns have been expressed on the possibility that the transgenic DNA may resist the digestion process, leading to HGT to bacteria living in the gastrointestinal (GI) tract, or translocation and accumulation into the human body and food products from livestock animals. Some considerations can help to put this issue in context:

(a) transgenic DNA is enormously diluted by the total amount of ingested DNA (from 0.00006% to 0.00009%) and is digested like any other DNA (Parrot et al., 2010). In addition, food processing (e.g. baking, frying, boiling)

usually results in DNA degradation (Gryson, 2010; Rizzi et al., 2012) further reducing the amount of intact DNA;

(b) HGT of transgenic DNA to GI bacteria of human and animals is estimated to be an extremely rare event, as confirmed by all the experiments conducted so far (Rizzi et al., 2012). In the unlikely case that this event occurs, the worst scenario is characterized by the HGT of antibiotic resistance genes to GI bacteria, making them resistant to clinical therapies. However, the antibiotic resistance genes found into GE crops today do not present any significant risk to human or animal health (Ramessar et al., 2007), and they are already naturally present into the environment and/or the human/animal GI (EFSA, 2011; Wilcks & Jacobsen, 2010).

(c) DNA fragments can be transferred across the GI barrier. This natural phenomenon has been demonstrated only for high-copy-number genes that have been detected in internal organs, tissues and blood of different animals and even in cow milk (Parrot et al., 2010; Rizzi et al., 2012; van de Eede et al., 2004 and references therein). In humans, the transfer through the GI tract of a high-copy-number gene from rabbit meat has been reported (Forsman et al., 2003).

(d) Transgenic DNA transfer through the GI tract has been reported in the literature in pig, lamb and rainbow trout (Chainark et al., 2006, 2008; Mazza et al., 2005; Sharma et al., 2006;), but in micro quantities and in the case of pigs and lambs with questionable reproducibility due to possible cross contamination (Walsh et al., 2011).

(e) In most studies conducted so far, no fragments of transgenic DNA were detected in any animal-derived products (ILSI, 2008). Only in one case, the presence of transgenic DNA in both "organic" and "conventional" cattle milk has been reported (Agodi et al., 2006).

(f) No evidence has been obtained to date that DNA absorbed through the GI tract can be integrated into the cells of the host organism and lead to a germ line transfer.

It can be concluded that transgenic DNA does not differ intrinsically or physically from any other DNA already present in foods and that the ingestion of transgenic DNA does not imply higher risks than ingestion of any other type of DNA (European Commission, 2010).

RNA. Along with the DNA also the corresponding transcribed RNAs are ingested and in general the content of DNA and RNA in foods are roughly comparable (Parrot et al., 2010). In the light of recent scientific evidence (Zhang et al., 2012a discussed below) concerns have been expressed about the potential effects that certain types of RNA (small double-strand RNAs, dsRNAs) introduced in some GE crops (e.g. virus resistant, altered oil composition) could have on human/animal health.

The function of such dsRNAs is not to be translated into proteins but to mediate gene regulation through a mechanism termed RNA interference (RNAi). The general mechanism of RNAi is conserved across eukaryotes and is triggered by different types of dsRNAs including small interfering RNA (siRNAs) and microRNAs (miRNAs) (Melnyk et al., 2011).

Recently, Zhang et al., (2012a) reported the first evidence of transfer, through the mouse GI tract, of a food-derived exogenous miRNA (MIR168a) naturally abundant in rice and previously detected also in human blood. This study highlights the unexpected resistance of the rice MIR168a to heat treatment during cooking and to digestion during the transit through the GI tract in the mouse. Moreover, the authors showed significant activity of the MIR168a on the RNAi-mediated regulation of a protein involved in the removal of low-density lipoprotein (LDL) in liver cells (Zhang et al., 2012a). This evidence is still the object of debate at the scientific level and a summary of the major issues are reported here:

(a) miRNAs are naturally present in both animal and plant derived foods/feeds and with a reported similarity to human genes (Ivashuta et al., 2009; Petrick et al., 2013);

(b) Petrick et al. (2013) pointed out that previous studies on feeding rats with rice (Zhou et al., 2011, 2012) failed to provide evidence on any alteration on LDL. However, such studies may be difficult to compare as they were conducted on another species of rodent and with different methodological approaches (e.g. different fasting of the animals and composition of the diet);

(c) although the systemic transmission of dsRNAs has been demonstrated in plants, worms and insects, such transport in mammals is still largely unknown (Melnyk et al., 2011). In humans, the presence of endogenous miRNAs has been documented in microvesicles circulating in the bloodstream and their role in intercellular communication is currently under investigation (Mittelbrunn & Sánchez-Madrid, 2012 and references therein);

(d) the results presented by Zhang et al. (2012a) are not in agreement with that documented in numerous clinical trials involving oral delivery of small RNA molecules. The stability of the dsRNAs in the GI tract and an efficient absorption through the mucosa in order to reach the active concentration of the molecule in the bloodstream, are still the limiting factors in this therapeutic approach (Petrick et al., 2013 and references therein);

(e) some miRNAs are active even at low concentrations and plant miRNAs seem to differ structurally from mammalian miRNAs (Yu et al., 2005; Zhang et al. 2012a; http://www.the-scientist.com/?articles.view/articleNo/31975/title/Plant-RNA-Paper-Questioned/);

(f) interestingly, Zhang et al. (2012b) detected the MIR168a sequence as predominant or sole plant miRNA in public animal small RNA datasets including insects. The authors point out that this may be an artifact due to the sequencing methodology employed (i.e. cross-contamination of the multiplexed libraries).

It can be concluded, that the RNA in general has the same "history of safe use" as DNA, since it is a normal component of the diet (Parrot et al., 2010). However, further investigations are necessary to clarify whether the evidence about the MIR168a is due to its unique properties or such conclusions can also be extended to other dsRNAs molecules contained in food/feed.

Safety of the proteins encoded by the transgenes

The expression of the introduced gene(s) leads to biosynthesis of one or more proteins. The ingestion of transgenic proteins has posed some questions about their possible toxic or allergenic effects in humans and animals. The safety of each transgenic protein is evaluated by means of the following analyses:

- bioinformatic analysis to assess the similarity with known allergens, toxic proteins and bioactive peptides;
- functional stability to pH and temperature;
- *in vitro* digestibility using simulate mammalian gastric fluid and simulated mammalian intestinal fluid, following the principle that a digested protein is less likely to be allergenic and absorbed in a biologically active form;
- protein expression level and dietary uptake, to estimate exposure of humans or animals to the protein;
- single dose (acute) toxicity testing and repeated dose (sub-chronic) toxicity testing in rodents using the purified transgenic protein, to predict *in vivo* possible toxic outcome in humans (Delaney et al., 2008; EFSA, 2008).

The results of these analyses are usually part of the documentation that GE crops developers submit to the competent authorities during the approval phase (risk assessment) that precede the commercialization of a GE crop. These data are not always made accessible by the companies or the competent authorities or published on peer-reviewed journals (Jaffe, 2004). However, as indicated by the significant increment of the publications after 2006, it seems that the GE crop developers acknowledged the necessity of an improved transparency (Domingo & Bordonaba, 2011). The experimental data collected so far on authorized GE crops can be summarized as follows:

(a) there is no scientific evidence of toxic or allergenic effects;
(b) some concern has been raised against GE corn MON 810, MON863 and NK603 (de Vendômois et al., 2009; Séralini et al., 2007, 2012), but these experimental results have been deemed of no significance (EFSA 2007, 2012; Houllier, 2012; Parrot & Chassy, 2009);
(c) only two cases are known about the potential allergenicity of transgenic proteins, the verified case of the brazil-nut storage protein in soybean, which has not been marketed (Nordlee et al., 1996) and the not verified case of maize Starlink (Siruguri et al., 2004);
(d) during the digestion process the proteins generally undergo degradation that leads to the loss of activity (Delaney et al., 2008);
(e) even though there are examples of some ingested proteins that are absorbed in minute quantities in an essentially intact form (e.g. ovalbumin, ovomucoid, β-lactoglobulin) (Kier & Petrick, 2008) or proteins that are hydrolyzed into smaller absorbed bioactive peptides (Udenigwe & Aluko, 2012), the consumption of transgenic proteins contained in the authorized GE crop does not result in any detectable systemic uptake (Kier & Petrick, 2008) and transgenic proteins are usually rapidly degraded and not detectable in animal derived products (e.g. milk, meat, eggs) (Ramessar et al., 2007);
(f) pre-screening of transgenic proteins through bioinformatic analyses contributes to avoid the introduction of potentially toxic, allergenic or bioactive proteins into food and feed crops (Delaney et al., 2008; Gibson, 2006; Ladics et al., 2011);
(g) the application of the concept of "history of safe use" to the choice the transgene donor organisms may increase intrinsic safety and simplify safety assessment procedures.

Safety of the intended and unintended changes of crop composition

Safety of the introduced change in the GE crop is usually evaluated during the determination of compositional equivalence (Section "Substantial equivalence"). However, on a case-by-case basis, additional analyses can be requested, such as that of processed foods or feeds, nutritional equivalence and 90-day rodent feeding tests with whole GE food or feed (EFSA, 2008, 2011).

A useful distinction can be introduced here between GE crops modified for input traits (e.g. herbicide or insect resistance) and GE crops with enhanced nutritional characteristics (e.g. increased vitamin content). For the former, the experience suggests that, once the compositional equivalence has been verified, little can be added by the other types of analysis, and nutritional equivalence can be assumed (EFSA, 2011).

On the contrary, for GE crops with improved nutritional characteristics, the nutritional equivalence cannot be assumed, and a nutritional animal feeding test using rapidly growing animals (e.g. broilers) should be conducted to demonstrate the intended nutritional effect. The high sensitivity of rapidly growing animals to toxic compounds may also help to detect unintended effects. The 90-day rodent feeding test is generally performed when the composition is modified substantially or if there are indications of potential unintended effects.

Only GE crops modified for agronomic traits have been authorized for commercialization so far, with the only exception of the "Amflora" potato (event EH92-527-1), intended for industrial purpose but authorized also for feed and nonintended consumption (http://ec.europa.eu/food/dyna/gm_register/gm_register_auth.cfm?pr_id=39).

It is noteworthy that, at the moment, the route to the authorization of GE crops intended only for industrial purposes is not fully clarified by the legislation. However, the results of animal tests are routinely presented to the European safety assessment authorities, even if not explicitly required (http://www.gmo-compass.org/eng/safety/human_health/41.evaluation_safety_gm_food_major_undertaking.html).

Recently, Podevin & Jardin (2012) pointed out that the viral promoter P35S, isolated from the cauliflower mosaic virus (CaMV) and used in several GE crops to achieve strong and constitutive expression of the transgene/s, partially overlaps with the CaMV viral gene VI. In some long variants of the P35S promoter this could potentially lead to the production of a residual viral protein. The use of the short version of the promoter is therefore recommended, even if the

bioinformatics analysis of the viral protein has not revealed any relevant similarity with known allergens (Podevin & Jardin, 2012).

An issue emerged about whether the combination of more GE traits in a single crop (GE stacks) may introduce changes that require additional safety assessment. Once safety of the single traits has been established independently, their combination should be evaluated in terms of stability, expression and possible interactions (EFSA, 2011). Weber et al. (2012) pointed out that GE stacks do not impose any additional risks in terms of transgene stability and expression, whereas attention should be focused only on the possible interactions between different traits.

Traceability

This is clearly a "hot topic" in **GE food&feed** (39.6%) (Table 1), with the publication rate after 2005 being high and constant (Figure 2). Traceability is defined in the EU General Food Law Regulation 178/2002/EC, inspired to the ISO standard, as the "ability to trace and follow food, feed, food producing animals and other substances intended to, or expected to, be incorporated into food or feed, through all stages of production, processing and distribution".

Traceability is a concept already widely applied to non-GE food/feed and it is not connected with their safety (Davison & Bertheau, 2007). It may include mandatory or voluntary labeling for the foods or feeds that contain or consist of GE crops or derived products. Labeling implies the definition of a threshold value, above which the food/feed is labeled according to the regulations in force.

The EU developed the most stringent regulatory framework for traceability of GE crops food/feed and derived products in the world. They have adopted mandatory labeling for unintentional presence of GE material in food or feed, with the lowest threshold value (0.9% based on the number of haploid genomes) compared to other countries (Davison & Bertheau, 2007; Ramessar et al., 2008). Labeling requires the detection and quantification of the GE food/feed or derived product in the tested food/feed or seeds or any other product when applicable. The scientific literature compiled about traceability largely deals with the following issues:

(a) sampling procedures – there are no universally acknowledged sampling procedures (Davison & Bertheau, 2007); this has been the object of a EU funded research programme (Paoletti et al., 2006);
(b) detection method – a large consensus has been established on qPCR (real-time quantitative PCR) -based methodologies that allows detection and quantification at the same time. Other experimental strategies and analytical methods have been proposed (e.g. microarray, Luminex XMAP), but they need further evaluation (Querci et al., 2010);
(c) definition of reference systems – the measurement unit of the GE product concentration depends on the unit used for the certified reference material (CRM) chosen for the analysis. At the moment, in the EU, mass fraction percentages are used for the CRMs, whereas a later recommendation from the EU suggested to use the "copy number of transgenic DNA in relation to haploid genomes", the unit of the legal threshold, so the development of suitable CRMs is necessary (Trapmann et al., 2009);
(d) detection of transgenes in mixtures composed by different ingredients, stacked transgenes and unauthorized events: all these issues require specific approaches and strategies have been proposed. The detection of the unauthorized events is very complex, because it could involve an already known transgene that did not receive authorization or a totally unknown GE event. Unfortunately, asynchronous authorization of GE crops or derived products in different countries does not improve this scenario: a higher degree of international harmonization would be beneficial (Holst-Jensen et al., 2012).

Conclusions

The technology to produce GE plants is celebrating its 30th anniversary. It has brought about a dramatic increase in scientific production over the years leading to high impact findings either in basic research (such as RNAi-mediated gene silencing) and applied research (GE crops), but the adoption of GE plants in the agricultural system has raised issues about environmental and food/feed safety.

We have reviewed the scientific literature on GE crop safety for the last 10 years that catches the scientific consensus matured since GE plants became widely cultivated worldwide, and we can conclude that the scientific research conducted so far has not detected any significant hazard directly connected with the use of GM crops. The analysis of the record list shows that the Biodiversity topic dominated, followed by Traceability and GE food/feed consumption, which contributed equally in terms of the number of records (Table 1; Figure 3).

It is noteworthy that the number of papers on Traceability has increased over the years, overcoming those on Biodiversity in 2011, clearly indicating an increasing demand for methods and protocols for transgene detection (Figure 3). The Gene flow issue also received increasing attention by the scientific community, as a response to the demands of the consumers connected with the coexistence of different productive systems (Figure 3).

It appears that knowledge on Gene flow and GE food/feed consumption would have benefited from a higher number of publications considering their high impact on both environmental and food/feed risk assessment. The difficulties of experimental design and, in the case of Gene flow, the public opposition to field trials, may have discouraged researchers, at least in the EU.

The literature about Biodiversity and the GE food/feed consumption has sometimes resulted in animated debate regarding the suitability of the experimental designs, the choice of the statistical methods or the public accessibility of data. Such debate, even if positive and part of the natural process of review by the scientific community, has frequently been distorted by the media and often used politically and inappropriately in anti-GE crops campaigns. In this regard, Houllier (2012) pointed out that, when

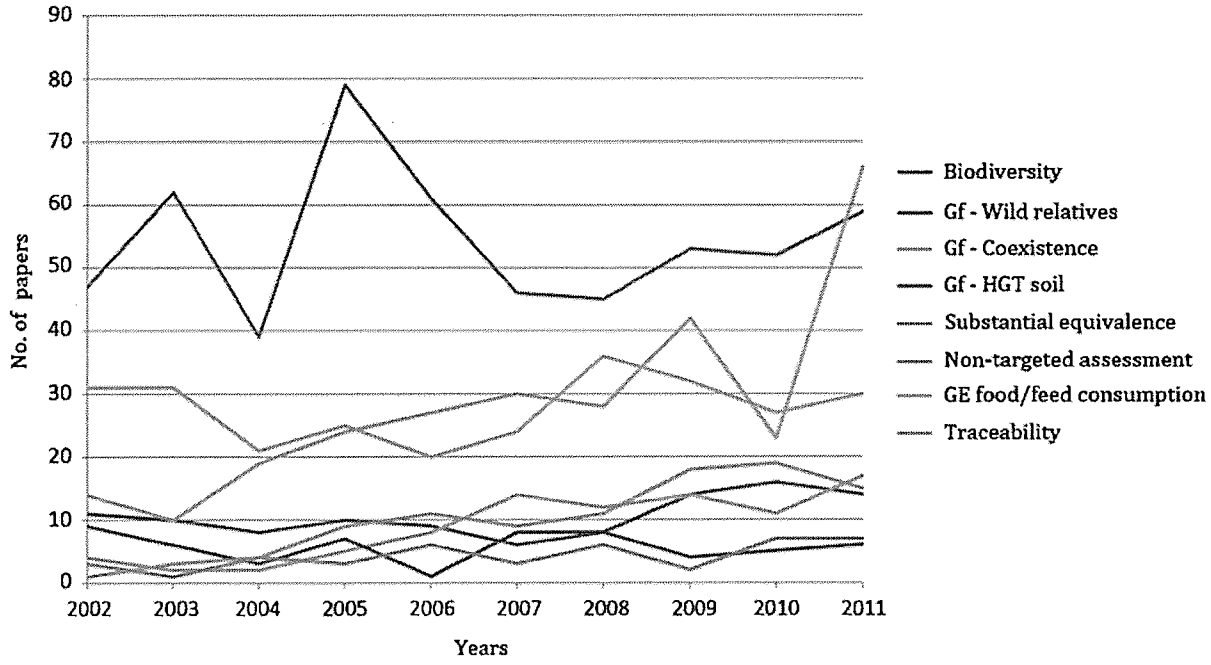


Figure 3. Distribution of the collected scientific papers. Records classified under the *General literature* are not shown.

dealing with "hot issues", researchers should take special care in following rigorous scientific standards, avoiding the publication of data not sufficiently peer reviewed by the scientific community.

It is interesting to note that the recent increase of scientific publications about Traceability and Non-targeted assessment (Figure 3) indicates considerable attention to the detection systems and the search for new safety evidence about a relatively low number of new approved GE crops. This likely reflects the consolidation of a situation in which the EU plays mainly the role of the importer of GE crop products from other countries, and enforces a stringent regulatory system.

In the EU, the regulatory burdens for GE crop approval are extremely heavy (Kalaitzandonakes et al., 2007), *de facto* excluding the public sector and minor crops from the development of GE technology. As a result, the number of experimental releases of GE crops is rapidly decreasing (Löchte, 2012) and even large companies are abandoning GE (Dixelius et al., 2012; Laursen, 2012). This scenario is the result of the interaction of complex sociological and psychological factors, risk/benefit ratios, political aspects and an unbalanced scientific communication.

All these factors have to be considered globally and taken into account in a constructive debate on whether the GE crops represent a strategic resource for the future. An improvement in the efficacy of the scientific communication to stakeholders, as clearly demonstrated in the case of the recent case of GE wheat field trials in the UK (Löchte, 2012), could have a significant impact on the future of agricultural GE.

We believe that genetic engineering and GE crops should be considered important options in the efforts toward sustainable agricultural production. Our collection of scientific records is available to researchers, communicators and teachers at all levels to help create an informed and balanced public perception on the hot issue of GE use in agriculture.

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Declaration of interest

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Supplementary material available online

Supplementary Table S1

New Biotechnology Volume 30, Number 4 May 2013 RESEARCH PAPER

Assessment of GE food safety using '-omics' techniques and long-term animal feeding studies

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Despite the fact that a thorough, lengthy and costly evaluation of genetically engineered (GE) crop plants (including compositional analysis and toxicological tests) is imposed before marketing some European citizens remain sceptical of the safety of GE food and feed. In this context, are additional tests necessary? If so, what can we learn from them? To address these questions, we examined data from 60 recent high-throughput '-omics' comparisons between GE and non-GE crop lines and 17 recent long-term animal feeding studies (longer than the classical 90-day subchronic toxicological tests), as well as 16 multigenerational studies on animals. The '-omics' comparisons revealed that the genetic modification has less impact on plant gene expression and composition than that of conventional plant breeding. Moreover, environmental factors (such as field location, sampling time, or agricultural practices) have a greater impact than transgenesis. None of these '-omics' profiling studies has raised new safety concerns about GE varieties; neither did the long-term and multigenerational studies on animals. Therefore, there is no need to perform such long-term studies in a case-by-case approach, unless reasonable doubt still exists after conducting a 90-day feeding test. In addition, plant compositional analysis and '-omics' profiling do not indicate that toxicological tests should be mandatory. We discuss what complementary fundamental studies should be performed and how to choose the most efficient experimental design to assess risks associated with new GE traits. The possible need to update the current regulatory framework is discussed.

Introduction

Safety assessment is structured, step-wise, and based on a comparative approach. The substantial equivalence concept according to the principles outlined in the Organization for Economic Cooperation and Development (OECD) consensus documents [1] encompasses a comparison of biochemical composition with a non-GE line considered to be safe. The GE line is compared to its near isogenic counterpart, according to specific determinants such as molecular characteristics, and agronomic and phenotypic traits [2]. Moreover, public consultation procedures have been established.

Despite the fact that an extensive and robust compositional assessment for evaluating the substantial equivalence of GE crop plants is currently imposed before market introduction (including current toxicological tests), some citizens remain sceptical of the safety of GE food and feed in the EU [3]. The first question is: may the improvement of a plant variety through transgenesis result in unintended effects which may be triggered by the insertion of a transgene? If so, could it impact on consumer and animal health? The second question regards the safety of animals: can long-term studies as well as multigenerational feeding studies detect potential unintended effects in animals? These questions have prompted new studies, carried out by public research laboratories using alternative evaluation techniques (i.e. not part of the regular evaluation process), namely high-throughput '-omics' profiling of

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GE varieties and long-term animal feeding studies as well as multi-generational feeding studies.

The aim of the present study was to investigate the data of these articles in the context of detection of possible unintended effects in GE plants.

Materials and methods

Our database 'BergeRicrochGMlibrary' of studies using transgenic plants is built from selected references from journal sites (such as Ingenta, PubMed, WoK). Search terms include 'transgen*' OR 'transgenic' OR 'GM' OR 'GE' OR 'engineered' OR 'modified' OR 'genetic* engineer*' OR 'genetic* modifi*' OR overexpress* OR 'insect* resist*' OR 'herbicide tolerant' OR 'glyphosate tolerant' AND 'plant' OR 'species' OR 'organism' OR 'crop'. This database is maintained by the analyses of tables of contents of journals from collections such as BioOne, Elsevier, ScienceDirect, Springer, etc. (507 journals); by the monitoring of 352 journals not covered by these collections; and by the monitoring of 530 other journals [4].

To perform a systematic review using an evidence-based decision approach, we followed a decision tree (Flow of Included Studies). We selected relevant search terms to sort a set of publications from potentially relevant studies identified and screened for retrieval to studies included in a literature review. For this review, the references were sorted using search terms related to 'omics'; 'transcriptomics'; 'proteomics'; 'metabolomics'; 'long-term'; 'mutigenerational'; 'feeding studies', 'Food safety', and 'Feed safety', 'food and feed'. Then selected papers on feeding studies are sorted using EndNote6 with a search of 'feeding studies', 'feed', 'fed' terms in titles and in abstracts separately. Long-term studies, that is longer than 90 days, extending over most of the lifetime of the test species, are used to assess the potential chronic toxicity and/or carcinogenicity for single defined substances [2].

Results

High-throughput profiling studies

Recent developments in biotechnology include the emerging technologies of '-omics' – transcriptomics, proteomics and metabolomics. Transcriptomics measures the steady-state mRNA abundance from a given tissue source. Proteomics is a technology for both qualitative and quantitative analyses of proteins, and investigations into protein posttranslational modifications. Metabolomics refers to the complete set of metabolites synthesized through a series of multiple enzymatic steps from various biochemical pathways.

High-throughput '-omics' profiling techniques have been suggested as a nontargeted approach to detect unintended effects in GE plants. The application of proteomics in food science [5] can address the safety issue of food of various origins, including transgenic food, in parallel to the transcriptomic and metabolomic approaches. We examined recently published profiling studies concerning major crop plants [6]. Here we update the evaluation of GE crops, so in total 60 profiling studies were published. The over-arching objective of our investigation is to explore the possibility, or not, of developing a new generation of '-omics' profiling for the assessment of commercial GE crop plants with regard to their nutritional equivalence and food safety. Studies using *Arabidopsis thaliana* as the laboratory model plant were not examined in this paper (for further details see [6]).

GE plants with new agronomic traits, but without deliberate modifications to metabolic pathways

These 36 studies concerned crops such as barley (1 study), cabbage (1), maize (11 and [7–9]), pea (2), potato (1), rice (4 and [10–16]), soybean (2 and [17]) and wheat (3). Here we do not discuss the studies on GE lines of tomato and tobacco producing pharmaceuticals nor the use of '-omics' to identify food allergens (see [6]).

Our examination reveals that '-omics' profiling of GE varieties were similar to non-GE counterparts, although some minor differences exist between GE lines and their comparator conventional control lines. For example, the amounts of some specific metabolites were higher or lower in the GE glyphosate-tolerant soybean line [18]; these differences could be explained by modification in the regulation of the shikimate pathway. Gene expression in leaves differs more between conventional varieties than between two GE glyphosate-tolerant varieties [19]. A study on wheat found that the different genetic background of the control lines resulted in a quantitatively different flavonoid content compared to the GE fungal-resistant line whereas different GE lines showed only minor differences relative to their non-GE counterparts [20]. Natural plant-to-plant variability also exists: in a comparison of GE insect-resistant MON810 and control maize lines, some 2DE-separated protein spots showed a high variability between individual samples from the same line [7]. Some differences observed between GE lines and their counterparts can be limited to a given developmental stage as shown in a Bt (*cry1Ab/1Ac* gene) rice study [10]. In rice, herbicide-resistant Bar68-1 carrying *bar* gene and insect-resistant 2036-1a carrying *cry1Ac/sck* gene events did not substantially alter proteome profiles as compared with conventional genetic breeding and natural genetic variation [11]. Another proteomics analysis showed that these GE events (Bar68-1 carrying *bar*, *cry1Ac* and *sck* genes) in rice do not substantially alter proteome profiles as compared with conventional genetic breeding and natural genetic variation [12]. In a metabolic study of the same rice event (*cry 1Ac* and *sck* genes), a slight difference in concentrations of phytosphingosine, palmitic acid, 5-hydroxy-2-octadenoic acid and three other unidentified metabolites was due to gene modification while environmental factors played a greater role than gene modification for most metabolites [13]. Proteomics analysis indicated no significant differences in Bt-rice seeds containing the *cry1ab* and *cry1/ac* genes compared to its isogenic controls [14]. Significant changes in some metabolites were found both in bacterial blight-resistant rice varieties obtained by conventional breeding or transgenesis compared to the parental non-GE variety. However, the line obtained by conventional breeding possesses a distinctive metabolite profile and shows more differences versus the parental than the transgenic line [15]. In comparison with a non-GE comparator, Bt rice showed differences in antioxidant system and signalling regulation as a response to insecticide stress [16].

Data analyses revealed variations related to factors such as variety and environment. Several environmental factors (such as field location, planting and sampling time, or crop management practices) were shown to exert a greater influence than transgenesis.

These profiling studies consistently indicate that transgenesis has fewer unintended impacts than conventional breeding. Interestingly, one study showed that transcriptome alteration was

greater in mutagenized plants than in transgenic plants [21]. Unlike transgenic lines, mutagenized lines are not subjected to food safety assessment in the EU.

None of these published assessments using new '-omics' profiling points to new safety concerns about marketed GE crop varieties.

GE plants with altered metabolic traits

These 24 studies concerned GE crops such as barley (1 study), grapevine (2), maize (1), potato (5), rice (5), tomato (6 and [22]), and wheat (3).

GE lines with altered metabolic traits do not necessarily exhibit pleiotropic changes. However, some changes in compounds do occur when certain pathways are modified. As expected, several metabolism pathways for example in tomato can be altered, either in conventional mutants or in GE lines, when regulatory genes are affected [23,24].

Some differences in wheat expressing glutenin subunit genes in the endosperm are found in metabolites between GE and parental lines, but generally, they fall in the range of differences caused by environmental factors (growth in fields in different years and on different sites) [25]. Thus, larger differences were observed between two wheat parental lines than between the GE and control lines. Some changes in seed compounds of two high-Trp rice lines are found due to altered pathways which were predictable as a result of altered biosynthetic pathway but no major changes were observed for other phenolic compounds [26]. In potato, depending on genotype, somaclonal variation may be responsible for an unknown proportion of differences [27].

Long-term and multigenerational feeding studies

The inclusion of GE plants in animal feed and for direct human consumption has increased consistently since the first commercial production in 1996. However, the increased use of GE plants for human consumption and feed for livestock has led to public concern related to a perceived risk for health, toxicity and allergenicity of the transgenic proteins.

When 'molecular, compositional, phenotypic, agronomic and other analyses have demonstrated equivalence of the GM food/feed, animal feeding trials do not add to the safety assessment' (EFSA [28]). However, valuable information can be added to the safety assessment of GE food and feed safety by animal feeding studies, especially if doubt remains on the equivalence of GE food [2]. In these comparative feeding studies, 33% of the feed consists of GE material or control material (see recommendations of the French Agency for Food, Environmental and Occupational Health and Safety [29]); the remaining part is composed of a balanced diet. The results of 90-day rodent feeding trials performed with GE maize, rice and soybean did not lead to any unintended effects in animals (see [30]). However, we decided to address the following question: can long-term studies as well as multigenerational feeding studies detect potential unintended effects in animals (that are not detected in 90-day subchronic tests)? We examined recently 33 published studies regarding the long-term effects of GE plants, that is studies significantly longer than the 90-day tests (17 studies), as well as multigenerational studies (16 studies). These studies have been compared to the already performed 90-day studies (for further details see [30]).

We explored the issue whether GE plants may reveal any long-term effects of GE exposure not identified during the short-term premarket risk assessment.

Long-term studies

A detailed discussion on long-term studies (longer than 90–96 days) is available in [30]. Here, we update this investigation with a 16-week study on pigs fed with Bt-maize [31] (see also the short-term feeding trial [32]), a 22-week study on Japanese quail fed with Bt-maize [33], a 32-week study on Atlantic salmon [34] fed with Bt-maize and glyphosate soybean, and a 35-week study on beef cattle fed with Bt-maize [35].

All the 17 studies were financially supported by public funds. The duration of GE-based diet feeding times vary between 110 days (16 weeks on pigs fed with Bt-MON810 maize [31]) and 728 days (104 weeks on rats fed with glyphosate-tolerant (CP4-EPSPS) soybeans [30]). Rat (Fischer 344 and Wistar strains) was the predominant model (used in four studies, two in both strains). Various animal models were additionally used such as Swiss mice (five studies), salmons (three), beef cattle (one), dairy cows (one), macaques (one), pigs (one), and quail (one). Several parameters have been examined (detection of transgenic DNA, body and organ weight, blood and urine analyses, enzyme activities, biochemistry, histopathology and immunology). Most of these studies utilized major commercial products, namely glyphosate-tolerant (CP4-EPSPS) soybean (ten rodent studies along with a feeding study on salmons [34]) and insect-resistant (Cry1Ab) maize (five feeding studies on cows for 100 weeks [30], beef cattle [35], pigs [31], quail [33] and salmons [34]). In addition, one study concerned rice containing human T-cell epitope from Japanese cedar pollen allergens fed to macaques for 26 weeks [30].

Recently, a study claimed that the glyphosate-tolerant GE maize NK603 and a related herbicide formulation caused organ damage, tumors, and early death among Sprague-Dawley rats on rats fed with maize NK603 during two years [36]. However, numerous agencies of food safety, namely the German agency 'Bundesinstitut für Risikobewertung' [37], the European authority 'EFSA' [38,39], the Australian and New Zealand agency 'Food Standards Australia and New Zealand' [40], the Danish agency 'Danmarks Tekniske Universitet' [41], the Netherlands agency [42], the French agency 'ANSES' [43], the French High Council of Biotechnologies 'HCB' [44], the Belgian Biosafety Advisory Council [45], the Health Canada and Canadian Food Inspection Agency (CFIA) [46] and the Brazilian National Biosafety Technical Commission [47] refuted these claims.

A diverse range of animal models and various feeding durations and feed composition were used in these studies. No new safety concerns were raised and no supplementary information, in addition to previously performed 90-day feeding studies, were apparent. The new study carried out on pigs [32] also showed no long-term effects after 110 days (16 weeks) of feeding with maize containing Cry1Ab protein (MON810 event). Differences observed in serum biochemistry were all within the normal reference intervals for pigs; according to the authors these differences were the result of a lower enzyme-resistant starch in the GE compared to non-GE maize, which had been previously reported [31]. Changing from the non-GE maize to the GE maize diet may have resulted in a lack of satiety in pigs fed the non-GE/GE treatment.

The enzyme resistant starch content of food is known to influence satiety. The authors concluded: *'Long-term feeding of GM maize to pigs did not adversely affect growth or the selected health indicators investigated.'* [32]. Previous work by the same group also found that short-term (31 days) feeding of GE maize had no adverse effects on growth [31]. No significant influence on feed intake of Bt-maize, fattening and slaughtering results were observed in a 35-week beef cattle study [35]. Feeding of Bt-maize did not impair the laying intensity and the specific and nonspecific immune response in a 22-week quail study [33] and differences in zinc serum concentrations range within the normal variation of in quail. In a 32-week salmon study, no differences were observed between Bt-MON810 and non-GE maize feed, while GE and non-GE diets resulted in higher LAP activity compared to a standard diet and activity of maltase and AcP was higher in this standard diet [34].

It is important that comparison of the GE diet is done with the non-GE isogenic counterpart [2]. The studies on maize and rice comply with these required standards to compare GE and non-GE lines. Unfortunately, six studies using a soybean-based diet do not declare whether an isogenic line was used (in five studies the event is not mentioned; see discussion in [30]).

Multigenerational studies

The main goal of these studies was to assess whether feeding a generation (n) with a GE-based diet had adverse effects on the next generation ($n + 1$). These 16 multigenerational studies were performed on animals fed with GE-based diets throughout their life or only on short-term (less than 90 days). In both cases these animals were bred to produce future generations (studies on two to ten generations) (for further details see [30,48–53]). The longest multigenerational study consisted of feeding quail with a diet containing up to 50% Bt176 maize over ten generations. The duration of GE-based diet feeding varies between 1 and 188 weeks. Rodents were predominantly used (mice in five studies (see [30,49]) and Sprague-Dawley and Wistar rats in four studies [30,52]). The farm animals used were pigs (two studies), bulls (one), dairy cows (one), goats (one), sheep (one), hens (one), and quail (one). Parameters measured included transgene detection, body and organ weight, feed intake, enzyme concentrations or activities, lactation, histopathology and hematology, reproductive factors and performance.

The GE-material in the diets utilized Bt-insect-resistant maize (in eight studies including [49–51]), glyphosate-tolerant (T25) maize [52], glyphosate-tolerant (*cp4 epsps* gene) soybean (three studies), glufosinate-ammonium-tolerant triticale (two studies), potato containing the phosphinothricin acetyltransferase (*bar* gene) and lysine-rich rice [53].

However, in two studies using a maize diet, an isogenic line was not used. The event was not mentioned in one Bt-maize study. In two studies using a maize diet and a soybean diet an insufficient number of animals was used (see discussion in [30]).

All these 16 studies were financially supported by public funds. No mutigenerational effects were reported in a majority of studies. However, effects were reported in three studies, but it should be noted that no isogenic lines were used. These differences concerned the level of LDH enzyme of target animals such as goats fed with glyphosate-tolerant soybean [54] and changes in immune responses of mice fed with glufosinate-ammonium-tolerant triticale in the fifth generation of mice [55]. However, these differences seem to be minor, especially because the authors do not conclude that they constitute a health hazard. The authors suggest that these changes may fall within the normal range of variation but should be further investigated. It should especially be determined whether they are reproducible. An inadequate number of animals were used in a study on soybean [52]. When comparing Bt176-maize to the non-GE maize fed to sheep, some minor metabolic changes were reported with no demonstration of any health hazards [30]. The authors suggest that these changes should be further investigated to check if they are reproducible or not.

Bt-MON810 maize did not significantly influence production and reproductive performances of animals compared with a diet containing 50% isogenic maize when using pig offspring at birth [50] and pigs for 115 days postweaning [51]. No impact of glufosinate-ammonium-tolerant T25 maize on reproductive function of Wistar rats and on progeny development were found in two consecutive generations [52]. A lysin-rich rice was found as safe as its near-isogenic non-GE rice in three consecutive generations of Sprague-Dawley rats [53].

Discussion

What lessons can be drawn from the use of new 'omics' techniques on the food safety?

The 60 '-omics' profiling publications comparing GE and non-GE crop varieties, with or without intentional metabolic changes, converge to show that transgene insertions produce few unintended effects [4].

Currently, the risk assessment of GE crops includes the analysis of 50–150 analytes identified by OECD consensus documents [1]. This number depends on the crop species. In the literature, metabolomics is the prevalent '-omic' approach to assess GE crops, followed by transcriptomics. To a lesser extent proteomics is also used to detect unintended effects in plants due to the genetic modification itself. Metabolic profiling of crops is becoming increasingly popular in assessing plant phenotypes and genetic diversity [56]. The use of metabolomics for regulatory GE crop assessment would be a change of paradigm (measuring more analytes, a few hundred analytes, but with less precision). Proteomics (through a 2-DE protein analysis) can be used for qualitative and quantitative estimation of the allergen levels, including new ones, with recent improvements in sensitivity, mass accuracy and fragmentation [57].

Few public laboratories have used different '-omics' approaches in a comparative approach. Therefore, an exhaustive comparative assessment of these techniques is not yet possible. These '-omics' profiling studies are highly heterogeneous (depending on plant tissues, growth parameters, range of comparators and methods). There is a need to conduct normalized, validated approaches before these techniques can be used for the routine safety of new GE crops.

Large effects due to the environment were observed in gene expression, protein, and metabolite levels in some studies, illustrating the need for exposure to the same environmental conditions, pairwise differences between GE lines and their progenitor lines. Larger differences were often observed between two conventional lines, between years of sampling, and between different field sites than between the GE and control lines. Many

methodological shortcomings are identified with '-omics' approaches, a paucity of reference materials, and a lack of focused strategy for their use that currently make them not conducive for the reglementary safety assessment of GE crops [58]. For determining unintended effects in GE crop varieties, a validation work is needed before these '-omics' technologies could gain full recognition by regulatory authorities and agencies.

What lessons can be drawn from the use of long-term and mutigenerational studies?

Very few published long-term feeding studies use the same animal model or the same crop model. Moreover, the parameters studied varied. Hence no studies have been carried out twice in the same conditions by different research teams. Therefore, improvements in the protocols should be made, particularly focusing on reproducibility of data.

No new safety concerns were raised in these mutigenerational studies. However, some studies suffer from weaknesses such as lack of an appropriate control group and the number of animals or the correct number of animals, lack of precision regarding duration of the study and the event studied. Statistical criticisms can also be raised: weak definition of factor levels and absence of a complete combination of factors inside experimental designs. No evaluation of the statistical power as well as few multivariate approaches were reported in these studies. Future studies should be undertaken according to EFSA recommendations which have underlined the necessity of an improved methodology when statistics are involved [59] and the distinction between statistical significance and biological relevance [60].

Conclusions

We addressed the question whether alternative techniques, such as '-omics' assessments of GE plants or long-term animal feeding studies, can provide useful clues for unintended effects of GE food/feed. The application of the precautionary principle and stricter regulations have failed to convince certain consumers that EU regulations are tough enough regarding food and feed safety. Long-term and multigenerational studies should only be conducted in a case-by-case approach for GE food/feed safety and nutritional regulatory assessment if some reasonable doubt remains after a 90-day rodent feeding trial. Thus, considering distrust in data provided by seed companies and sceptical opinion on GE crops, it is important that new approaches such as '-omics' have been used by public research laboratories. However, none of these '-omics' assessments have raised new safety concerns about marketed GE crop varieties. This is not surprising considering the experience acquired after 15 years of growing and consuming GE food and feed. Our review does not provide evidence that more food safety testing is necessary for GE crop varieties. These long-term and multigenerational data and '-omics' data taken together suggest that, apart from specific cases, their risk assessment could be lowered.

Despite these scientific data, allegations against food safety of GE crop varieties are probably to remain in the public debate in the EU. However, it can be noticed that the French and German governments, which launched a procedure called 'safeguard clause' to ban cultivation of GE maize, did not use food safety arguments to justify it (in the EU a procedure called 'safeguard clause' allows a Member State providing valid reasons to consider that a GE crop plant constitutes a risk to human/animal health or to the environment, to provisionally restrict or prohibit the use and/or sale of that product on its territory [61]). Instead these governments tried to demonstrate environmental risks for the cultivation of GE maize, arguments which also failed to provide scientifically valid data [62].

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Evaluation of Genetically Engineered Crops Using Transcriptomic, Proteomic, and Metabolomic Profiling Techniques[W]

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A major principle and guiding tool for the food safety assessment of genetically engineered (GE) crops is the concept of "substantial equivalence" according to principles outlined in the Organization for Economic Cooperation and Development (OECD) consensus documents (OECD, 2006) and further elaborated by the Food and Agriculture Organization of the United Nations/World Health Organization. In this safety assessment, GE crop-derived foods and feeds are compared with their counterparts from parental or near isogenic lines in order to identify differences, which are subsequently evaluated with respect to safety for humans and animals as well as nutritional quality. The question addressed is: may the improvement of a plant variety through the acquisition of a new desired GE trait lead to unintended effects (i.e. going beyond that of the original genetic modification) and, if so, does this have an impact on health? Possible mediators of such pleiotropic effects could be altered expression of untargeted genes or metabolic effects of a novel gene product. Current tools to assess the food safety of GE crops include extensive multisite and multiyear agronomic evaluations, compositional analyses, animal nutrition, and classical toxicology evaluations. In the 2000s, new methodologies were developed to allow, in theory, a holistic search for alterations in GE crops at different biological levels (transcripts, proteins, metabolites). These methodologies include cDNA microarrays, microRNA fingerprinting, proteome, metabolome, and toxicological profiling. The term "omics" in relation to food and feed safety appeared for the first time in 2005 (Li et al., 2005). This review highlights the knowledge generated by recently published profiling studies regarding the effect of genetic modification itself, compared with environmental and intervariety variation, for major crops (44 studies) and for Arabidopsis (*Arabidopsis thaliana*) as a reference plant.

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THE LESSONS TO BE LEARNED FROM ARABIDOPSIS

Arabidopsis is a well-established model plant that offers comprehensive resources such as the entire genome sequence, a large collection of natural variants, a number of molecular tools, and several information platforms and databases. In addition, as illustrated below, Arabidopsis provides valuable information about the potential impact of transgenesis.

The first question to be addressed is whether the insertion of genes that are not believed to alter biological processes in plants will lead to transcriptome changes. To answer this question, El Ouakfaoui and Miki (2005) used selectable marker (*nptII*) and reporter (*uidA*) genes. Under controlled growth conditions, they found no reproducible changes for the approximately 24,000 genes screened when comparing transgenic lines with their wild-type progenitor. Their conclusion was that the stable insertion of T-DNA did not cause detectable pleiotropic effects to the transcriptome. This finding was not obvious since, due to the gene density on the Arabidopsis genome, insertion could have been anticipated to cause major disturbances altering gene expression. Strikingly, under abiotic stresses (salt, drought, cold, and heat), the authors found approximately 8,000 genes (35% of the genome) with changed expression in both wild-type and transgenic plants.

In contrast, Ren et al. (2009a) attributed some unintended effects to the presence of a selectable marker gene (*bar*, encoding phosphinotricin acetyl transferase). Metabolic fingerprinting revealed that the major contributors distinguishing the wild type and four transgenic lines were modified levels of Ala and Thr. The authors attributed this trend to the *bar* gene, since it was common to all lines. However, protein analysis by two-dimensional electrophoresis (2DE) on 12 *bar*-containing lines showed no consistent differences (four to 14 protein spots did change in intensity depending on the line, but most of them were different;

Ren et al., 2009b). In that study, cold treatment triggered changes in only 10 protein spots. In another study, Abdeen and Miki (2009) found only four genes differentially expressed in transgenic lines expressing *bar*.

A second question to be examined is whether expression of a protein affecting regulatory processes (e.g. a transcription factor affecting drought tolerance; Abdeen et al., 2010) will necessarily have pleiotropic effects. These authors found no effect on the transcriptome in such plants without drought. As can be expected, in response to drought, changes in the level or timing of expression of some drought-responsive genes occurred between transgenic and wild-type plants.

A third question to address is whether deliberate modification of a metabolic pathway using transgenesis will have pleiotropic effects. Kristensen et al. (2005) inserted one to three genes from a pathway converting Tyr to a cyanogenic glucoside (dhurrin). They found only marginal inadvertent effects on the transcriptome and metabolome when the whole pathway or only the first enzyme was inserted. However, the combination of the first two genes leads to the predicted synthesis of a toxic cyanohydrin intermediate. In this case, plants responded by metabolism and detoxification reactions, as was evident from an altered metabolite profile showing the accumulation of detoxification products and changes in the transcriptome.

Metzdorff et al. (2006) developed and characterized six independent lines transformed with an antisense chalcone synthase gene to decrease flavonoid biosynthesis. The lines differed in the type of integration (site and copy numbers, level of gene silencing). Unintended effects on gene expression included few genes (up to 15 in flower and up to 13 in leaf out of the 1,500 analyzed), and the affected genes were involved in stress response and photosynthesis. Lines differed with respect to the affected genes, and analyses of one such gene by PCR did not show a consistent trend with the microarray data, which the authors explain by a large biological variation in expression for this gene. One conclusion of Metzdorff et al. (2006) is that "it is crucial to have substantial information on the natural variation of crop plants in order to be able to interpret 'omic' data correctly."

Interestingly, Arabidopsis also provides some insight concerning the above-mentioned issue. Ruebelt et al. (2006) qualitatively and quantitatively analyzed its seed proteome and showed that existing natural variability can be important. When various ecotypes were grown side by side in a growth chamber under controlled conditions, the authors found that nearly half of the 2DE-resolved spots were present or absent depending on the ecotype and that 95% of the spots present in all ecotypes varied quantitatively. Twelve transgenic lines were also compared with their parental line as well as with 12 ecotype lines: the genetic modification of Arabidopsis using three different genes and three different promoters did not cause unintended changes to the analyzed seed proteome.

In conclusion, these data on a model plant for research point to a greater influence of genetic background and stress (from the environment or new metabolites) than of transgene insertion itself. To determine whether these conclusions are also valid for crop plants, the following two sections examine the conclusions of profiling strategies in a systematic species-by-species approach.

CROP PLANTS: COMPARISON OF GE VARIETIES WITH IMPROVED AGRONOMIC TRAITS (WITHOUT INTENTIONAL METABOLIC CHANGES) WITH NON-GE VARIETIES

The main data from the publications discussed below are listed in Supplemental Table S1, which also includes data from earlier publications or on other species (cabbage [*Brassica capitata*] and potato [*Solanum tuberosum*]) and on GE plants producing bioproducts (such as antibodies), which are not discussed below. The search strategy used to find these references is presented in Supplemental Table S1.

Barley

Using field-grown barley (*Hordeum vulgare*) lines expressing either a chitinase or a β-glucanase, Kogel et al. (2010) compared changes in the leaf transcriptome and metabolome caused by transgenes, cultivar, or biotic interactions in the root. Transgene effects were negligible in the first case and low in the second, while the difference caused by the genetic background of cultivars (even if down to a low number of alleles) was of a greater magnitude. Effects of exposing roots to the spores of mycorrhizal fungi could be visualized by metabolome but not transcriptome analysis. Based on this result, the authors conclude that the metabolome represents a more immediate probe of the physiological status of the plant.

Maize

When performing transcriptomic studies using in vitro- or field-grown maize (*Zea mays*) plants, Coll et al. (2008, 2009) found differential expression for a minority of transcripts between in vitro-grown MON810 (insect-resistant of *Bt* type) and control lines, and most of these differences were not observed in the field. In real agricultural conditions, under two farming practices (conventional and low-nitrogen fertilization), Coll et al. (2010a) found differential expression for only 0.14% of the analyzed sequences (approximately one-third of the maize genome). Analysis of the expression of a subset of sequences in a different MON810/non-GE pair indicated that varietal differences had the highest impact on gene expression patterns, followed by nitrogen availability, while the MON810 characteristic had the lowest impact.

Coll et al. (2010b) found the grain proteome of two field-grown MON810/non-GE variety pairs to be vir-

tually identical, with very few spots showing variations in the 1- to 1.8-fold range, which were all variety specific. Previously, Albo et al. (2007) had also found limited changes in the grain proteome of two different MON810 varieties (also field grown). Zolla et al. (2008) also used two MON810 variety pairs but found more differences, although environment (field versus growth chamber) induced more changes. To explain the differences resulting from genetic modifications, these authors speculated about genome rearrangement induced by the transformation method but did not consider the possibility that the control lines were certainly not fully isogenic. The discrepancy between these results remains unexplained, especially since one of the two pairs used by Coll et al. (2010b) was the MON810/non-GE pair used by Zolla et al. (2008).

In a first grain metabolome analysis, carried out on a greenhouse-grown MON810 line, Manetti et al. (2006) found differences in the levels of compounds from primary nitrogen metabolism in transgenic grain samples. Using a different MON810 line, grown in a growth chamber, Piccioni et al. (2009) identified 40 water-soluble metabolites and found a higher concentration for five compounds in the GE extracts (all different from those of Manetti et al. [2006]). Leon et al. (2009) found increases in some metabolites from specific metabolisms (purine, amino acid, arachidonic acid, linoleic acid) in three field-grown MON810 lines compared with their controls. There were only 10 metabolites with increased levels when two different technologies were compared. One of them, carnitine, had been proposed in a previous study by the same team (Levandi et al., 2008) to be a biomarker for *Bt* maize (note, however, that both studies analyzed the same samples, which provides no additional perspective). It should be pointed out that these various teams did not find similar results, which may be explained by their use of different genetic backgrounds and/or different growth conditions and also different technologies.

In this context, the work of Barros et al. (2010) is important. Using transcriptome, proteome, and metabolome profiling to compare two GE maize lines (MON810 and glyphosate tolerant) with the respective control lines, they found that the environment (plants were grown over three seasons in one location) affected gene expression, protein distribution, and metabolite content more strongly than the genetic modification. In addition, the authors found distinct profiles for the three locations that were also part of their comparisons during one season.

Natural plant-to-plant variability also exists. Using MON810 and control lines, Batista and Oliveira (2010) compared 2DE-separated protein spots from samples obtained either from individual plants (five different ears of five different maize plants) or from pooled plants. For some spots, they noticed a high variability between individual samples from the same line and that these differences were masked in the pools. For other spots, variability was observed between individual samples and also between pools. The authors concluded that differences not related to the genetic engineering, such as natural plant-to-plant variability, need to be eliminated when using omics.

Harrigan et al. (2010) reviewed compositional data for GE maize and soybean (*Glycine max*) varieties (seven GE crop varieties) from a total of nine countries and 11 growing seasons. From their analysis, which is not based on omic technologies but represents the most comprehensive compilation of GE crop composition data to date, the authors conclude that compositional differences between GE varieties and their conventional comparators are "encompassed within the natural variability of the conventional crop and that the composition of GM and conventional crops cannot be disaggregated."

Pea

Analyzing two pea (*Pisum sativum*) cultivars producing a bean (*Phaseolus vulgaris*) α-amylase inhibitor (AI1), Islam et al. (2009) found around 30 seed protein spots showing changes in abundance in each transgenic/control pair (generally not the same spots, although AI1 was produced at similar levels in both cultivars). While differences were minor for one pair, they were more pronounced quantitatively and qualitatively (appearance and disappearances of 36 protein spots) for the second pair. The authors suggest that differences of "similar magnitude" occur between cultivars. In a different cultivar, Chen et al. (2009) reported that 33 proteins differentially accumulated in AI1-expressing lines compared with the parental line, three of which were associated with the expression of AI1. The remaining 30 proteins were associated with the transformation events. A number of the increased spots corresponded to seed storage proteins. Since such proteins are common food allergens, the authors suggested that these increases might be linked to food antigens detected in mice fed with GE peas (attempts to use 2DE of proteins and proteomics to detect new allergens are listed in Supplemental Table S2).

Rice

Montero et al. (2010) found around 0.40% transcriptomic differences in leaves of in vitro-grown experimental rice (*Oryza sativa*) lines producing an antifungal protein. They could distinguish differences due to transgene insertion (15%), transgene expression (50%), and regeneration (35%). Around half of the genes whose expression was affected by the transgene itself also had their expression affected in non-GE plants after wounding.

Zhou et al. (2009) compared profiles of compounds from primary metabolism in three GE lines (each independently transformed with the same two insect resistance genes; their data were averaged) with those of the wild-type line (field grown side by side). They found three metabolites to be present in greater

amounts in the GE group (up to 3-fold). Differences in other metabolites were within the same range as those of the wild type under various growth conditions (location and/or sowing time). It should be mentioned that wild-type lines planted at different times contained varying amounts of trehalose (up to 40-fold) and change in location influenced the levels of four compounds.

The work by Jiao et al. (2010) provides some perspective on transgenic changes in the context of varietal changes in rice. Comparing two lines with different sets of antifungal genes and one with two insect resistance genes with their respective controls, the authors found decreases or increases, inconsistent between lines, ranging from 20% to 74% for amino acids, 19% to 38% for fatty acids, 25% to 57% for vitamins, and 20% to 50% for elements. These changes were all within the range occurring among varieties (according to OECD values). A 25% reduction in protein content was observed for one antifungal GE line, which was therefore considered by the authors to be less nutritious.

Batista et al. (2008) addressed the following question: which of the mutagenized or transgenic plants are more susceptible to present unintended modification? Gene expression was analyzed in duplicated samples of four types of rice plants (irradiated stable mutants and transgenic plants producing an antibody or developed for improved stress tolerance) and their respective controls. In all cases studied, the modification in transcriptome was greater in mutagenized than in transgenic plants. Since these results were obtained with seedlings grown on tissue culture medium, wider confirmation is necessary.

Soybean

Cheng et al. (2008) found that gene expression in leaves (grown in a growth chamber) differs more between conventional varieties than between two GE glyphosate-tolerant varieties (carrying the same transgenic event) and their closest conventional varieties. The authors also note that the older the soybean variety, the larger the difference in gene expression (recently developed cultivars are more inbred), which raises the question of which varieties should be chosen to create a reference set for the crop species. Also using a glyphosate-tolerant variety (not specified) grown in a growth chamber, but analyzing seeds, García-Villalba et al. (2008) identified and quantified the main metabolites: in general, the same metabolites, in similar amounts, were found in GE glyphosate-resistant soybean and in its corresponding parental line. However, significant differences were observed in some specific cases: among the 45 metabolites examined, higher amounts were found for three and lower amounts for five (one was not detected) in the GE line. At least some of these differences could be explained by modification in the regulation of the shikimate pathway in GE soybean (glyphosate tolerance is conferred by a transgenic 5-enolpyruvylshikimate-3-phosphate synthase enzyme that bypasses the endogenous glyphosate-sensitive enzyme).

The study on natural variation in soybean crop composition and the impact of transgenesis by Harrigan et al. (2010) has been mentioned above.

Using 2DE protein analysis, soybean endogenous allergen expression was found not to be altered after genetic modification (see related refs. in Supplemental Table S2).

Wheat

Gregersen et al. (2005) found that the strong expression of a phytase gene had no significant effect on the overall gene expression patterns in the developing wheat (*Triticum aestivum*) seed. Samples from greenhouse-grown plants were taken at three different seed development times. The slight differences observed concerned primarily genes strongly expressed over a shorter period of seed development. This highlights the necessity of careful interpretation of microarray results when extensive progressive developmental changes occur, as is the case for seeds, and when minor asynchrony is hard to avoid. Ioset et al. (2007) analyzed lines with either a combination of three transgenes or a single one (KP4, of viral origin) for increased defense against fungal pathogens. For greenhouse-grown plants, they found only minor differences in the flavonoid profile between GE lines and their conventional control lines. In contrast, the different genetic background of the control lines resulted in a quantitatively different (up to 2-fold for some compounds) flavonoid content. In a field test, KP4 did not influence flavonoid content either, whether the lines were infected by pathogens or not.

Conclusion

These profiling studies are highly heterogeneous (plant tissues, growth parameters, range of comparators, technologies). They have to be considered as exploratory (i.e. not normalized validated approaches for the routine assessment of GE plants).

This survey on the profiling of GE crop lines with agronomic traits, but without deliberate modifications to metabolic pathways, reveals that some differences exist when compared with control lines. However, the available data on various conventional lines consistently show more differences. This has to be linked to the fact that GE lines have been selected by a process based not only on the suitable expression of a new trait but also on phenotypic and compositional equivalence with a close comparator, followed by a number of crosses to introgress the new trait into elite lines. A number of environmental factors (field location, sampling time during the season or at different seasons, mineral nutrition) have also been shown, consistently, to exert a greater influence than transgenesis.

TOWARD ADAPTING THE SUBSTANTIAL EQUIVALENCE CONCEPT TO GE PLANTS WITH ALTERED METABOLIC TRAITS

The substantial equivalence concept encompasses a comparison of biochemical composition with a non-GE line considered to be safe. However, many GE crop lines have been developed to obtain improved feed or food composition. Before examining whether this concept can be used to address the need to assess the safety of these new crops, the following section examines systematically the conclusions of available omic studies. Further details are given in Supplemental Table S3. Some publications not intended to study the unintended effects of transgenesis per se, but nevertheless providing relevant information, are discussed below or listed in Supplemental Table S3.

Maize

Huang et al. (2005) generated maize lines with an elevated content of free and total Lys in the kernels due to the combined deregulation of its synthesis and reduced levels of a Lys-poor storage protein. Kernels from field-grown plants showed, in addition, strong increases in the content of two Lys metabolites and up to 2-fold higher content of other free amino acids but with only marginal changes for total amino acids.

Potato

Lehesranta et al. (2005) demonstrated major qualitative and quantitative differences in the tuber proteome of field-grown varieties and landraces but found only limited quantitative differences between GE lines (affected either in cell wall structure or ethylene/polyamine metabolism) and their controls. Using the same lines, plus related ones as well as lines expressing a sense and antisense fructokinase gene (all grown in pots), similar conclusions were reached using metabolic profiling (Defernez et al., 2004) or targeted compositional analysis (Shepherd et al., 2006). The most obvious differences were found between the two non-GE varieties. Differences were also found between tissue culture-derived tubers and tubers derived from transformation with the empty vector. This raises the possibility that somaclonal variation (known to occur significantly in potato, depending on genotype) may be responsible for an unknown proportion of differences.

Similarly, using field-grown tubers engineered to produce inulin-type fructans, Catchpole et al. (2005) found their metabolite composition to be similar to the progenitor line and variations to be within the range found in classical cultivars, apart from the predictable increase in fructans and derivatives. Baroja-Fernández et al. (2009) found numerous transcriptomic changes in tubers with altered levels of Suc synthase, but their data were not compared with varietal changes.

An additional perspective (i.e. influence of sampling time) is provided by Kim et al. (2009), who found that 1 week of storage significantly modified tuber metabolite patterns, but the constitutive expression of β-amyloid, curdlan synthase, or glycogen synthase triggered neither quantitative nor qualitative differences.

Rice

In seeds of two high-Trp rice lines (field grown), Wakasa et al. (2006) found an increase in the content of other free amino acids (but to a lesser extent than that of Trp) and of indole acetic acid, which was predictable given the relation between the Trp biosynthetic pathway and the production of this growth regulator. However, they found no major change for other phenolic compounds. The same laboratory (Dubouzet et al., 2007) also found limited metabolic and transcriptomic differences in 8-d-old seedlings of lines with high Trp. Beatty et al. (2009) reported limited transcriptional changes in roots and shoots of "nitrogen use-efficient" rice obtained by overexpression of Ala aminotransferase.

Tomato

Le Gall et al. (2003) analyzed metabolic profiles during tomato (*Solanum lycopersicum*) fruit ripening and the potential unintended effects when two transcription factors were simultaneously overexpressed to increase flavonol content. The levels of at least 15 other metabolites were found to be different between the red GE and non-GE tomato types, but according to the authors (who did not specify the growth conditions), these changes are within the natural variation normally observed in a field-grown crop.

Long et al. (2006) found no perturbation in phenolic metabolites in mutant and transgenic lines altered in structural genes for carotenoid biosynthesis, and reciprocally, the down-regulation of ferulate 5-hydroxylase did not affect carotenoid content in red fruit from greenhouse-grown plants.

In a more comprehensive study, but also limited to greenhouse conditions, Fraser et al. (2007) characterized the fruit metabolic changes associated with the overproduction of carotenoids. Specific sectors of metabolism were altered in green fruit, resembling some metabolic changes normally associated with ripening. Ripe fruit showed the least change in overall metabolites, although levels of 43% of the metabolites were altered. Thus, perturbation in carotenoid synthesis has profound regulatory implications for tomato fruit development, but these effects arise without altering the general phenotype of the plant and fruit ripening.

In addition, as expected, several metabolisms can be altered, either in conventional mutants or in transgenic lines, when regulatory genes are affected, such as those involved in light perception (Long et al., 2006; see other refs. in Supplemental Table S3) or growth regulator biosynthesis (Mattoo and Handa, 2008).

Wheat

Baudo et al. (2009) report the transcriptomic comparison of GE and conventionally bred lines (grown in a greenhouse) expressing a given set of seed storage proteins (glutelins) known to determine bread-making quality. Differences in endosperm and leaf transcriptome between GE and parent lines were rare (up to six genes). More differences (up to 527 genes in endosperm) were observed between this parent line and another conventionally bred line. The latter, although of different overall background, contains the same set of glutelins as the GE line and unexpectedly showed fewer differences (up to 154 genes) with the GE line than with the parent of the GE line. Baker et al. (2006) performed metabolomic comparisons also using lines differing in their set of glutelins. They found some differences in polar metabolites between GE and parental lines, but generally, they were in the range of differences caused by the environment (plants grown in fields on different sites and in different years). Larger differences were often observed between two parental lines, between years, and between different sites than between the GE and control lines. Additional articles analyzing wheat or barley lines with a modified set of seed storage proteins are listed in Supplemental Table S3.

Conclusion

Few of these studies brought their results in perspective with the potential effects of the environment. Nevertheless, the available data are noteworthy since they indicate that GE lines with altered metabolic traits do not necessarily exhibit pleiotropic changes. This is encouraging for the future use of transgenesis to improve food and feed quality. However, some pleiotropic effects do occur when certain pathways are modified.

A key consideration for crops with altered composition, in a substantial equivalence perspective, is the choice of a comparator for GE lines. The published omic studies did not yet examine the question of what the appropriate comparator should be (the progenitor or a crop that most closely resembles the new variety with respect to the intentionally altered metabolic trait). On the other hand, it can be stressed that, up to now, choosing a comparator has not posed a major problem. GE crops (as well as conventional varieties) with altered composition have already been assessed and approved by regulators (e.g. crops with high oleic acid content).

DISCUSSION

Divergent Views on Omics

Some authors (for a selection of refs., see Supplemental Table S4) consider that nontargeted profiling provides coverage of gene, protein, and metabolite analysis that cannot be matched by traditional targeted approaches. A so-called "unbiased" analysis of the metabolome, for example, certainly offers new possibilities for plant physiologists and holds promise for a better understanding of the variation in metabolites relevant to human health and nutrition. However, as Lay et al. (2006) pointed out, "bias" does occur with omics (i.e. systematic errors) as well as other problems with "statistics (e.g., number of replicates), methodology and method misuse."

As this review shows, there is an obvious lack of homogeneity in experimental design and methodology, sometimes even within the same laboratory. Most published omic studies lack a biological validation of observed differences between GE crops and their comparators. Some include no biological replicates. Variable patterns in transcriptome, proteome, or metabolome are reported depending on growth conditions, geography, season, or variety. Considering all sources of difficulties in data interpretation, it seems premature to infer precise conclusions from variations assigned to a GE variety, such as the definition of a given compound as a "biomarker" for a given type of GE crop (Levandi et al., 2008). However, as discussed below, the available data valuably point to general trends concerning transgenesis.

Metabolomics Versus Traditional Analytical Chemistry

Current risk assessment of GE crops includes the analysis of 50 to 150 analytes (depending on the crop species) identified by OECD consensus documents (OECD, 2006) as the key compounds for that crop, using validated analytical methods. Following these guidelines, current approaches allow the measurement of 80% of biomass in soybean seed and 95% of nonstarch biomass in maize grain. Metabolomics would measure a few hundred analytes (i.e. the same compounds, plus additional low-abundant metabolite pools, usually extremely variable, some of which are unidentified). Despite the recent publication of numerous omics studies in relation to GE crop assessment, it does not yet seem feasible to propose large-scale methods that can be internationally certified and accepted. Using metabolomics would be a change of paradigm (measuring more analytes but with less precision.) for GE crop assessment but would provide little or no added value for food safety (Chassy, 2010), since it does not yet surpass the currently used analytical methods (Harrigan et al., 2010). In addition, when studies have used different metabolomic technologies simultaneously, discrepancies in the results were obvious (Leon et al., 2009).

Which Omic Approach and When?

As can be seen in Table I, metabolomics is the prevalent approach. Some authors consider that transcriptomics can routinely establish substantial equivalence (Baudo et al., 2009). Others suggest combining

methods (Supplemental Table S4). However, few studies have used different omics side by side; therefore, a comparative assessment of these techniques is still required.

At present, published profiling studies of GE crops represent merely a compilation of data, and mandatory use of these techniques in GE food safety assessment would be pointless. Basic research should be carried out to improve methods and evaluate the reliability of the results. A weight-of-evidence approach for a better determination of the consistency of the observed differences, and determination of their nontransient nature and of their biological relevance, are all recommended. Modeling is needed to analyze observed differences in various pathways. Subsequently, a tiered approach to the potential use of omics could be proposed, which would follow a decision tree incorporating parameters from traditional safety assessments and establish, on a case-by-case basis, whether omics use is helpful or not.

Food safety-oriented cDNA microarrays could be constructed. van Dijk et al. (2009) used this approach to analyze the tuber transcriptome of two different non-GE potato varieties to detect variation due to genetic differences or environmental conditions. The extent of natural variation of gene expression was examined to help future biological and/or toxicological assessments.

Regarding allergenicity predictions, 2DE combined with immunoblotting are used to identify the allergenic spots that bind IgE antibodies. Proteomic and mass spectrometry methods are also able to provide qualitative and quantitative information on the levels of allergens, including new ones (Supplemental Table S2).

Transgenesis in the Context of Existing Variations

Before commercialization, GE crop lines have to be checked for phenotypic and compositional equivalence (for key nutrient, antinutrient, and toxicant contents) to existing varieties (apart from the new trait). Therefore, it seems unlikely from a plant physiology point of view that a new transgenic line that has equivalent key metabolite content, as well as similar growth, flowering, fruit development, seed production, etc., parameters, would exhibit extensively altered gene expression, protein, or metabolite profiles.

Nevertheless, not unexpected from a systems biology point of view, some differences attributed to transgenesis were reported in the published omics studies. However, when a larger set of references was included in the study (i.e. beyond the pairwise comparison of a GE line and its near isogenic line), the most pronounced differences were consistently found between the various conventional varieties, a trend linked to the crop diversity maintained or created by plant breeders. This should be put in perspective, taking into account that conventional breeding is generally regarded as safe, despite the fact that the nature of the changes in new conventional cultivars is usually unknown (Parrott et al., 2010).

Large effects due to the environment were also observed on gene expression, protein, and metabolite levels in some studies (Baker et al., 2006; Zolla et al., 2008; Zhou et al., 2009; Barros et al., 2010). The present knowledge created by profiling approaches illustrates the need to place pairwise differences between GE lines and their direct progenitor in a wider context.

What Conclusions Can Be Drawn Regarding the Substantial Equivalence Concept?

It is important to keep in mind that the standard proposed by the OECD/Food and Agriculture Organization of the United Nations/World Health Organization was substantial equivalence rather than total equivalence and that there is no specific statistical or biological basis to define "substantial" (Hoekenga, 2008). In other words, no "limits of concern" have

Table I. *Number of publications comparing GE and non-GE crop varieties without or with intentional metabolic changes according to omic profiling*

The total number of published studies and the number with transcriptomic (T), proteomic (P), or metabolomic (M) data are given. Some publications reported various profiling approaches.

Plant Species	GE with No Intentional Metabolic Changes				GE with IntentionalMetabolic Changes			
	Total	T	P	M	Total	T	P	M
Barley	1	1	0	1	—			
Cabbage	1	0	0	1	—			
Grapevine (*Vitis vinifera*)	—				2	1	1	0
Maize	11	4	4	5	1	0	0	1
Pea	2	0	2	0	—			
Potato	1	0	1	0	5	1	1	3
Rice	4	2	0	2	5	2	2	2
Soybean	2	1	0	1	—			
Tomato	—				6	2	0	6
Wheat	3	1	1	1	4	2	0	2
Total	25	9	8	12	19	8	4	14

been defined regarding differences. In addition, plant composition is usually variable even within a single variety. Pairwise differences between a GE line and its comparator are usually less than natural variability. Furthermore, near isogenic lines differ by a number of alleles, which could explain a number of differences attributed to transgenesis. Thus, the substantial equivalence concept cannot provide more than a guiding framework for evaluation.

Nevertheless, the experience acquired after 15 years of GE crop commercialization has comforted the validity of this framework. However, considering the highly polarized views on GE crops, it is important to notice that the opinions expressed previously by food safety agencies (i.e. general "equivalence" of authorized GE crops with non-GE comparators) have now been independently corroborated at the transcriptomic, proteomic, and metabolomic levels by recently published omic comparisons (Table I). None of the published omic assessments has raised new safety concerns about marketed GE cultivars.

Which Changes in Regulation for New Crops?

Based on their extensive comparison of compositional data of maize and soybean varieties, Harrigan et al. (2010) proposed that "if regulatory scrutiny is to be commensurate with the potential for compositional deviation, there is no reason to prioritize crops on the basis of genetic modification via transgenesis over crops genetically modified via conventional breeding, chemical mutagenesis or irradiation." Batista et al. (2008) showed, in the case studied, that the observed transcriptome alteration was greater in mutagenized than in transgenic plants. It should be mentioned that as far back as 1987, a report by the National Academy of Science (entitled Introduction of Recombinant DNA-Engineered Organisms into the Environment) had already stated that "there is no evidence that unique hazards exist in the use of recombinant DNA techniques or in the transfer of genes between unrelated organisms" and "that the risk[s]...are the same in kind as those associated with...other genetic techniques."

Today, the fast-accumulating data from targeted approaches as well as nontargeted profiling, consistently indicating that transgenesis has less impact than conventional breeding, should lead at least to a convergence of regulations for various crop breeding methods. Obviously, on a scientific basis, this should mean lowering the current regulatory burden for GE crops (Chassy, 2010). Considering that health problems have not been identified for GE crops after 15 years of commercialization, the time may have come to simplify the risk assessment of modern biotechnology products and therefore reduce cost. This would make risk assessment more affordable for small companies, academic institutions, or low-income countries.

However, considering that regulations ruling GE crop marketing have been strengthened continuously due to political pressure, especially in the European Union (Morris and Spillane, 2010), it is more likely that the non-GE authorization, and first of mutagenized crops, will be brought into line with the GE regulation. In addition, although there is no evidence that more food safety testing is necessary for GE crops, one can predict that a "whatever is possible should be done" policy will push for the use of omics technologies in their mandatory assessment.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. GE varieties with improved agronomic traits versus non-GE varieties.

Supplemental Table S2. References on the use of "omics" to identify food allergens.

Supplemental Table S3. GE varieties with altered metabolic traits versus non-GE varieties.

Supplemental Table S4. References discussing the use of "omics" in food safety assessment.

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Prevalence and impacts of genetically engineered feedstuffs on livestock populations[1]

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ABSTRACT: Globally, food-producing animals consume 70 to 90% of genetically engineered (GE) crop biomass. This review briefly summarizes the scientific literature on performance and health of animals consuming feed containing GE ingredients and composition of products derived from them. It also discusses the field experience of feeding GE feed sources to commercial livestock populations and summarizes the suppliers of GE and non-GE animal feed in global trade. Numerous experimental studies have consistently revealed that the performance and health of GE-fed animals are comparable with those fed isogenic non-GE crop lines. United States animal agriculture produces over 9 billion food-producing animals annually, and more than 95% of these animals consume feed containing GE ingredients. Data on livestock productivity and health were collated from publicly available sources from 1983, before the introduction of GE crops in 1996, and subsequently through 2011, a period with high levels of predominately GE animal feed. These field data sets, representing over 100 billion animals following the introduction of GE crops, did not reveal unfavorable or perturbed trends in livestock health and productivity. No study has revealed any differences in the nutritional profile of animal products derived from GE-fed animals. Because DNA and protein are normal components of the diet that are digested, there are no detectable or reliably quantifiable traces of GE components in milk, meat, and eggs following consumption of GE feed. Globally, countries that are cultivating GE corn and soy are the major livestock feed exporters. Asynchronous regulatory approvals (i.e., cultivation approvals of GE varieties in exporting countries occurring before food and feed approvals in importing countries) have resulted in trade disruptions. This is likely to be increasingly problematic in the future as there are a large number of "second generation" GE crops with altered output traits for improved livestock feed in the developmental and regulatory pipelines. Additionally, advanced techniques to affect targeted genome modifications are emerging, and it is not clear whether these will be encompassed by the current GE process-based trigger for regulatory oversight. There is a pressing need for international harmonization of both regulatory frameworks for GE crops and governance of advanced breeding techniques to prevent widespread disruptions in international trade of livestock feedstuffs in the future.

Key words: genetic engineering, genetically modified organisms, livestock feed, safety

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INTRODUCTION

The first genetically engineered (**GE**) feed crops were introduced in 1996. Their subsequent adoption has been swift. In 2013, GE varieties were planted on more than 95% of sugar beet, 93% of soy, and 90% of all cotton and corn acres in the United States (USDA National Agricultural Statistics Service, 2013). Global livestock populations constitute the largest consumers of GE feed crops. Independent studies have shown the compositional equivalence of the current generation of GE crops (Cheng et al., 2008; Garcia-Villalba et al., 2008; Herman and Price, 2013; Hollingworth et al., 2003), and no significant differences in feed digestibility, performance, or health have been observed in livestock that consume GE feed (Flachowsky et al., 2012). Similarly, it is not possible to detect differences in nutritional profiles of animal products after consumption of GE feed (Guertler et al., 2010; Tufarelli and Laudadio, 2013).

Despite these findings, some states have considered legislation that would require mandatory GE labeling

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of meat, milk, and eggs derived from animals that have eaten GE feed (CAST, 2014). Furthermore, some food companies are actively targeted by campaigns to promote products from animals that are fed non-GE diets. Given the widespread adoption of GE crops, the segment of animal agriculture that is currently feeding non-GE diets is relatively small. Approximately 0.8% of U.S. cropland and 0.5% of U.S. pasture were certified organic in 2011 (USDA National Agricultural Statistics Service, 2012), and only a portion of organic crops are used for animal feed.

Our objective was to briefly review the literature on livestock GE feeding studies and the composition of animal products derived from animals fed a GE diet. We gave special attention to health studies of animals, including an analysis of publicly available data on the health of commercial livestock populations since the introduction of GE crops in 1996. Also, we summarized the global usage and trade of GE feedstuffs along with the estimated size of GE-sensitive markets. Finally, we discussed issues regarding pipeline and regulation of GE crops with modified output traits, asynchronous regulatory approvals, and novel breeding technologies.

Livestock Feeding Studies with Genetically Engineered Feed

A total of 165 GE crop events in 19 plant species, including those used extensively in animal feed (alfalfa, canola, corn, cotton, soybean, and sugar beet), have been approved in the United States (James, 2013). Before approval, each new GE crop goes through a comprehensive risk assessment. The risk analysis of GE organisms is governed by internationally accepted guidelines developed by the Codex Alimentarius Commission (www.codexalimentarius.org). One leading principle is the concept of substantial equivalence, which stipulates that any new GE variety should be assessed for its safety by comparing it with an equivalent, conventionally bred variety that has an established history of safe use. Over the past 20 yr, the U.S. Food and Drug Administration found all of the 148 GE transformation events that they evaluated to be substantially equivalent to their conventional counterparts, as have Japanese regulators for 189 submissions (Herman and Price, 2013). By contrast, plant varieties developed through other processes of achieving genetic changes (e.g., radiation mutagenesis) go through no formal risk assessment before being placed on the market. There have been instances where plants bred using classical techniques have been unsuitable for human consumption. For example, the poison α-solanine, a glycoalkaloid, was unintentionally increased to unacceptable levels in certain varieties of potato through plant breeding resulting in certain cultivars being withdrawn from the U.S. and Swedish markets due to frequently exceeding the upper safe limit for total glycoalkaloid content (Petersson et al., 2013).

The difficulties associated with the safety and nutritional testing of whole foods/feed derived from GE crops, which contain thousands of bioactive substances, are well known (reviewed in Bartholomaeus et al., 2013). These include the fact that the quantity of the GE food that can be included in the diet of test animals is limited by the potential to generate nutritional imbalances and might not be high enough to detect adverse effects. Substantial differences in composition could be present without producing a recognizably meaningful difference between treatment groups fed whole foods. Many toxicologists concur that animal feeding trials of whole GE food have a low power to detect adverse effects and contribute little, if anything, to the safety assessment of whole foods (Kuiper et al., 2013). Far more sensitive analytical, bioinformatical, and specific toxicological methods exist to identify unintended effects resulting from plant breeding and provide more precise and quantifiable data for the safety evaluation of whole foods.

In 2013, the European Union (**EU**) Standing Committee on the Food Chain and Animal Health (Brussels, Belgium) adopted a regulation mandating a 90-d subchronic rodent feeding study (OECD, 1998) for every single GE transformation event. This is despite the fact that the European Food Safety Authority (2008; Parma, Italy) states that such testing is only warranted when driven by a specific hypothesis indicated by molecular, compositional, phenotypic, agronomic, or other analysis (e.g., metabolic pathway considerations) of the particular GE event. This mandate is seen by some as interference in the risk assessment of GE foods based on pseudoscience or political considerations (Kuiper et al., 2013). The United States and Australia/New Zealand explicitly do not require a 90-d subchronic rodent feeding study or actively discourage their conduct due to their negligible scientific value.

Studies in which GE crops are fed to target (food-producing) animals have focused less on GE risk assessment and more on evaluating the nutritional properties of the GE crop as well as resulting animal performance and health as compared to the results when fed an isogenic counterpart. Clear guidelines on experimental design for these types of studies have been developed (International Life Sciences Institute, 2003, 2007).

Multiple generations of food animals have been consuming 70 to 90% of harvested GE biomass (Flachowsky et al., 2012) for more than 15 yr. Several recent comprehensive reviews from various authors summarize the results of food-producing animal feeding studies with the current generation of GE crops (Deb et al., 2013; Flachowsky, 2013; Flachowsky et al., 2012; Tufarelli and Laudadio, 2013; Van Eenennaam, 2013). Studies have

been conducted with a variety of food-producing animals including sheep, goats, pigs, chickens, quail, cattle, water buffalo, rabbits, and fish fed different GE crop varieties. The results have consistently revealed that the performance and health of GE-fed animals were comparable with those fed near-isogenic non-GE lines and commercial varieties. Many authors came to the same conclusion a decade ago (Aumaitre et al., 2002; Faust, 2002), suggesting that little contradictory data has emerged over the past 10 yr, despite the increased global prevalence of GE feed.

A number of long-term (of more than 90 d and up to 2 yr in duration) feeding trials and multigenerational studies conducted by public research laboratories using various animal models including pigs, cows, quail, and fish have also been reviewed (Ricroch, 2013; Ricroch et al., 2013; Snell et al., 2012). Significant among these studies are 2 thorough multigenerational studies that examined the long-term effects of feeding a GE corn variety (MON810, expressing the insecticidal Cry1Ab protein from *Bacillus thuringiensis* [**Bt**], one of the few GE corn varieties approved for cultivation in the EU) to food-producing animals, specifically, a German study in dairy cattle and an Irish study in pigs (Guertler et al., 2010, 2012; Steinke et al., 2010; Walsh et al., 2011, 2012 a, b, 2013; Buzoianu et al., 2012 a, b, c, d, 2013 a, b). The results from the multiple papers resulting from these 2 studies are summarized in Table 1. These studies were notable in that they included appropriate controls consuming isogenic non-GE lines of corn, and both comprehensively examined a range of phenotypes and indicators of growth and health and also used sophisticated techniques to look for the presence of recombinant DNA (**rDNA**) and Bt protein in the tissues and products derived from these GE-fed animals.

Results from these comprehensive studies revealed the compositional and nutritional noninferiority of GE corn to its isogenic control and an absence of long-term adverse effects from GE corn consumption. Organ pathology and function were similar between animals fed GE and non-GE corn, and there were no adverse effects of feeding GE corn on small intestinal morphology or the gut microbiota. Antibodies specific to the GE corn protein (Cry1Ab) were not detected in the blood, indicating the absence of an allergic-type immune response to the protein. Neither the *cry1Ab* gene nor the Cry1Ab protein was found in the blood, organs, or products of animals fed GE corn, indicating that neither the intact rDNA nor the intact recombinant protein migrated from the digestive system of the animal into other body tissues or edible animal products.

Even though these 2 comprehensive studies overwhelmingly revealed that a diet of Bt corn was not associated with long-term deleterious effects on the immune systems or animal performance, there were statistically significant differences in some of the parameters measured. Although the authors concluded that these differences were not of biological relevance, significant findings in any parameter in animal feeding studies have been interpreted by some as evidence of harm (Dona and Arvanitoyannis, 2009). Others have pointedly responded that statistical differences per se are not "adverse effects" and need to be considered in terms of their biological importance (Rickard, 2009). The European Food Safety Authority clarified the difference between statistical significance and biological relevance (European Food Safety Authority, 2011). In the absence of some predefined understanding of what changes might be of biological relevance, studies risk becoming "hypothesisless fishing trips." Post hoc analysis of a large number of variables in a data set with a small sample size can lead to spurious conclusions because such studies "are fraught with differences that are not biologically significant between groups from simple variation and probability" (DeFrancesco, 2013).

The Federation of Animal Science Societies maintains an extensive bibliography of food-producing animal GE feeding studies (FASS 2014). Given the large number of 90-d subchronic rodent and food-producing animal GE feeding studies that currently exist in the literature, it is worth questioning the value of more animal feeding studies as part of a GE risk assessment for crops that are substantially equivalent to conventional comparators (Flachowsky, 2013). The rationale for conducting long-term feeding trials and multigenerational studies need to be explicitly stated, especially given that GE proteins are digested in the gut and no intact GE protein has been found in the bloodstream. Once compositional equivalence has been established for a GE crop, animal feeding studies add little to the safety assessment (Bartholomaeus et al., 2013).

There are less than 100 long-term (>90 d) and multigenerational target animal GE feeding studies in the peer-reviewed literature, which has prompted some to call for more of these types of feeding studies (DeFrancesco, 2013). Although such studies may seem intuitively appealing, they must result in novel useful data to justify the additional time, expense, and animal experimentation. Objective analyses of available data indicate that, for a wide range of substances, reproductive and developmental effects observed in long-term studies are not potentially more sensitive endpoints than those examined in 90-d rodent subchronic toxicity tests (European Food Safety Authority, 2008). There is no evidence that long-term and multigenerational feeding studies of the first generation of GE crops that have been conducted to date have uncovered adverse effects that were undetected by short-term rodent feeding studies (Snell et al., 2012). In the context of GE feed risk assessment, they argue that the decision to conduct long-term and

Table 1. Summary results of 2 comprehensive evaluations of target animal effects of long-term feeding of genetically engineered feed (Bt-MON810 corn) to dairy cattle and pigs[1]. Table adapted from Ricroch et al. (2013)

A. Dairy cattle study				
Study Design	Methods	Results	Conclusions	Reference
36 Simmental dairy cows (9 primiparous and 9 multiparous per treatment group) were assigned to 2 feeding groups and fed with diets based on whole-crop silage, kernels, and whole-crop cobs from GE corn (Bt-MON810) or its isogenic non-GE counterpart as main components. The 765-d study included 2 consecutive lactations.	Feed intake, milk production and composition, and body condition over 25 mo	There were no consistent effects of feeding GE corn or its isogenic control on milk composition or body condition. All changes fell within normal ranges.	Compositional and nutritional equivalence of GE corn to its isogenic control. No long-term effects.	Steinke et al. (2010)
	Gene expression pattern of markers for apoptosis, inflammation, and cell cycle from gastrointestinal tract and samples from liver	Statistical analysis of the examined gene expression pattern revealed no significant difference in the gene expression profile of cows fed transgenic or near-isogenic feed ration	Genetically engineered maize MON810 does not have any effect on major genes involved in apoptosis, inflammation, and cell cycle in the gastrointestinal tract and in the liver of dairy cows.	Guertler et al. (2012)
	Fate of *cry1Ab* DNA and recombinant protein	All blood, milk, and urine samples were free of recombinant DNA and protein. The *cry1Ab* gene was not detected in any fecal samples; however, fragments of the Cry1Ab protein were detected in feces from all cows fed transgenic feed.	Milk of dairy cows fed GE corn for 25 mo should be classified not different from milk of cows fed non-GE corn.	Guertler et al. (2010)
B. Pig study				
Large white × landrace cross-bred male 40-d-old pigs (n = 40) were fed 1 of the following treatments: 1) isogenic corn-based diet for 110 d (isogenic), 2) Bt corn-based diet (MON810) for 110 d (Bt), 3) isogenic corn-based diet for 30 d followed by Bt corn-based diet for 80 d (isogenic/Bt), and 4) Bt corn-based diet (MON810) for 30 d followed by isogenic corn-based diet for 80 d (Bt/isogenic).	Feed intake, growth, characteristics, and body composition. Heart, kidneys, spleen and liver weight and histological analysis. Blood and urine analysis.	No difference in overall growth, body composition, organ weight, histology and serum and urine biochemistry. A significant treatment × time interaction was observed for serum urea, creatinine, and aspartate aminotransferase.	Serum biochemical parameters did not indicate organ dysfunction; changes were not accompanied by histological lesions. Long-term feeding of GE maize did not adversely affect growth or the selected health indicators investigated.	Buzoianu et al. (2012a)
	Effect on intestinal microbiota	Counts of the culturable bacteria enumerated in the feces, ileum, or cecum were not affected by GE feed. Neither did it influence the composition of the cecal microbiota, with the exception of a minor increase in the genus *Holdemania*.	Feeding Bt corn to pigs in the context of its influence on the porcine intestinal microbiota is safe.	Buzoianu et al. (2012d)
	Hematological analysis, measurement of cytokine and Cry1Ab-specific antibody production, immune cell phenotyping, and *cry1Ab* gene and truncated Bt toxin detection	On d 100, lymphocyte counts were higher ($P < 0.05$) in pigs fed Bt/isogenic than pigs fed Bt or isogenic. Erythrocyte counts on d 100 were lower in pigs fed Bt or isogenic/Bt than pigs fed Bt/isogenic ($P < 0.05$). Neither the truncated Bt toxin nor the *cry1Ab* gene was detected in the organs or blood of pigs fed Bt corn.	Perturbations in peripheral immune response were thought not to be age specific and were not indicative of Th 2 type allergenic or Th 1 type inflammatory responses. No evidence of *cry1Ab* gene or Bt toxin translocation to organs or blood following long-term feeding.	Walsh et al. (2012b)
Large White × Landrace cross-bred male pigs (9 per treatment group) fed diet containing 38.9% GE or non-GE isogenic parent line corn for 31 d.	Growth performance, intestinal histology, and organ weight and function.	Short-term feeding of Bt MON810 corn to weaned pigs resulted in increased feed consumption, less efficient conversion of feed to gain, and a decrease in goblet cells/mum of duodenal villus. There was a tendency for an increase in kidney weight, but this was not associated with changes in histopathology or blood biochemistry.	The biological significance of these findings is currently being clarified in long-term exposure studies in pigs.	Walsh et al. (2012a)
	Effects on the porcine intestinal microbiota were assessed through culture-dependent and -independent approaches.	Fecal, cecal, and ileal counts of total anaerobes, Enterobacteriaceae, and Lactobacillus were not significantly different between pigs fed the isogenic or Bt corn-based diets. Furthermore, high-throughput 16S rRNA gene sequencing revealed few differences in the compositions of the cecal microbiotas.	*Bacillus thuringiensis* corn is well tolerated by the porcine intestinal microbiota.	Buzoianu et al. (2012c)
	Immune responses and growth in weanling pigs. Determined the fate of the transgenic DNA and protein in vivo.	Interleukin-12 and interferon gamma production from mitogenic stimulated peripheral blood mononuclear cells decreased in GE-fed pigs. Cry1Ab-specific IgG and IgA were not detected in the plasma of GE corn-fed pigs. The detection of the *cry1Ab* gene and protein was limited to the gastrointestinal digesta and was not found in the kidneys, liver, spleen, muscle, heart, or blood.	No evidence of *cry1Ab* gene or protein translocation to the organs and blood of weaning pigs. The growth of pigs was not affected by feeding GE corn. Alterations in immune responses were detected; however, their biologic relevance is questionable.	Walsh et al. (2011)

continued

Table 1. (cont.)

Large White × Landrace cross-bred female pigs (12) – Fed for approximately 143 d throughout gestation and lactation F_0 + 1 generation (offspring at birth). Large White × Landrace cross-bred pigs (10) – Corn dietary inclusion rate identical between treatments (isogenic parent line corn from service to weaning and GE corn from service to weaning [Bt]) and ranged from 86.6% during gestation to 74.4% during lactation). Offspring (72) fed in 4 dietary treatments as follows: 1) non-GE corn-fed sow/ non-GE corn-fed offspring (non-GE/non-GE), 2) non-GE corn-fed sow/GE corn-fed offspring (non-GE/GE), 3) GE corn-fed sow/non-GE corn-fed offspring (GE/non-GE), and 4) GE corn-fed sow/GE corn-fed offspring (GE/GE) for 115 d.	Hematological and immune functions to detect possible inflammatory and allergenic responses at various times. Attempts to detect Cry1Ab protein in blood and feces at various times.	Cytokine production similar between treatments. Some differences in monocyte, granulocyte, or lymphocyte subpopulations counts at some times, but no significant patterns of changes.	No indication for inflammation or allergy due to GE corn feeding. Transgenic material or Cry1Ab-specific antibodies were not detected in sows or offspring.	Buzoianu et al. (2012b)
	Pig growth performance, BW, and feed disappearance recorded at the time of each dietary change (at weaning [d 0] and on d 30, 70, and 100) and at harvest (d 115). At harvest, organ weight, histological observations, and cold carcass weight. Serum biochemistry.	No pathology observed in the organs. Offspring of sows fed Bt corn had improved growth throughout their productive life compared to offspring of sows fed non-GE corn, regardless of the corn line fed between weaning and harvest. Some minor differences in average daily gain, carcass and spleen weights, dressing percentage, and duodenal crypt depths for offspring from GE fed or in average daily feed intake for offspring from sows fed GE and for GE-fed pigs or in liver weight for pigs in the GE/GE.	Feeding GE Bt corn from 12 d after weaning to slaughter had no adverse effect on pig growth performance, body composition, organ weights, carcass characteristics, or intestinal morphology. Transgenerational consumption of GE corn diets not detrimental to pig growth and health.	Buzoianu et al. (2013a)
	Sequence based analysis of the intestinal microbiota of sows and their offspring fed GE corn	At d 115 postweaning, GE/non-GE offspring had lower ileal Enterobacteriaceae counts than non-GE/non-GE or GE/GE offspring and lower ileal total anaerobes than pigs on the other treatments. Genetically engineered corn-fed offspring also had higher ileal total anaerobe counts than non-GE corn-fed offspring, and cecal total anaerobes were lower in non-GE/GE and GE/non-GE offspring than in those from the non-GE/non-GE treatment. The only differences observed for major bacterial phyla using 16S rRNA gene sequencing were that fecal Proteobacteria were less abundant in GE corn-fed sows before farrowing and in offspring at weaning, with fecal Firmicutes more abundant in offspring.	While other differences occurred, they were not observed consistently in offspring, were mostly encountered for low-abundance, low-frequency bacterial taxa, and were not associated with pathology. Therefore, their biological relevance is questionable. This confirms the lack of adverse effects of GE corn on the intestinal microbiota of pigs, even following transgenerational consumption.	Buzoianu et al. (2013b)
	The effects of feeding GE corn during first gestation and lactation on maternal and offspring health serum total protein, creatinine and gamma-glutamyltransferase activity, serum urea, platelet count, and mean cell Hb concentration	Genetically engineered corn-fed sows were heavier on d 56 of gestation. Offspring from sows fed GE corn tended to be lighter at weaning. Sows fed GE corn tended to have decreased serum total protein and increased serum creatinine and gamma-glutamyltransferase activity on d 28 of lactation. Serum urea tended to be decreased on d 110 of gestation in GE corn-fed sows and in offspring at birth. Both platelet count and mean cell Hb concentration (MCHC) were decreased on d 110 of gestation in GE corn-fed sows; however, MCHC tended to be increased in offspring at birth.	There was a minimal effect of feeding GE corn to sows during gestation and lactation on maternal and offspring serum biochemistry and hematology at birth or BW at weaning.	Walsh et al. (2013)

[1]GE = genetically engineered; Bt = *Bacillus thuringiensis*; Hb = hemoglobin.

multigenerational studies should be reserved for cases where some reasonable doubt remains following a 90-d feeding trial triggered by a potential hazard identified in the compositional analysis of the GE crop or other available nutritional or toxicological data.

Field Datasets of Livestock Populations Fed with Genetically Engineered Feed

Although a small number of controlled long-term and multigenerational feeding trials of commercialized GE crops in food-producing species are available in the peer-reviewed literature, large numbers of livestock in many countries have been consuming GE feed for over 15 yr. Hence, a very large and powerful set of GE-fed target animal data has been quietly amassing in public databases. United States agriculture feeds billions of food-producing animals each year, with annual broiler numbers alone exceeding the current size of the global human population (Table 2). During 2011, less than 5% of U.S. animals within each of the major livestock sectors were raised for certified National Organic Program (**NOP**) markets that specifically prohibit the feeding of GE feed (Table 2). Given the increase in GE adoption rates between 2000 and 2013, it can be predicted that the vast majority of conventionally raised livestock in

Table 2. Organic livestock production statistics in the United States (2011)

Industry	Number of organic farms in the United States[1]	Number of animals on organic farms[1]	Total number of livestock animals in the United States[2]	Organic livestock numbers as percent of the U.S. total[3]
Broilers	153	28,644,354	8,607,600,000	0.33%
Layers	413	6,663,278	338,428,000	1.97%
Turkeys	70	504,315	248,500,000	0.20%
Beef cows	488	106,181	30,850,000	0.34%
Dairy cows	1,848	254,711	9,150,000	2.78%
Hogs	97	12,373	110,860,000	0.01%

[1]USDA National Agricultural Statistics Service, 2012.

[2]USDA Economics, Statistics, and Market Information System, 2013.

[3]USDA Economic Research Service, 2013.

the United States consumed feed derived from GE crops over the past decade. Cumulatively, this amounts to over 100 billion animals consuming some level of GE feed between 2000 and 2011 (Table 3).

The duration and level of exposure to GE feed would be expected to vary depending on the animal industry. For example, in a typical U.S. broiler operation, chickens are fed for 42–49 d on diets that are composed of approximately 35% soybean meal and 65% corn grain, whereas in others species, longer-term exposure would be the norm (e.g., dairy cows over recurrent lactations). The average U.S. dairy cow has a productive life of 5 yr with 3 conceptions, 3 gestations, and 3 lactations. A typical U.S. dairy diet contains 50% corn silage, 20% corn grain, and 10% dehulled soybean meal. Also, many cows receive large portions of their rations as ground corn grain, fuzzy cottonseed (no processing except for removal of the lint), or roasted full-fat soybeans. Other GE sources of animal feed include alfalfa hay, sugar beet pulp, corn distillers grains or other coproducts from corn processing, cottonseed meal, canola meal, and soy hulls. A beef cow on the range might consume only some GE alfalfa hay, but her progeny entering the feedlot might be expected to consume a ration containing high quantities of GE feed during their 120 d in the feedlot before harvest. Depending on the feeding stage and relative feed prices, feedlot rations will consist of about 80 to 85% grain (usually corn); distillers' grains and/or other sources of starch/energy; and 10 to 15% hay, silage, or other forage. The remaining share of the ration will include some protein source such as soybean or cottonseed meal (Mathews and Johnson, 2013), also likely to be of GE origin.

Table 3. Estimated cumulative number of livestock raised in the United States during the period from 2000 to 2011

Industry[1]	United States
Broilers	94,683,600,000
Layer Hens	3,722,708,000
Turkeys	2,733,500,000
Beef cattle	339,350,000
Dairy Cows	33,550,000
Hogs	1,219,460,000
Total	102,732,168,000

[1]Numbers for broilers, hogs (barrows and gilts), and beef cattle (steers) are for slaughtered animals during calendar year. Dairy animals are number of dairy cows in a calendar year divided by 3 to account for 3 lactations per animal.

It would be reasonable to hypothesize that if animal feed derived from GE crops had deleterious effects on animals consuming GE feed, then animal performance and health attributes in these large commercial livestock populations would have been negatively impacted. To examine this hypothesis further, in October 2013, data on livestock health were collated from publicly available sources in the United States from before the introduction of GE crops in 1996 through 2000 through 2011, a decade when high levels of GE ingredients would be expected to be present in livestock feed based on the known extent of GE crop cultivation. Data were collected for the broiler, dairy, hog, and beef industries. In general, USDA data sets were from the Economics, Statistics, and Market Information System (2013). Additional data for broilers were available from the National Chicken Council (2011) and were 1) days to market, 2) feed efficiency (feed to meat gain ratio), and 3) percent mortality.

Yearly data on cattle condemnation rates were available for 1999 through 2002 from the USDA Food Safety and Inspection Service (**FSIS**) website (USDA Food Safety and Inspection Service, 2003) and from 2003 through 2007 based on a Freedom of Information Act request as reported (White and Moore, 2009). Data from 1994 was collected from the National Non-Fed Beef Quality Audit as reported (Boleman et al., 1998). Non-fed beef is from culled cows and bulls (i.e., animals that do not spend a significant amount of time being "fed" in a feedlot). Data were analyzed to compare trends before and after the introduction of GE feed into livestock diets. Regression analyses were performed for the period 1983 through 1994 as representative of a period with no GE feed and for the period from 2000 through 2011 as a period with high levels of GE feed based on high rates of GE crop adoption. Where data were available for both time periods, the slope of the regression lines between periods was compared using an unpaired t test.

Table 4. Livestock production statistics in the United States before and after the introduction of genetically engineered feed in 1996

						Broiler				Cattle postmortem condemned, %			
										Fed cattle		Non-fed cattle	
Year	Milk yield, kg	Somatic cell count, cells/mL, 1,000s	Carcass wt, kg, broiler	Carcass wt, kg, hog	Carcass wt, kg, cattle	Condemned, %	Market age, d	Mortality rate, %	Feed to gain	Steers	Heifers	Cows	Bulls
1983	5,708		1.82	75.3	318.8	1.54							
1984	5,667		1.85	75.7	317.5	1.60							
1985	5,910		1.87	76.6	329.3	1.74	49	5	2				
1986	6,029		1.89	77.1	327.4	1.90							
1987	6,252		1.91	77.6	325.2	1.91							
1988	6,446		1.92	78.5	330.2	1.95							
1989	6,460		1.93	78.0	336.1	1.95							
1990	6,640		1.95	79.4	336.1	1.83	48	5	2				
1991	6,742		1.97	79.8	343.3	1.87							
1992	6,995		2.01	79.8	344.7	1.72							
1993	7,054		2.03	81.2	338.8	1.58							
1994	7,315		2.06	81.6	351.9	1.68						2.6	
1995	7,461	304	2.08	82.1	348.8	1.79	47	5	1.95				
1996	7,485	308	2.12	82.1	347.4	1.80							
1997	7,671	314	2.14	83.9	346.5	1.82							
1998	7,797	318	2.16	83.9	357.8	1.86				0.09	0.10	2.22	0.26
1999	8,059	311	2.22	84.8	359.6	1.74				0.11	0.20	2.11	0.31
2000	8,256	316	2.22	86.6	361.9	1.56	47	5	1.95	0.13	0.17	2.71	0.32
2001	8,226	322	2.24	87.5	361.9	1.31				0.09	0.10	2.67	0.31
2002	8,422	320	2.28	87.5	373.2	1.07				0.08	0.09	2.77	0.24
2003	8,503	319	2.31	88.0	359.2	1.00				0.09	0.08	2.92	0.75
2004	8,597	295	2.34	88.0	361.0	1.13				0.08	0.08	2.44	0.35
2005	8,878	296	2.39	89.3	370.5	1.04	48	4	1.95	0.07	0.07	2.59	0.30
2006	9,048	288	2.44	89.8	377.8	1.22	48	5	1.96	0.06	0.07	2.34	0.30
2007	9,191	276	2.45	89.8	376.4	1.16	48	4.5	1.95	0.05	0.06	2.21	0.28
2008	9,250	262	2.48	89.8	380.0	1.10	48	4.5	1.93				
2009	9,332	233	2.48	90.7	384.1	0.91	47	4.1	1.92				
2010	9,591	228	2.53	91.2	378.7	0.88	47	4.0	1.92				
2011	9,680	217	2.58	92.1	381.4	0.87	47	3.8	1.91				

Livestock production statistics for the United States before and after the introduction of GE feed crops in 1986 are summarized in Table 4. In all industries, there were no obvious perturbations in production parameters over time. The available health parameters, somatic cell count (an indicator of mastitis and inflammation in the udder) in the dairy data set (Fig. 1), postmortem condemnation rates in cattle (Fig. 1), and postmortem condemnation rates and mortality in the poultry industry (Fig. 2) all decreased (i.e., improved) over time.

All animals arriving at USDA-inspected slaughter facilities undergo both antemortem and postmortem inspections to identify abnormalities. Carcasses are condemned postmortem if there are visible lesions or tumors present on organs and carcasses. Of the more than 163 million cattle arriving at USDA-inspected slaughter facilities for the years 2003 through 2007, a total of 769,339 (0.47%) were condemned (White and Moore, 2009). Cattle fed or finished in feedyards, typically for 120 d before slaughter on high concentrate diets containing corn and soy as major ingredients, made up the majority (82%) of the cattle at harvest but represented a minority (12%) of the cattle condemned. Condemnation rates for non-fed cattle, particularly cows, were higher than for fed cattle, but the rate in 2007 (2.49%), the last year for which data are available, was similar to that reported in cattle in 1994 (2.6%; Boleman et al., 1998), before the introduction of GE crops.

The broiler data are particularly important due to the large number of animals involved (approximately 9 billion broilers are processed annually in the United States) and the fact that there are several variables that are indicative of health (Fig. 2). The rate of broiler carcass condemnation decreased significantly over time and was at its lowest in 2011. Moreover, mortality was essentially unchanged throughout the years presented and was also at its lowest in 2011. Although broilers are exposed to large amounts of corn and soybean meal during their 42- to 49-d lifespan, they increase their body size 60-fold during this period, making them very sensitive to

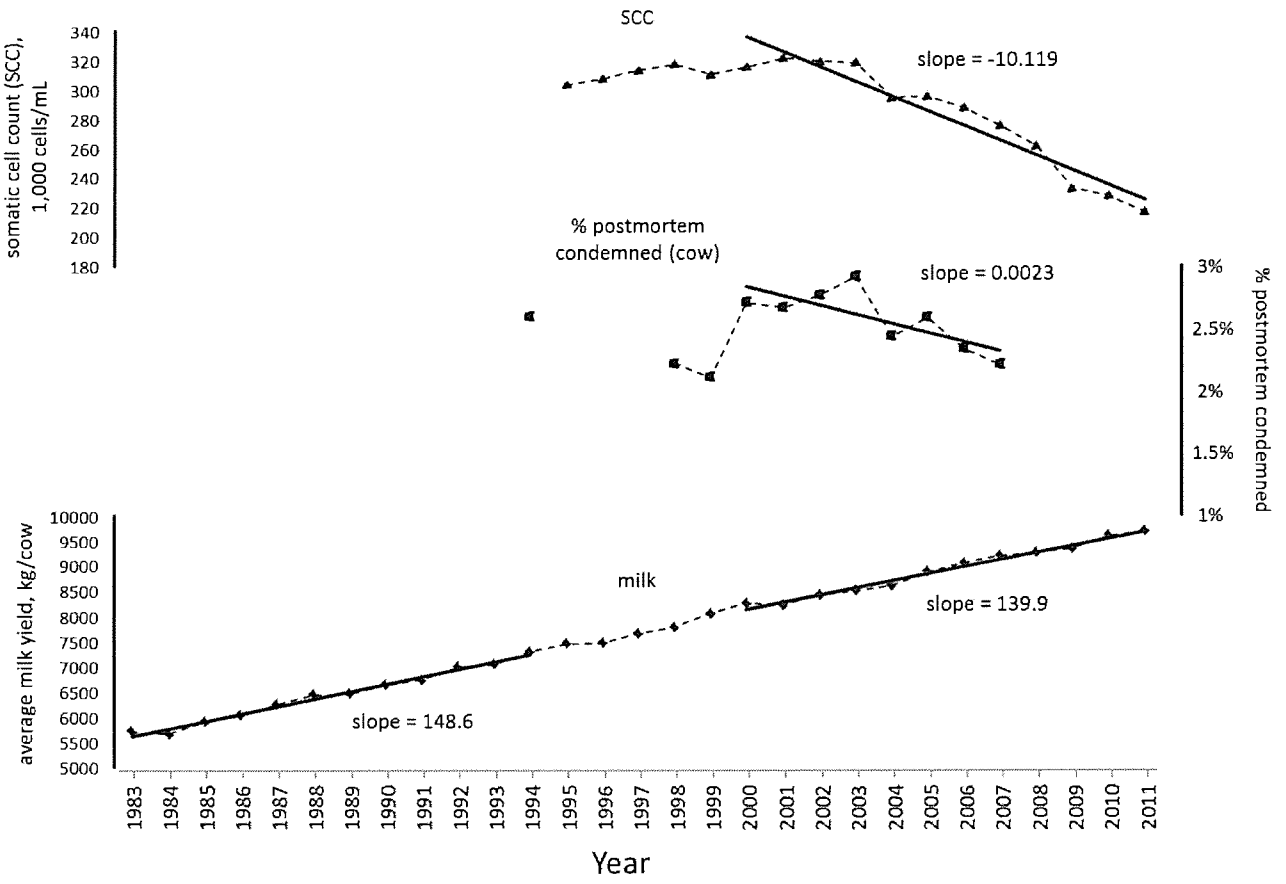

Figure 1. Milk production, percent postmortem condemned, and somatic cell counts for the United States before and after the introduction of genetically engineered crops in 1996. Sources: USDA National Agricultural Statistics Service, 2013; USDA Food Safety and Inspection Service, 2003; White and Moore, 2009; Boleman et al. (1998). Slope does not differ significantly between time periods 1983 through 1994 and 2000 through 2011.

dietary perturbations (European Food Safety Authority, 2008; International Life Sciences Institute, 2003). The conversion of feed to gain continuously decreased from 5 in 1985 to 3.8 in 2011, attributable most likely to improved genetics (Havenstein et al., 2003) and management, but this ratio is something that would be expected to worsen (i.e., increase) if the health of these animals was deteriorating following exposure to GE feed. An estimated 24 consecutive generations of broilers would have been consuming GE feed during the time period 2000 to 2011.

These field data sets representing billions of observations did not reveal unfavorable or unexpected trends in livestock health and productivity. The available health indicators from U.S. livestock suggest that these rates actually improved over time despite widespread adoption of GE crops in U.S. agriculture and increasing levels of GE content in livestock diets. There was no indication of worsening animal health after the introduction of GE feed, and productivity improvements continued in the same direction and at similar rates as those that were observed before the introduction of GE crop varieties in 1996.

A small number of experimental animal feeding studies have generated highly controversial results suggesting deleterious health effects of GE feed. Some of these reports were published and then retracted (Séralini et al., 2012), although recently and controversially republished without further peer review (Séralini et al., 2014), and others were never subjected to peer review (Ermakova, 2005; Velmirov et al., 2008). Adverse effects, including high rates of tumorogenesis, sterility, premature mortality, and histopathological abnormalities have been reported. These studies have been criticized for nonadherence to Organisation for Economic Co-operation and Development (Paris, France) consensus documents and standard protocols. Methodological flaws variously include the use of control feed that was not derived from near-isogenic lines, insufficient animal numbers to enable appropriate statistical power, lack of dose response or insufficient or no information on natural variations in test parameters, overinterpretation of differences that lie within the normal range of variation (i.e., the biological significance of differences is more important than their mere presence), and poor toxicological and/or statistical

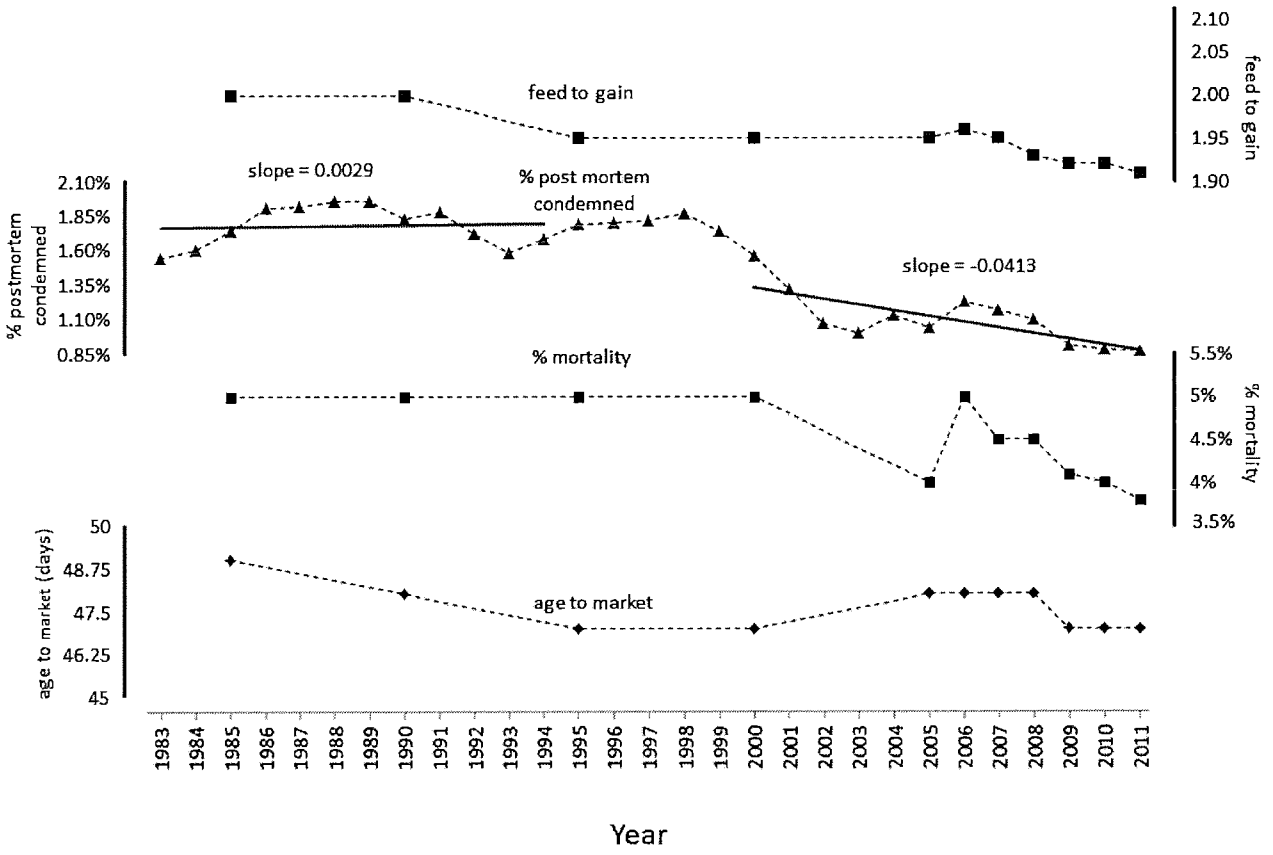

Figure 2. United States broiler statistics before and after the introduction of genetically engineered crops in 1996. Sources: USDA National Agricultural Statistics Service, 2013; National Chicken Council, 2011. Slope differs between time periods 1983 through 1994 and 2000 through 2011 ($^*P < 0.05$).

interpretation of the data (Bartholomaeus et al., 2013; European Food Safety Authority, 2012; Marshall, 2007; Schorsch, 2013; The Australian and New Zealand Food Standards Agency, 2013, 2012). A particularly succinct summary of the methodological design flaws is presented in Table 5 (Bartholomaeus et al., 2013).

Despite a wealth of studies and literature to the contrary, these isolated and poorly designed studies have resulted in the promulgation of new regulations, including a mandatory 90-d rodent subchronic toxicity feeding study for all new GE approvals in the EU (Kuiper et al., 2013), and have generated a great deal of media attention (Arjó et al., 2013). They are also contrary to the field experience as documented by the health and production data collected on the billions of commercial food-producing animals that have primarily been consuming GE feed for over a decade. The media attention devoted to these sensational studies is exacerbating the continued controversy associated with the safety of GE food and feed and is bolstering arguments calling for the mandatory labeling of milk, meat, and eggs from GE-fed animals.

Summary of Data on Recombinant DNA/protein in Milk, Meat, and Eggs from Animals Fed Genetically Engineered Feed

Studies have concluded that animals do not digest transgenic and native plant DNA differently and that rDNA from GE crops has not been detected in animal products (Einspanier, 2013). Fragments of highly abundant plant DNA (e.g., chloroplast genomes) have been found in the digestive tracts and tissues of some species (Einspanier et al., 2001); however, neither recombinant DNA nor protein has ever been found in milk, meat, or eggs from animals that have eaten GE feed with the exception of a single study that reported the presence of fragments of transgenic DNA in both "organic" and "conventional" milk in Italy (Agodi et al., 2006). The organic milk was derived from animals not fed GE crops, so the authors postulated that the rDNA was due to feed and fecal contamination during milking of cows offered GE diets. This result has not been repeated despite recent studies using more sophisticated techniques that have looked for the presence of transgenic material in animal products (Buzoianu et al., 2012b; Deb et al., 2013; Guertler et al., 2010; Tufarelli and Laudadio, 2013). It is important to

Table 5. Examples of limitations in experimental design, analyses, and interpretation in some whole food toxicity studies with genetically engineered (GE) crops (Bartholomaeus et al., 2013). Table reproduced with permission

Best practices	Deficiencies observed	References
Experimental design		
Identity of test and control substances	The identity of the GE test substance was not confirmed through an appropriate analytical method. Confirmation of correct control and test crop presence in diet was not conducted.	Brake and Evenson (2004), Ermakova (2005), Ewen and Pusztai (1999), Kilic and Akay (2008), and Malatesta et al. (2002a,b, 2003, 2005, 2008)
Use of appropriate control crops	The control crop was not of similar genetic background to the GE test crop. In some studies the control was simply identified as a "wild" variety.	Ermakova (2005), Ewen and Pusztai (1999), Malatesta et al. (2002a,b, 2003, 2005, 2008), and Rhee et al. (2005)
	The test and control substances were not produced under similar environmental conditions and/or no information was provided on the production of test and control substances.	Ermakova (2005), Ewen and Pusztai (1999), and Malatesta et al. (2002a,b, 2003, 2005, 2008)
Acceptable levels of contaminants (e.g., pesticides, mycotoxins, other microbial toxins) in control and test crops	Study results were not interpreted in light of differences in antinutrient or mycotoxin levels in test and control diets.	Carman et al. (2013) and Velmirov et al. (2008)
Nutritionally balanced diet formulations for control and test diets	Compositional analyses were not performed on the test and control substances to confirm that test and control diets had similar nutrient content and were nutritionally balanced.	Ewen and Pusztai (1999)
Description of study design, methods, and other details sufficient to facilitate comprehension and interpretation	Inadequate information was provided on the source of animals used, age, sex, animal husbandry practices followed, collection, and evaluation of biological samples to confirm that the procedures followed met accepted practices.	Ermakova (2005), Ewen and Pusztai (1999), and Séralini et al. (2012, 2014)
Statistical analyses and study interpretation		
Use of appropriate statistical methods for the design of the study	Statistical methods were sometimes not provided in sufficient detail to confirm if they were conducted appropriately for the data that were collected; statistical methods were documented but were not appropriate. Estimates of statistical power were based on inappropriate analyses and magnitudes of differences.	de Vendomois et al. (2009), Ewen and Pusztai (1999), Malatesta et al. (2003, 2005), and Séralini et al. (2007, 2012, 2014)
Appropriate interpretation of statistical analyses	Statistical differences were not considered in the context of the normal range for the test species, including data from historical and/or concurrent reference controls; the toxicological relevance of the difference was not considered (i.e., the reported finding is not known to be associated with adverse changes). Observed differences were not evaluated in the context of the entire data collected to determine if changes in a given parameter could be correlated with changes in related parameters.	Carman et al. (2013), de Vendomois et al. (2009), Ewen and Pusztai (1999), Kilic and Akay (2008), Malatesta et al. (2002a,b, 2003, 2005), and Séralini et al. (2007, 2012, 2014)
Adequate numbers of animals or test samples collected to be able to make meaningful comparisons between test and control groups	Too few animals/group were used to make meaningful comparisons; tissue sampling did not follow acceptable guidelines and was too limited to provide an accurate assessment of what was occurring in the organ being examined.	Ermakova (2005), Malatesta et al. (2002a,b, 2003, 2008), and Séralini et al. (2012, 2014)
Study publication and availability		
Publication of studies in peer-reviewed journals	Circumvention of the peer-review process removes a level of review that may contribute to ensuring that WF studies are appropriately designed and interpreted.	Ermakova (2005) and Velmirov et al. (2008)

note that animals and humans regularly ingest DNA and RNA as part of traditional diets without consequence. The DNA from GE crops is chemically equivalent to DNA from other sources and both are thoroughly broken down in the gastrointestinal tract during digestion (Beever and Kemp, 2000; Jonas et al., 2001; CAST, 2006).

Intact recombinant proteins have never been detected in tissues or products of animals fed GE crops (Alexander et al., 2007). This is particularly important when considering the prospect of labeling secondary products such as milk, meat, and eggs. In some countries, mandatory food labeling regulations target the presence of GE components in the finished product (e.g., Australia, New Zealand, and Japan), whereas in other countries, regulations target foods that use GE technology as a part of the production process (e.g., the EU, Brazil, and China). It should be noted, however, that only Brazil currently requires mandatory labeling of products from animals that consume GE feed. Technically, the Brazilian law requires the label to state "(name of animal) fed with rations containing a transgenic ingredient" or "(name of ingredient) produced from an animal fed with a ration containing a transgenic ingredient.", but has yet to fully implement these laws. Given that there are no detectable and reliably

quantifiable traces of GE materials in milk, meat, and eggs, any proposed labeling of animal products derived from GE-fed livestock would have to be based on documenting the absence of GE crops in the production chain, thereby necessitating the need for identity preservation and segregation requirements for producers and importers (Bertheau et al., 2009). This difference is important for verification: a product-based system can be enforced with testing equipment to analyze for the presence of GE materials and can filter a cheater, whereas a tracking system segregating indistinguishable products cannot guarantee the absence of products from animals that might have eaten GE feed (Gruère and Rao, 2007).

In 2012 the USDA's FSIS approved a voluntary process-based label for meat and liquid egg products that allows companies to label that they meet the Non-GMO Project's standard (<0.9% tolerance for GE presence) for the avoidance of GE feed in the diet of the animal producing the product. The FSIS allows companies to demonstrate on their labels that they meet a third-party certifying organization's standards, provided that the claims are truthful, accurate, and not misleading. A similar approach of certifying the absence of prohibited methods in the production chain, rather than testing for some quantifiable attribute in the end product, is used for other voluntary process-based labels such as certified organic and the USDA's Agricultural Marketing Service (**AMS**) Process Verified Never Ever 3 (**NE3**) Program which requires that animals are never treated with antibiotics or growth promotants or fed animal byproducts. Again, because the products raised using these methods are indistinguishable from conventional animal products, the USDA Process Verified Program ensures that the NE3 requirements are supported by a documented quality management system.

2013 Data on Global Production and Trade in Genetically Engineered Feedstuffs and Sources of Non-Genetically Engineered Feedstuffs

Global grain production is currently 2.5 billion t, of which approximately 12% (300 million t) is traded. Soy and corn make up two-thirds of global grain trade and these are the main players in commercial animal feed. Figure 3 illustrates the major global producers of these 2 crops and the proportion of global production that is from GE crop varieties. It is estimated that approximately 85% of soybean and 57% of corn grain production (USDA Foreign Agricultural Service, 2014b) are used in global livestock diets annually. The demand for livestock products has been increasing in response to population growth and income, particularly in developing countries. In Asia alone, consumption of meat and dairy products has been increasing annually by approximately 3 and 5%, respectively (Food and Agriculture Organization of the United Nations, 2012). Increase in demand for animal products, especially meat, will drive demand for grain and protein feeds (USDA Economic Research Service, 2008). The Food and Agriculture Organization of the United Nations (Rome, Italy) predicts that by 2050 global grain trade will double to 600 million t (Bruinsma 2009).

Of the protein sources available, soybean meal has one of the best essential AA profiles for meeting the essential AA needs of livestock and poultry. It is a good source of both lysine and methionine, which are the first limiting AA for swine and poultry, respectively. It is estimated that 79% (85 million ha) of global soybean hectarage is planted to GE varieties (Fig. 3). In 2013, 36.5% of global soybean production (97.2 million t) was exported and 97% came from 3 countries that grow GE soybeans—the United States, Brazil, and Argentina (Fig. 4).

Soybean meal is also an important component of animal feed globally (Fig. 5). In the 2011 to 2012 marketing year, domestic animal agriculture used 27.6 million t of U.S. soybean meal. Poultry continue to be the single largest domestic user of soybean meal, consuming about half of all meal, followed by swine. Soybean meal is a very important protein source for animal feeds in the EU, supplying 46% of the lysine supply overall. The EU imports 65% of its protein-rich feedstuffs, for which there are no alternative sources grown in the EU (Popp et al., 2013), and is the largest importer of soybean meal and the second largest importer of soybeans after China (Fig. 4 and 5). About 70% of soybean meal consumed in the EU is imported and 80% of this meal is produced from GE soybeans.

Corn is an important subsistence crop in many parts of the world and hence the majority of production is consumed within the country of production. Although only 32% (57 million ha) of global corn hectarage is planted with GE varieties (Fig. 3), 71% of global trade came from those countries that grow GE corn varieties (Fig. 6). Approximately 11.6% (100 million t) of global corn production was internationally traded in 2013. Three of the top 5 corn exporting countries—the United States, Brazil, and Argentina—currently grow GE corn. The remaining 2 countries—Ukraine and India—do not have officially registered and approved GE corn varieties.

Of the top 5 corn importing countries—Japan, Mexico, the EU, South Korea, and Egypt—only 5 countries within the EU (Spain, Portugal, Romania, Czechoslovakia, and Slovakia) grew a small amount (148,013 ha) of Bt-MON810 corn (USDA Foreign Agricultural Service, 2014a). Corn is the second largest category of GE products imported into the EU after soy. Unlike soybean, EU corn production is sufficient to meet most of its own corn consumption, with imports accounting for only 10% of total supply. Annual EU imports of corn products include US$1.8 billion of corn, $151 million of corn seed for

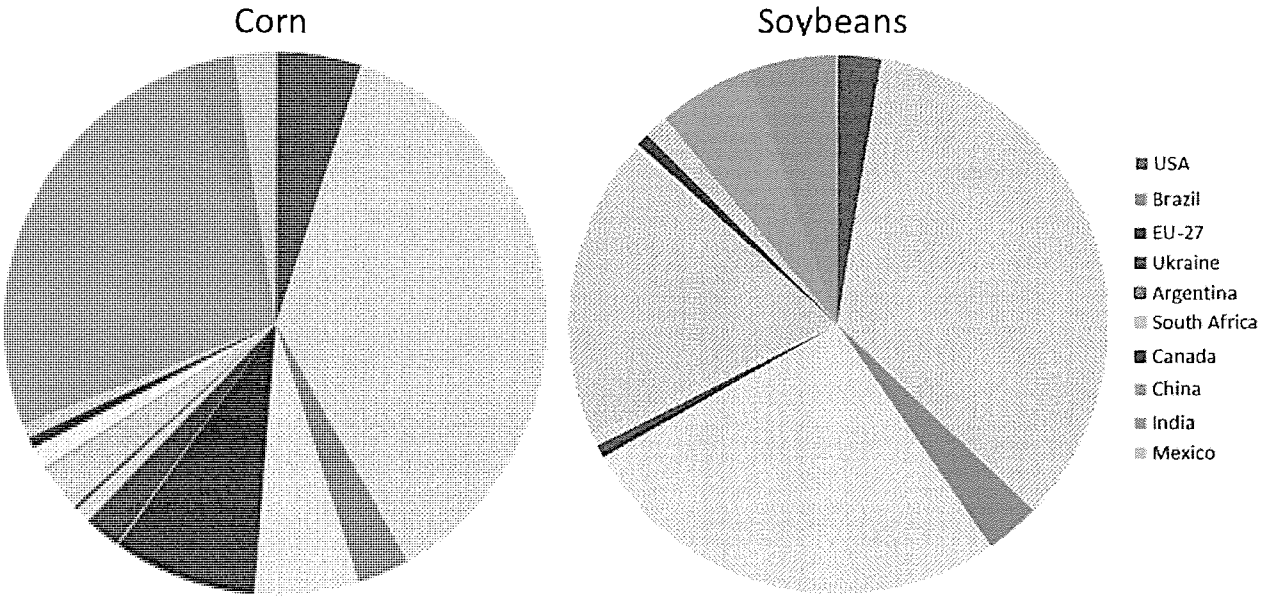

Figure 3. Genetically engineered (GE) and conventional corn and soy produced (million t) by selected countries 2012. Pattern represents production from GE varieties and solid slices represent conventional varieties. Sources: United States Department of Agriculture Foreign Agricultural Service; individual country Global Agricultural Information Network reports 2013; Food and Agriculture Organization of the United Nations (FAOSTAT). EU-27 = the 27 member states of the European Union (EU); production and trade database searches (faostat3.org/faostat-gateway/go/to/download/Q/*/E).

planting, and $87 million of dried distillers grains (USDA Foreign Agricultural Service, 2013a).

Prevalence of Markets Sourcing Non-Genetically Engineered Feed Globally for Livestock Populations as Compared to Conventional

World markets for grains can be separated into 4 segments: the conventional market (non-GE grain that is not certified as such), the mixed market (GE and conventional undifferentiated), the identity-preserved (certified non-GE) market, and the organic market. It is diffi-

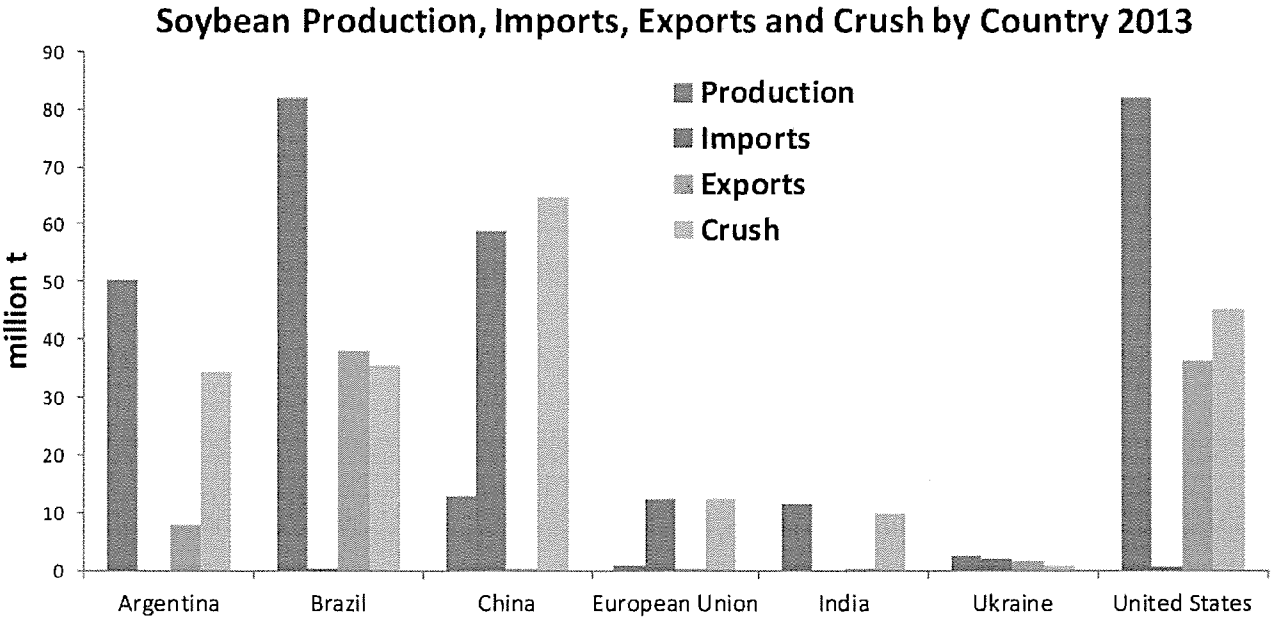

Figure 4. Soybean production, imports, exports, and crush (million t) by major import and export countries, 2013. Source: United States Department of Agriculture Foreign Agricultural Service; Production and trade database searches (http://faostat3.fao.org/faostat-gateway/go/to/download/G1/*/E).

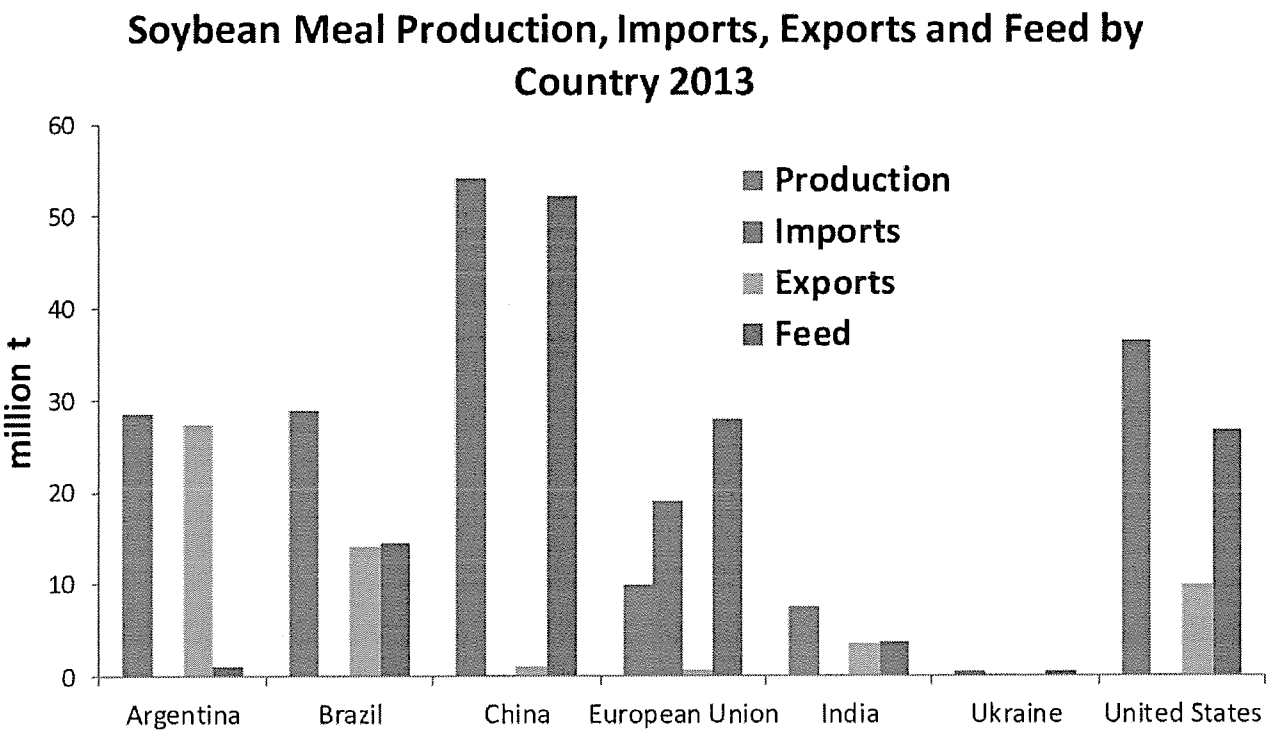

Figure 5. Soybean meal production, imports, exports, and feed (million t) by major import and export countries, 2013. Source: United States Department of Agriculture Foreign Agricultural Service; production and trade database searches (http://faostat3.fao.org/faostat-gateway/go/to/download/G1/*/E).

cult to determine exact size estimates for these different markets, although it can be stated that the conventional and mixed markets are much larger than the remaining 2.

Of the top 5 soybean meal exporting countries in 2013—Argentina, Brazil, the United States, India, and Paraguay—only India does not allow the cultivation of GE soybeans. Of the top 5 soybean meal importing countries in 2013—the EU, Indonesia, Thailand, Vietnam, and Iran—none grow GE soybeans (USDA Foreign Agricultural Service, 2014a). It is estimated that between 4.0 and 4.5% of global trade in soybeans is required to be identity-preserved certified non-GE, and if it is assumed that this volume of traded soybeans is segregated from supplies that may contain GE soybeans, then the GE share of global trade is in the range of 93 to 96% (Table 6). A similar pattern occurs in soybean meal, where 88% of globally traded meal likely contains GE material (Table 7).

The estimated size of the export market requiring certified non-GE corn is 7.3 million t or 7% (Table 6). This excludes countries with markets for certified non-GE corn for which all requirements are satisfied by domestic production (e.g., corn in the EU). Farm animal feed in the 27 member states of the European Union (EU-27) is composed of 50% roughages and 10% grains produced on farm, 10% purchased feed materials, and 30% industrial compound feed. It has been estimated that in the EU, less than 15% of the animal feed market is identity-preserved certified non-GE, although there are great variations between countries. The main driver for non-GE feed is the poultry sector (17%) followed by the cattle (9%) and pig sectors (2%; European Feed Manufacturers' Federation, 2013).

The United States used to be a major supplier of corn to the EU in the 1990s but GE corn plantings in the United States caused a drastic decline in corn exports to the EU because of trade disruptions due to asynchronous approvals (i.e., cultivation approvals of specific GE varieties in the United States occurring before food and feed import approvals in the EU). The result is that the United States is no longer a major supplier of corn to the EU. Similarly, in 2007 there was a problem with asynchronous approval of a GE corn variety approved for cultivation in Argentina but unapproved for food and feed use in the EU. This concentrated demand on corn grown in Brazil, which increased prices an estimated €50/million t for compound feed producers in the EU (Popp et al., 2013).

China, which imported an estimated 5 million t of corn in 2013, making it the sixth largest corn importer, began rejecting shipments of U.S. corn in November 2013 after tests found a GE variety of corn that had been approved for cultivation in the United States, Argentina, and Brazil since 2011 but was not approved for food and feed import into China, despite a 2010 regulatory submission requesting such approval. China has a zero-tolerance policy for unapproved events. Since these trade disruptions began, a total of 3.3 million t of U.S. corn have been subject to re-

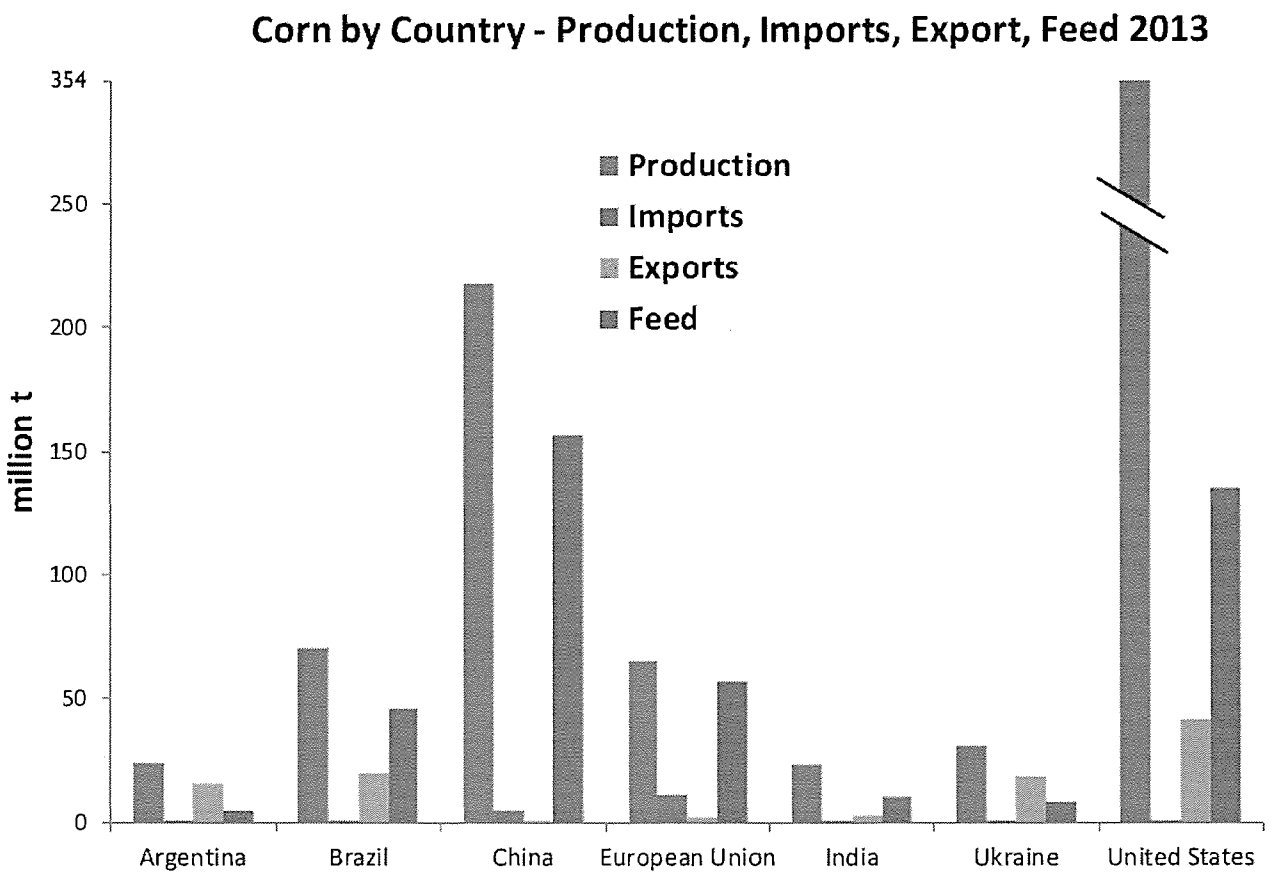

Figure 6. Corn production, imports, exports, and feed (production and trade database searches (http://faostat3.fao.org/faostat-gateway/go/to/download/G1/*/E)) by major import and export countries, 2013. Source: United States Department of Agriculture Foreign Agricultural Service; production and trade database searches(http://faostat3.fao.org/faostat-gateway/go/to/download/G1/*/E).

jection and diverted shipments (1.4 million t) or canceled or deferred sales. It has been estimated that up to $2.9 billion in economic losses were sustained by the U.S. corn, distillers' grains, and soy sectors in the aftermath of the zero-tolerance enforcement policy on U.S. export shipments to China (National Grain and Feed Association, 2014).

Interestingly, Ukraine signed a 3-yr agreement with China in 2013 for the delivery of 4 to 5 million t of corn per year. Ukraine does not export or import GE products as none are officially registered and approved for commercial use or sale in the country. However, private sources estimate approximately 60% of the Ukraine soybean crop and 30% of the corn crop consist of GE varieties (USDA Foreign Agricultural Service, 2013b). China only accepts GE-positive cargo if the shipment is marked accordingly and contains only those GE events that are approved for import in China as well as cultivation in the country of origin. Given asynchronous regulatory approvals and the realities of agricultural production systems where harvesting machinery and storage facilities are shared among different production systems, trade disruption appears almost unavoidable if importing countries enforce a "zero-tolerance" policy for unapproved events that have been approved for cultivation in exporting countries.

Reliance on imported animal feed is becoming increasingly complicated for countries that wish to source non-GE products due to the significant GE adoption rate worldwide. In 2013, 4 major United Kingdom food supermarket groups—Tesco, Cooperative, Marks and Spencer, and Sainsbury's—ceased requiring that poultry and egg suppliers use only non-GE feed (Popp et al., 2013). Likewise, in 2014, the German poultry industry, which feeds 0.8 million t of soybean meal annually, abandoned its commitment to use only non-GE soybeans in poultry feed (USDA Foreign Agricultural Service, 2014c). This was largely due to the fact that Brazil is growing more GE soybeans and therefore has less identity-preserved certified non-GE soybeans available for export. As the global production of GE feed crops continues to rise, the EU's stringent GE tolerance levels (0.9% GE material limit plus 0.05% measuring uncertainty tolerance) and zero tolerance for unapproved events are complicating the maintenance of non-GE supply chains (Popp et al., 2013).

Table 6. Share of global crop trade accounted for by genetically engineered (GE) crop production 2012/2013 (million t; Brookes and Barfoot, 2014c). Table reproduced with permission

Variable	Soybeans	Corn	Cotton	Canola
Global production	266	862.9	26.8	62.6
Global trade (exports)	97.2	100.1	10.0	12.0
Share of global trade from GE producers	94.6 (97.3%)	71.3 (71.2%)	6.9 (69%)	10.2 (85%)
Estimated size of market requiring identity-preserved (certified non-GE) market (in countries that have import requirements)[1]	4.0–4.5	7.3	Negligible	0.1
Estimated share of global trade that may contain GE (i.e., not required to be segregated)	90.1–93.2	64–92.8	6.9	10.1
Percentage of global trade that may be GE	92.75–95.9%	64–92.7%	69%	84.2–85%

[1]Estimated size of market requiring certified conventional in countries with import requirements excludes countries with markets for certified conventional for which all requirements are satisfied by domestic production (e.g., corn in the European Union [EU]). Estimated size of certified conventional market for soybeans (based primarily on demand for derivatives used mostly in the food industry): main markets: EU, 2.5 to 3.0 million t bean equivalents, and Japan and South Korea, 1 million t.

Current U.S. Options for Products from Non-Genetically Engineered Fed Livestock

Consumers wishing to purchase products from animals fed non-GE diets in the United States currently have that choice available through certified NOP products, the FSIS-approved Non-GMO Project verified label claim for meat and liquid eggs, and other non-genetically modified organism certification programs. Additionally, some private retailers are pursuing voluntary labeling. For example, in March 2013, the retail chain Whole Foods Market set a deadline that by 2018, animal products sold in its U.S. and Canadian stores must be labeled to indicate whether or not they came from animals that had consumed GE feed (Whole Foods Market, 2013). These voluntary process-based labels, in effect, verify that GE crops were not used in the production process, rather than testing for the presence of GE content in the animal products themselves as such products contain no detectable and quantifiable traces of GE materials.

Given the high rates of GE adoption in major feed crops, U.S. producers wishing to purchase non-GE feed for their livestock likely contract with growers or source identity-preserved (certified non-GE) or organic feed. In 2011, the United States had 1.26 million ha of certified organic cropland and 0.93 million ha of certified organic pasture and range (USDA National Agricultural Statistics Service, 2012). This translates into roughly 0.8 and 0.5% of total U.S. cropland and pasture/rangeland, respectively (Fig. 7). The availability and cost of certified organic feeds is a major challenge for U.S. organic livestock producers. The costs of certified organic feedstuffs are 2 to 3 times greater than non-organically-grown feeds (Hafla et al., 2013).

United States feed grain distributors and soy product manufacturers report sourcing organic soybeans from other countries. Organic farmers and handlers anywhere in the world are permitted to export organic products to the United States if they meet NOP standards and are certified by a USDA-accredited organic certification body. In 2007, USDA-accredited groups certified 27,000 producers and handlers worldwide to the U.S. organic standard, with approximately 16,000 in the United States and 11,000 in over 100 foreign countries (Grow and Greene, 2009). In 2007, approximately half of the accredited foreign organic farmers and handlers certified to NOP standards were in Canada, Italy, Turkey, China, and Mexico. Organic farming is often labor intensive, and developing countries with lower farm labor costs may have a competitive advantage in the production of some organic products.

In 2009, Canada was the main market for U.S. organic exports, while countries in Latin America, including Mexico, Brazil, Argentina, and Uruguay, along

Table 7. Share of global crop derivative (meal) trade accounted by genetically engineered (GE) product 2012/2013 (million t; Brookes and Barfoot, 2014c). Table reproduced with permission

Variable	Soymeal	Cottonseed meal	Canola/rape meal
Global production	179.3	20.5	34.9
Global trade (exports)	57.2	0.6	5.6
Share of global trade from GE producers	50.4 (88%)	0.29 (46%)	3.6 (64%)
Estimated size of market requiring identity-preserved (certified non-GE) market (in countries that have import requirements)[1]	2.1	Negligible	Negligible
Estimated share of global trade that may contain GE (i.e., not required to be segregated)	48.3	0.63	3.6
Percentage of global trade that may be GE	84.4%	45%	64%

[1]Estimated size of certified conventional market for soymeal: European Union, 2 million t, and Japan and South Korea, 0.1 million t (derived largely from certified conventional beans referred to in Table 6).

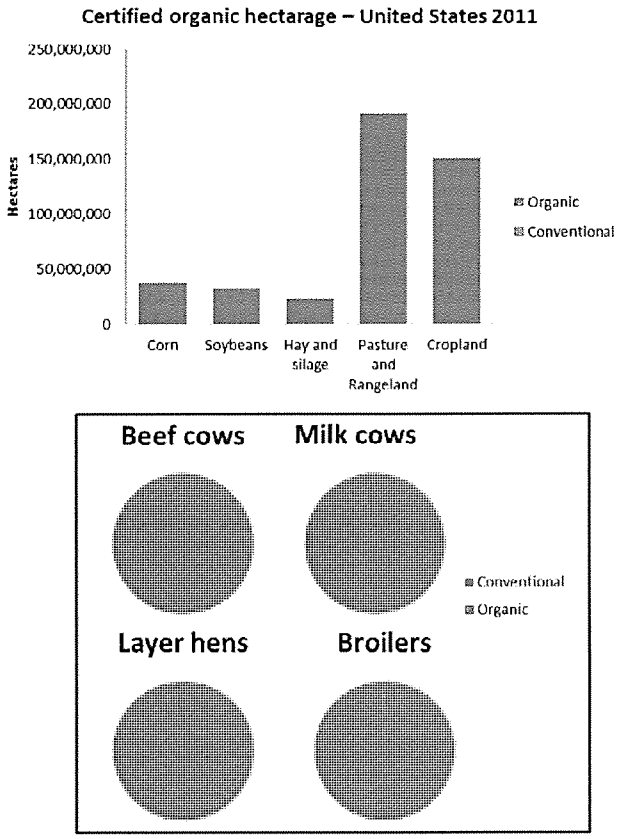

Figure 7. Certified National Organic Program hectarage and livestock numbers as a percentage of conventional U.S. numbers, 2011. Source: USDA National Agricultural Statistics Service, 2012. www.ers.usda.gov/datafiles/Organic_Produc tion/National_Tables_/CertifiedandtotalUSacreageselectedcropslivestock.xls. See online version for figure in color.

with China and other countries in Asia are major sources of organic imports (Grow and Greene, 2009). The countries with the fastest growth in organic production are those that produce organic products for export including China, Bolivia, Chile, Uruguay, and Ukraine. The amount of organic farmland increased well over 1,000% in these countries between 2002 and 2006, while organic farmland in Europe and North America showed slower (27–80%) expansion rates (Grow and Greene, 2009). In 2013, the United States imported over $100 million of organic soybeans primarily from China and India (Fig. 8; Global Agricultural Trade System online [GATS] organic products www.fas.usda.gov/commodities/organic-products). The proportion of organic imports used for livestock feed versus human food purposes is unavailable as import product codes do not distinguish between these uses. Improved data collection is necessary to better describe international trade patterns in organic and identity-preserved (certified non-GE) feed.

Dairy

Organically raised livestock accounted for $1.31 billion in sales in 2011, the last year with a complete set of data on production and sales. Organic milk led livestock commodities, accounting for $765 million, or 58%, of organic animal product sales; however, less than 2% of U.S. dairy production is currently organic (Hafla et al., 2013). During 2011, approximately 254,700 dairy cows (2.78% of the total U.S. dairy herd; Table 2) on 1,848 dairy operations were certified organic. Production costs for organic dairies are greater than for conventional dairies due to the increased cost of organic feed and the increased use of labor and capital, which is not scale neutral as the total costs per unit of production drops sharply as herd size increases. Using pasture as a source of dairy forage is more common on organic dairies, which can help to reduce feed costs per cow but also contributes to lower production per cow. The U.S. organic dairy systems depend on the willingness of consumers to pay a premium (Hafla et al., 2013). The retail price for organic milk between 2004 and 2007 averaged 3 times the cost of conventional milk (USDA Economic Research Service, 2012b), and in 2013, organic milk made up 4.38% of total U.S. fluid milk market sales.

Beef

Natural, organic (grain-fed or otherwise), and grass/forage-fed (including cattle finished on grasses/forages to a specific quality standard) account for about 3% of the U.S. beef market (Mathews and Johnson, 2013). The term "natural" is not associated with an official production process standard so natural beef may come from animals that have consumed GE feed. Likewise, the USDA NE3 Process Verified Program does not mandate or specify the use of non-GE feed.

Beef from grass-fed ruminants can be labeled with a "grass (forage) fed" marketing claim through the AMS Process Verified Program if fed according to USDA standards. Under this verification standard, grass or forage must be the exclusive feed source throughout the lifetime of the ruminant animal except for milk consumed before weaning. The animal cannot be fed grain or any grain byproduct before marketing and must have continuous access to pasture during the growing season. However, silage is an accepted feed that can consist of relatively large portions of grain. For example, corn silage, which averages 10 to 20% grain and can consist of up to a third or more grain, blurs the distinction between grain fed and forage fed (Mathews and Johnson, 2013).

In a survey of certified organic beef producers in the United States, 83% reported that cattle were raised exclusively or predominantly on grass and hay until

slaughter, while the remaining 17% reported using a grain finishing system (Hafla et al., 2013). Organic beef cattle may be finished in feedlots for no more than 120 d and must have access to pasture during this time. In 2011, 106,181 beef cows (0.34% of the total U.S. beef cows; Table 2) and 113,114 unclassified cows and young stock were raised in certified organic production systems. The price of natural/organic beef averaged $12.08/kg in the first quarter of 2011, which represented a premium of $3.75/kg.

Poultry

The largest volume of organic meat sales is for poultry. In 2011, the number of certified organic broilers totaled more than 28 million (0.33% of the total U.S. broilers; Table 2), layer hens totaled more than 6.6 million (1.97% of the total U.S. layers), and turkeys totaled 504,000 (0.20% of the total U.S. turkeys). In 2011, sales of U.S. organic broilers and eggs totaled $115 million and $276 million, representing 0.5 and 3.7% of total sales, respectively. The retail price for organic poultry and eggs between 2004 and 2006 was approximately twice that of conventional products (USDA Economic Research Service, 2012a).

Currently, the size of the market for products derived from animals raised in production systems that use either identity-preserved certified non-GE or organic feed is less than 5% (Fig. 7). Voluntary labeling programs and market premiums exist for products derived from animals that have not consumed GE feed. Mandating the labeling of products derived from animals that have eaten GE-feed at the current time would result in labeling essentially all products derived from conventionally raised livestock (i.e., >95% of all animal products) in the United States.

If suppliers and marketers respond to mandatory labeling of products from animals fed GE feed by increasing the offering of products from animals fed non-GE feed, an increase in the non-GE feed supply would be required. This could come from non-GE feed sources (e.g., wheat and barley), from contracting with U.S. growers to plant non-GE crop varieties, or from imported feed sources. Reversion from GE to conventional crop varieties would require the adoption of altered agronomic practices to manage those crops and relinquishment of the documented environmental and economic benefits associated with the adoption of GE crops (Areal et al., 2013; Fernandez-Cornejo et al., 2014; Green, 2012; NRC, 2010). The prices received by U.S. non-GE corn and soybean producers in recent years have averaged 15% more than the prices received by conventional commodity producers (CAST, 2014), and globally traded non-GE soybean meal is roughly at a 13% premium to conventional soybean meal prices. Given the importance of feed costs in overall animal production costs, the cost of animal products from animals fed non-GE feed would be more expensive.

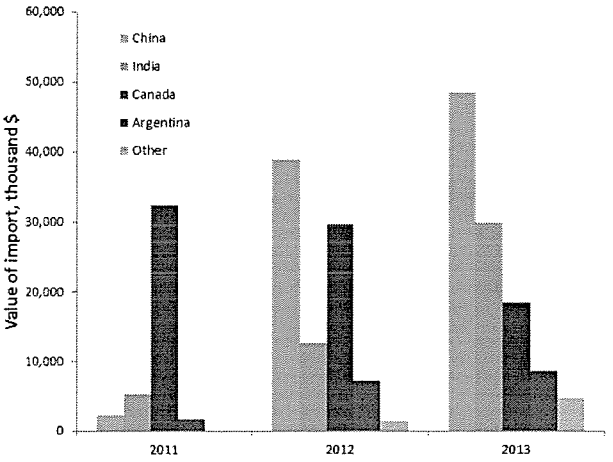

Figure 8. Value of certified National Organic Program soybeans imported into the United States, 2011 through 2013. Source: United States Department of Agriculture Foreign Agricultural Service (2014a). See online version for figure in color.

Impact of Genetically Engineered Feedstuffs on the Sustainability of Livestock Production

Feedstuffs are a major contributor to life cycle assessments in the production of meat, milk, and eggs on a national and global scale. By 2020, developing countries will consume 107 million t more meat and 177 million t more milk than the annual average of the years 1996 through 1998. The projected increase in livestock production will require annual feed consumption of cereals to rise by nearly 300 million t by 2020 (Delgado, 2003). Despite the fact that the first generation of GE crops with so-called "input" traits (those that potentially alter inputs needed in production) were not designed to increase crops yields per se, GE technology has added an estimated 122 and 230 million t to the global production of soybeans and corn, respectively, since the introduction of GE varieties in the mid 1990s (Brookes and Barfoot, 2014a).

In 2013, approximately 175.2 million ha of GE crops were cultivated worldwide (James, 2013) by 18 million farmers. Over 90% (>16.5 million) were small-scale, resource-poor farmers in developing countries. This planting was greater than a 100-fold increase from the 1.7 million ha that were planted in 1996, making GE the fastest-adopted crop technology in recent history. India cultivated 11.0 million ha of Bt cotton with an adoption rate of 95%. In China, 7.5 million farmers cultivating an average of approximately 0.5 ha collectively grew 4.2 million ha of Bt cotton, an adoption rate of 90%. Farmers have planted these GE varieties to enable the adoption of improved agronomic practices (e.g., reduced insecticide applications) providing environmental, economic,

and food security benefits in various countries (Ali and Abdulai, 2010; Burachik, 2010; Fernandez-Cornejo et al., 2014; Huang et al., 2010; Kathage and Qaim, 2012; Qaim and Kouser, 2013).

During the period 1996 through 2012, it has been estimated that the cumulative economic benefits from cost savings and added income derived from planting GE crops was $58.15 billion in developing countries and $58.45 billion in industrial countries (Brookes and Barfoot, 2014a). The adoption of the technology also reduced pesticide spraying by 499 million kg (–8.7%), and has decreased the environmental impact of these crops by 18.1% (as measured by the indicator the Environmental Impact Quotient [a method that measures the environmental impact of pesticides]; Kovach et al., 1992) as a result of the use of less-toxic herbicides and reduced insecticide use (Brookes and Barfoot, 2014b). As a result of fuel savings associated with making fewer spray runs, the adoption of production systems with reduced tillage, and additional soil carbon sequestration, GE crops have also resulted in a significant reduction in the release of greenhouse gas emissions, which, in 2012 alone, was equivalent to removing 11.88 million cars from the roads (Brookes and Barfoot, 2014b).

Although some weed resistance has developed as a result of poor pest management practices and overreliance on a single herbicide (i.e., glyphosate), which may impact future benefits, the adoption of GE technology by the major livestock feed producing countries over the past 16 yr has had a positive sustainability outcome both in terms of increased global yield as a result of improved pest control and reduced overall environmental impacts per kilogram of animal feed produced.

The Future

There are numerous GE crops enhanced for animal nutrition in the research and development pipeline, with almost 100 events under research in many countries of the world (Tillie et al., 2013). This reflects both the importance of feed markets for GE crops and the potential nutritional improvements that can be brought to the quality of feedstuffs using this technology. There are 2 ways in which plant breeding might increase the efficiency of livestock production; the first is by raising the crop yield per hectare (e.g., improved drought tolerance or N use efficiency) and the second is by improving the rate of conversion of vegetable calories into animal calories (e.g., altered output traits or crop composition). Genetic engineering offers new possibilities for approaching both of these objectives, including improving the nutritional value of feed (e.g., AA content; Huang et al., 2006), lowering N and P pollution through altered crop composition (e.g., low phytate; Chen et al., 2008), and reducing manure excretion through a higher NE value (e.g., reduced lignin; Jung et al., 2012). Several of these crops are far advanced in the regulatory pipeline (Table 8; Tillie et al., 2013)

These so-called "second generation" crops modified for output traits will pose some regulatory and commercialization challenges. The first is that they will not, by definition, be substantially equivalent to isogenic non-GE varieties. Protocols have been developed to address the safety testing of these crops (International Life Sciences Institute, 2007). However, given the different regulatory approaches that are in place for crops that are compositionally equivalent, it is unclear how regulatory requirements may vary between countries in terms of the number and length of target animal feeding studies for these crops with altered output traits. Additionally, if the benefits derived from growing these crops accrue to the livestock producer or feeder and not directly to the farmer growing the crop, there will need to be some form of supply chain segregation in place to ensure a price premium is obtained for the value-added output trait.

An additional concern is the increasing problem of asynchronous regulatory approval, or regulatory asynchronicity. Currently, 33 countries have regulatory systems that handle approval for the cultivation or importation of new GE crops (International Service for the Acquisition of Agri-Biotech Applications, 2014). There are considerable discrepancies in the amount of time required to review and approve new GE crops in different countries. This leads to a situation where GE crops may be cultivated and marketed in some countries and remain unapproved in others. As discussed previously, this has resulted in trade disruptions, especially when countries use a "zero-tolerance" policy for unapproved events, meaning that even minute traces of unapproved GE crops are illegal and must be withdrawn from the market. Under a zero-tolerance policy, trade of relevant commodities between asynchronous countries will likely cease as importing and exporting firms will act to avoid the risk associated with a positive test (Kalaitzandonakes et al., 2014). Countries with zero-tolerance policies will be perceived as risky export markets, and importers will pay higher prices and insurance premiums to offset risks taken by the supplier.

Currently, the most accepted techniques for the detection of rDNA and protein products are PCR and ELISA, respectively. Various analytical methods have been developed and are routinely used for the monitoring of GE origin in raw materials and processed foods and have been reviewed elsewhere (Alexander et al., 2007; Marmiroli et al., 2008). Although efforts have been taken to harmonize analytical methodology for the detection of GE products at national, regional, and international levels, no international standards have yet been established (Holst-Jensen et al., 2006). Sampling, testing, and cer-

Table 8. Summary of genetically engineered crops modified for output traits in the latest stages of the pipeline. Modified from Tillie et al. (2013).

Crop	Identifier	Stage[1]	Commercial name	Trait	Developer[2]	Regulatory approval status					
						United States	Argentina	Brazil	China	European Union	Japan
Soybean	DP-305423-1	1	Treus-Plenish	High oleic acid	Pioneer	All uses – 2009	None	None	Food and feed – 2011 (expires 2014)	Food and feed application; additional data request – 2012	All uses – 2010
Safflower		1	Sonova 400	Omega-6	Arcadia BioSciences	Grown under permit; dietary supplement	None	None	None	None	None
Corn	BVLA430101	2		Phytase expression	CAAS/Originally in Agritech	None	None	None	None	None	Cultivation – 2009
Corn	REN-00038-3	2	Mavera	High lysine	Monsanto	All uses – 2006	None	None	None	Application withdrawn – 2009	All uses – 2007
Corn	REN-00038-3 × MON00810-6	2	Mavera YieldGard	High lysine + herbicide tolerance	Monsanto	All uses – 2006	None	None	None	Application withdrawn – 2009	All uses – 2007
Soybean	DP-305423-1 × MON04032-6	2		High oleic acid + herbicide tolerance	Pioneer	All uses – 2009	None	None	None	Food and feed application; additional data request – 2012	All uses – 2012
Soybean	MON-87705-6	2	Vistive Gold	High oleic acid	Monsanto	All uses – 2011	None	None	None	Imports and domestic use – 2012	Food and feed – 2013
Soybean[3]	DD-026005-3	2		High oleic acid	Pioneer	All uses – 1997	None	None	None	None	All uses – 2007
Alfalfa	MON-00179-5	3	None	Low lignin	Forage Genetics/ Monsanto	Food and feed – 2013	None	None	None	None	None
Rapeseed	MPS961-5	3	PhytaSeed	Phytase expression	BASF	Food and feed – 1999	None	None	None	None	None
Soybean	MON87769	3	None	Omega-3	Monsanto	All uses– 2011/2012	None	None	None	Food and feed application; additional data request – 2012	None

[1]Development stage: 1 = commercialized; 2 = commercial pipeline; 3 = regulatory pipeline.

[2]Pioneer, Johnston, IA; Arcadia Biosciences, Davis, CA; CAAS, Beijing, China; Monsanto, St. Louis, MO; Forage Genetics, Nampa, ID; BASF, Ludwigshafen, Germany.

[3]Events whose development is currently discontinued. The information regarding the regulatory status of the events reported in this table was updated in May 2014.

tification depend on statistical processes, however, and hence all are subject to some error, which increases at very low tolerances (Lamb and Booker, 2011).

Kalaitzandonakes et al. (2014) succinctly summarizes some emerging trends in terms of likely increased regulatory asynchronicity in the future. These include 1) the expanding pipeline of novel GE crop events, including second generation crops modified for output traits; 2) the expanding range of GE crop species being grown and traded; 3) the expanding global hectarage of GE crops and the growing number of countries that raise them; and 4) the nascent and inexperienced regulatory expertise in many countries that will be called on to manage a large number of regulatory submissions for new GE crops in the future. Given the scope of trade of livestock feedstuffs and the increasing importance of GE crops in this supply, trade disruptions appear imminent, especially in countries that have slow approval processes for GE imports and yet are heavily dependent on commodity imports from exporting countries that are cultivating and developing a large number of GE crop varieties.

The emergence of precise gene-editing technologies (e.g., zinc finger nucleases [ZFN], meganucleases, transcription activator-like effector nucleases [TALEN], oligonucleotide-directed mutagenesis, and clustered regulatory interspaced short palindromic repeat [CRISPR]/Cas-based RNA-guided DNA endonucleases) that enable targeted editing of specific nucleotides in the endogenous genome (Kim and Kim, 2014) will further complicate this situation. Gene editing could be considered a form of directed mutagenesis and it is unclear whether gene-editing technologies for crops and animals will be encompassed by the GE regulatory system. This is especially uncertain where gene editing results in the substitution of 1 naturally occurring allelic form of a gene for another of the same gene or induces a mutation in an existing gene through a single base pair change analogous to the spontaneous mutation process (Wells, 2013). Whether these types of modifications should be subject to regulation is a topic of dis-

cussion among the global regulatory community (Bruce et al., 2013; Hartung and Schiemann, 2014; Lusser and Davies, 2013). Given that the regulatory process takes years and costs millions of dollars (Prado et al., 2014), the governance of emerging gene-editing technologies will have a great influence on the future development of crops carrying these genetic modifications and will significantly impact the ability of the public sector and small companies to bring gene-edited products to market.

Of particular practical importance is that there will be no way to differentiate a gene-edited DNA alteration from a naturally occurring mutation and hence no way to trace and track "genetically modified" gene-edited crops or differentiate them from genetic modifications resulting from spontaneous mutations. Many of the existing PCR-based tests for GE crops are designed using primers that amplify unique DNA sequences that are common to a variety of transgenic crops (e.g., exogenous promoter sequence or gene coding sequence). As new GE crops with multiple novel regulatory and coding region sequences are developed, it will be increasingly difficult to use PCR-based assays to detect all possible events. Furthermore, PCR-based screening methodology may be unable to detect the genetic modifications that are under development through precise breeding techniques (Lusser et al., 2012). Likewise, some gene-editing techniques generate genetic changes that cannot be distinguished from conventionally bred crops or from crops produced by natural genetic variation or unregulated radiation mutagenesis (Broeders et al., 2012). Process-based regulatory frameworks that rely on PCR-based detection of specific transgenic constructs will be unable keep pace with technological developments when the products of these advanced breeding techniques are indistinguishable from those produced using conventional breeding techniques.

These developments may lead to a revaluation of the current rDNA process-based regulatory trigger for GE organisms to a more scientifically defensible product-based approach centered on the novelty and any unique risks associated with the phenotype of the product rather than the process used to accomplish the genetic modification (Bradford et al., 2005; McHughen, 2007). The need for international coordination and synchronization of regulatory frameworks for GE products is becoming increasingly urgent as both research and development of GE crops and animals are proceeding at an accelerated rate in an ever increasing number of countries in the world. In the absence of international harmonization, costly trade disruptions are likely to become increasingly widespread in the future to the detriment of global food security.

Conclusions

Commercial livestock populations are the largest consumers of GE crops, and globally, billions of animals have been eating GE feed for almost 2 decades. An extensive search of peer-reviewed literature and field observations of animals fed diets containing GE crop products have revealed no unexpected perturbations or disturbing trends in animal performance or health indicators. Likewise, it is not possible to distinguish any differences in the nutritional profile of animal products following consumption of GE feed. Animal agriculture is currently highly dependent on GE feed sources, and global trade of livestock feed is largely supplied by countries that have approved the cultivation of GE crops. Supplying non-GE-fed animal products is likely to become increasingly expensive given the expanding global planting of GE crops and the growing number of countries that raise them. The market for animals that have not consumed GE feed is currently a niche market in the United States, although such products are available to interested consumers via voluntary process-based marketing programs. The cost of these products is higher than conventionally produced products due to both the higher cost of non-GE feed and the costs associated with certifying the absence of GE crops in the production process and product segregation. There is currently a pipeline of so-called "second generation" GE crops with improved output traits for livestock production. Their approval will further complicate the sourcing of non-GE feedstuffs. Additionally, recent developments in techniques to induce precise genetic changes in targeted genes offer both tremendous opportunities and a challenge for global regulatory oversight. Given these developments, there is an urgent need for international harmonization of both regulatory frameworks for GE crops and governance of advanced breeding techniques to prevent widespread disruptions in international trade of livestock feedstuffs in the future.

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Compositional differences between near-isogenic GM and conventional maize hybrids are associated with backcrossing practices in conventional breeding

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Summary

Here, we show that differences between genetically modified (GM) and non-GM comparators cannot be attributed unequivocally to the GM trait, but arise because of minor genomic differences in near-isogenic lines. Specifically, this study contrasted the effect of three GM traits (drought tolerance, MON 87460; herbicide resistance, NK603; insect protection, MON 89034) on maize grain composition relative to the effects of residual genetic variation from backcrossing. Important features of the study included (i) marker-assisted backcrossing to generate genetically similar inbred variants for each GM line, (ii) high-resolution genotyping to evaluate the genetic similarity of GM lines to the corresponding recurrent parents and (iii) introgression of the different GM traits separately into a wide range of genetically distinct conventional inbred lines. The F1 hybrids of all lines were grown concurrently at three replicated field sites in the United States during the 2012 growing season, and harvested grain was subjected to compositional analysis. Proximates (protein, starch and oil), amino acids, fatty acids, tocopherols and minerals were measured. The number of statistically significant differences ($\alpha = 0.05$), as well as magnitudes of difference, in mean levels of these components between corresponding GM variants was essentially identical to that between GM and non-GM controls. The largest sources of compositional variation were the genetic background of the different conventional inbred lines (males and females) used to generate the maize hybrids and location. The lack of any compositional effect attributable to GM suggests the development of modern agricultural biotechnology has been accompanied by a lack of any safety or nutritional concerns.

Keywords: Maize (*Zea mays*), marker-assisted breeding, compositional assessments, genetic modification, natural variability.

Introduction

Maize (*Zea mays* L.) is a major source of food and feed products, globally (James, 2012). Continuous improvements in traits such as herbicide tolerance, insect protection and stress tolerance are essential to the sustainable cultivation of this vital crop. Today, crop development relies extensively on the introduction of transgenic traits into plants, also referred to as genetic modification (GM). Over 70% of all maize crops are enhanced with GM traits, and this number continues to increase (James, 2012). Results from approximately 20 years of compositional studies on maize, many of which have been conducted as part of comparative safety assessments required for commercialization, have consistently shown no meaningful differences between GM crops and their conventional counterparts (Harrigan *et al.*, 2010; Herman and Price, 2013) In fact, the impact of GM trait introduction on composition is negligible relative to varietal/hybrid differences and environmental (geography, climate and agronomic practices) variability (Harrigan *et al.*, 2010; Berman *et al.*, 2011; Zhou *et al.*, 2011a,b; Harrigan and Harrison, 2012; Harrison *et al.*, 2013a,b). These results are hardly surprising and they stem from two key considerations. Firstly, domestication and breeding selection have placed some constraints on compositional variability in modern maize (Flint-Garcia *et al.*, 2009); this has led to suggestions that incorporation of more exotic alleles will be required to support continued germplasm improvements (Flint-Garcia, 2013). Secondly, both conventional breeding and the introduction of a new GM trait utilize the same type of multiple successive backcrossing steps to maximize the genetic similarity of any new line to commercially viable elite germplasm and to ensure desired agronomic characteristics (Figure 1). This backcrossing is driven primarily by breeding and agronomic considerations (Wehrhahn and Allard, 1965) but may have collateral regulatory and safety implications; as pointed out by European Food Safety Authority (EFSA) GMO Panel, 'the occurrence of unintended effects is not a phenomenon specific to genetic modification. In classical breeding extensive backcrossing, selection of favourable lines and discarding lines with unwanted properties is common practice to remove unintended effects' (EFSA, 2008).

Demonstrations of equivalence between GM and conventional counterparts have led to proposals that compositional studies are simply not required for regulatory assessments (Herman *et al.*, 2009; Herman and Price, 2013). Others have proposed that more

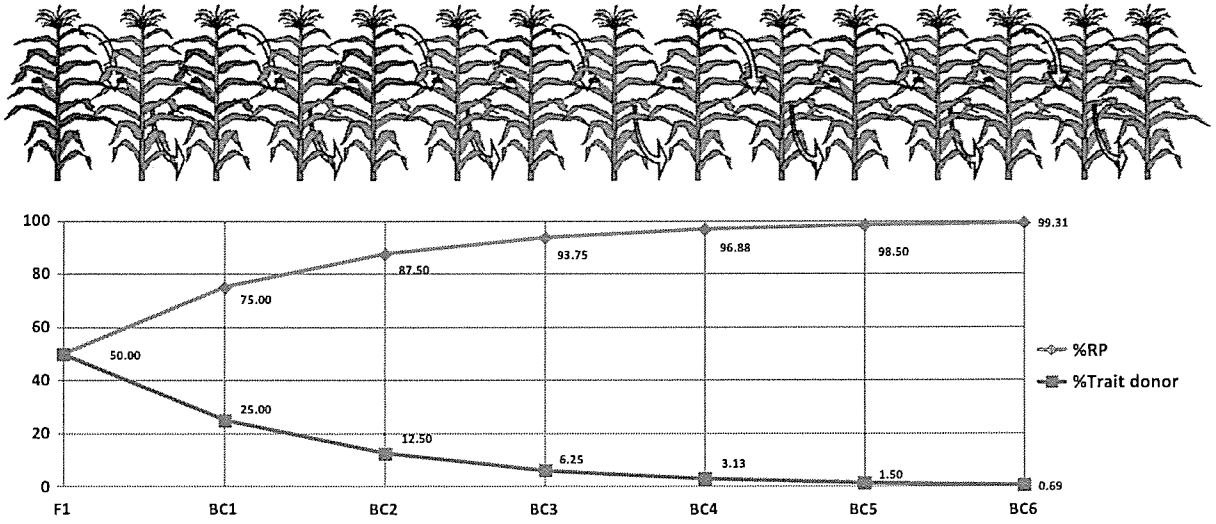

Figure 1 Overview of the successive conventional crossings involved in the GM trait integration process. Theoretically, six successive backcrosses will yield 99% genetic similarity to the desired elite germplasm. Marker-assisted backcrossing allows a reduction in the number of generations required to achieve this level of similarity. The process also generates multiple traited variants that are closely related to the elite germplasm as well as to each other.

innovative data analysis options (Harrison *et al.*, 2011, 2013a,b; Harrigan and Harrison, 2012) that acknowledge the inherent variability of crop composition would ensure that effective assessments of safety are conducted without imposing the prohibitive regulatory burdens that discriminate against smaller entrepreneurial organizations and may restrict agricultural innovation.

The purpose of this study was to provide for the first time an assessment of maize grain composition in the context of natural variability associated with conventional germplasm and with particular reference to the impact of the multiple backcrossing steps that drive the development of both conventional and GM maize products. There have been no studies on to what extent differences between GM and non-GM near-isogenic comparators are actually due to the GM trait. The study was designed specifically to distinguish quantitatively between the relative effects of a new GM trait and the residual genetic variation that distinguishes any near-isogenic lines from each other. A range of different GM traits (drought tolerance, MON 87460; herbicide resistance, NK603; insect protection, MON 89034) were included to assess the generality and robustness of study results, that is, were the observations reproducible regardless of GM trait?

Three features of the study design that are particularly informative included (i) marker-assisted backcrossing (MABC) to generate two genetically similar inbred variants for each GM line, (ii) high-resolution genotyping to evaluate the genetic similarity of GM lines to the corresponding recurrent parents during MABC and (iii) introgression of the different GM traits separately into a wide range of genetically distinct conventional inbred lines.

All F1 hybrids developed from the above breeding programme were grown concurrently at three replicated field sites in the United States during the 2012 growing season. Components analysed in the harvested grain included proximates (protein, starch and oil), amino acids, fatty acids, tocopherols and minerals, offering a comprehensive assessment of kernel composition and consistent with those used in regulatory assessments.

Results and discussion

Genetic characterization of germplasm used in maize hybrid production

The design of the experiment was founded on the use of backcrossing in developing and preserving elite germplasm. An overview of trait integration is shown schematically in Figure 1. Overall, the study included a total of four males (sometimes referred to as base inbreds) and two females (sometimes referred to as testers) to generate the GM (MON 87460, NK603 and MON 89034) hybrid sets, and selection of individuals from the backcrossing process allowed generation of near-isogenic GM variants. A summary of the hybrid and variant sets is presented in Table 1. As highlighted in Table 1, the GM trait that was to be incorporated into a hybrid was carried on either the male or female line. All traited lines were genetically fingerprinted on the Illumina (Diego, CA, USA) Infinium™ platform. The Infinium™ microarrays used for genotyping consisted of 35 000 SNPs markers. Results of the genetic similarity analysis (see Supporting Information) are presented in Table 2 and shown schematically in Figures 2 and 3. In this study, the similarity of all male and/or female inbred lines that contained a GM trait was calculated as greater than 93.7% for all comparisons to the corresponding conventional line (recurrent parent). This high degree of similarity is associated with the impact of backcrossing and is a critical feature of current conventional and GM commercial breeding practices. The genetic analysis also indicated subtle differences between the inbred variants themselves. This is the foundational concept of this study as such differences more broadly imply (i) a genetic basis for compositional differences between 'matched' variants and (ii) that differences observed between GM and non-GM comparators in many reported studies are not directly the effect of the GM trait. In other words, the results of the genetic fingerprinting allowed us to review whether compositional differences between near-isogenic GM and non-GM comparators are simply due to residual genetic variation associ-

Table 1 Overview of GM traits, hybrid sets (Female/Male) and hybrid entries (numbers 1–52)

Trait			NK603		MON 87460		MON 89034	
Female	Male	Control	A	B	A	B	A	B
A7196Z	T3653Z	1	2	3	4	5	6	7
A7196Z	T5927Z	8	9	10	11	12	13	14
A7196Z	A8389Z	15	16	17	18	19		
A7196Z	T5373Z	20			23	24	21	22
V0064Z	T3653Z	25	26	27	28	29	32	33
			30	31				
V0064Z	T5927Z	34	35	36	37	38	41	42
			39	*				
V0064Z	A8389Z	43	44	45	46	47		
V0064Z	T5373Z	48			51	52	49	50

For NK603, the GM trait was carried on the male lines except for entries 30, 31 and 39; for MON 87460, the GM trait was carried on the male line for all entries; for MON89034, the GM trait was carried on the female line except for entries 49–52. *The corresponding B variant was not available.

Table 2 Genetic similarity of traited males and female used in hybrid formation (see Materials and methods)

	% similarity
MON 87460	
T3653Z -A	98.54
T3653Z -B	97.63
T5927Z -A	94.06
T5927Z -B	94.19
A8389Z –A	96.98
A8389Z -B	97.42
T5373Z -A	99.35
T5373Z -B	97.66
NK603	
T3653Z -A	NA
T3653Z -B	97.19
V0064Z -A	98.07
V0064Z -B	98.07
T5927Z -A	98.74
T5927Z -B	95.97
A8389Z -A	97.63
A8389Z -B	97.56
MON 89034	
V0064Z -A	98.41
V0064Z -B	98.71
A7196Z -A	98.16
A7196Z -B	97.36
T5373Z -A	93.68
T5373Z -B	96.66

ated with the multiple conventional breeding steps required in the development of new GM products.

Compositional analysis

Compositional components analysed in the harvested grain included proximates (protein, starch and oil), amino acids, fatty acids, tocopherols and minerals. For all hybrids, least square mean values of each component were determined across all sites (the combined-site analysis) as well as separately for each of the three individual sites. Compositional values were consistent with those reported for maize hybrids elsewhere (Alba *et al.*, 2010). Given the large volume of data, tabulated results are presented in Tables S1–S12 and File S1. Table 3 (main text) provides a condensed overview of the data summarized by the female sets.

For the three GM traits, the following data analysis steps were taken:

1 comparisons of mean component values from the GM hybrids A and B, derived from their respective inbred variants (Table 1) to those of the conventional control hybrid derived from the respective recurrent parent as well as comparisons to each other (Table 4; Figure 4). Statistically significant differences between the mean values were declared at $\alpha = 0.05$. The purpose of this step was to compare the number of significant differences between the traited and control lines to that observed between corresponding traited variants (A and B).

2 assessment of magnitudes of differences in the comparisons of mean component values from the traited hybrids, A and B, (Table 1) with those of the conventional control hybrid derived from the respective recurrent parent (Figure 5 and File S2). This step involved determining the mean difference between each corresponding comparator at the individual sites and expressing that difference in percentages relative to the combined-site mean for the conventional control. This allowed a direct comparison of the range and distribution of component differences that could be associated with GM trait effects or with residual genetic variation.

Overall, for each trait, these two steps would elucidate any consistent trends in differences between a conventional control and the GM product when expressed in a range of genetic backgrounds. The use of a range of diverse traits would allow a robust general conclusion on the effect of genetic modification.

Finally, variance component analysis (VCA) to compare the effect of the GM trait on compositional variability relative to other experimental factors such as germplasm (the effect of different male and female lines) and location (Figure 6 and File S3) was performed across all traits.

MON 87460

MON 87460 contains a gene that encodes cold-shock protein B (CSPB) from *Bacillis subtilis*. Expression of this gene confers a yield advantage when water availability is limited (Castiglioni *et al.*, 2008). The compositional equivalence of MON 87460 to a conventional near-isogenic control has been reported (Harrigan *et al.*, 2009). In the current study, there were a total of 960 comparisons in the combined-site analysis (40 analytes × eight hybrid sets × three entries [control, hybrid A, and hybrid B] within each set). Of these, only 49 comparison (5.10%) were significantly different ($\alpha = 0.05$) and most differences were associated with comparisons between the respective hybrid variants and not between conventional and GM products. Given the paucity of observed differences, no meaningful trends were observed, that is, no analytes could be consistently associated with the GM trait, or with differences between the hybrid variants. The more meaningful interpretation of the data is that the differences between the MON 87460 and respective near-isogenic control hybrids were an unavoidable consequence of the conventional breeding steps required in developing all commercial maize, conventional or GM, that is, due to differ-

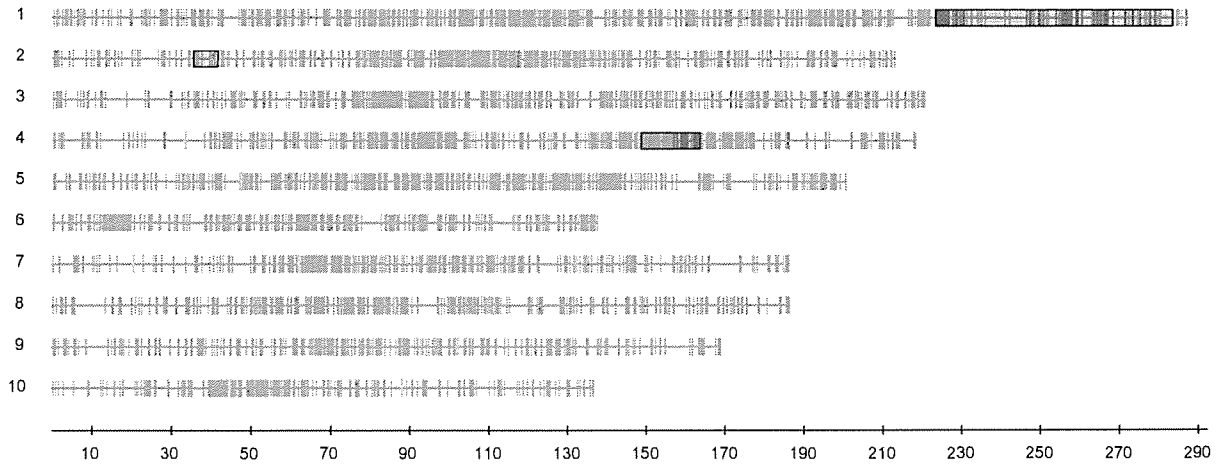

Figure 2 Comparison of the genetic profile of a MON 89034 inbred with its recurrent parent, SNP markers (vertical tics) on the ten chromosomes of the maize genetic map are coloured grey if the genotypes are the same, and either red or green if different; if recurrent parent is homozygous and the MON 89034 line is homozygous with the opposite allele, the marker is red; if recurrent parent is homozygous and the MON 89034 line is heterozygous, the marker is green. Mismatched genomic regions identified by a clustering algorithm are boxed above and account for about 4% of the genome allowing us to say the MON 89034 and recurrent parent is 96% similar. The region on chromosome 4 is near the event insertion site, corresponding to genomic segment from donor line that was selected with the event in backcross process. A large unconverted genomic region on chromosome 1 is also apparent.

Figure 3 Comparison of all genetic profiles of GM trait-containing inbred with its recurrent parent. The number at top indicates chromosome number. They yellow segments indicated unconverted regions. The similarity of the traited inbreds to the recurrent parent as well as to each other is listed on the left of the diagram.

ences in genetic background differences rather than to the MON 87460 trait.

In assessing magnitudes of difference, it was also evident that the range and distribution of values between the GM hybrid variants or between the GM and near-isogenic conventional comparators were essentially similar. This is exemplified in Figure 5 which shows difference associated with a single component (using linoleic acid as an example, see also File S2) as well as across all components.

In summary, it can be concluded that a GM trait that acts through a regulatory protein, such as an RNA chaperone, is no more likely to impact crop composition than a trait expressed through the function of a single enzyme (such as herbicide tolerance in NK603, see below). Drought tolerance has been associated with some osmoprotectant metabolites such as sugars (e.g. trehalose) and free proline (Bartels and Sunkar, 2005), and although these do not represent a safety endpoint, their measurement could contribute to a hypothesis-driven characterization of a stress-tolerant GM product.

NK603

NK603 contains a gene that encodes the CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein which confers tolerance to the family of Roundup® (Monsanto Company, St. Louis, MO, USA) herbicides. Earlier compositional assessments have been reported (Ridley *et al.*, 2002). In the study reported here, there were a total of 840 comparisons in the combined-site analysis (40 analytes × seven hybrid sets × three entries per set) of which only 38 (4.52%) were significantly different ($\alpha = 0.05$). As for MON 87460, no analytes could be consistently associated with the GM trait, or with differences between the variants. Assessments of magnitudes of difference were also essentially similar to that observed for the MON 87460 hybrids. This raises the question of whether nonhypothesis-driven compositional analysis is scientifically warranted for GM crop assessments. In other words, a hypothesis directly linking expected compositional changes to a GM trait would be required for meaningful interpretation otherwise

Table 3 Grain component mean values* combined by female

	Control		MON87460		MON89034		NK603	
Analyte	A7196Z	V0064Z	A7196Z	V0064Z	A7196Z	V0064Z	A7196Z	V0064Z
Proximates†								
Oil	4.09	3.81	4.13	3.82	4.09	3.75	4.27	4.06
Protein	8.36	7.71	8.52	7.86	8.41	7.72	8.52	7.84
Starch	73.95	74.12	73.91	74.16	73.94	74.28	73.68	73.69
Amino acids†								
Alanine	0.63	0.59	0.64	0.59	0.63	0.58	0.64	0.60
Arginine	0.46	0.45	0.46	0.45	0.47	0.45	0.46	0.45
Aspartate	0.57	0.54	0.58	0.54	0.58	0.54	0.57	0.55
Cysteine	0.20	0.20	0.19	0.19	0.21	0.20	0.19	0.19
Glutamate	1.73	1.61	1.75	1.62	1.75	1.59	1.74	1.64
Glycine	0.33	0.32	0.33	0.32	0.33	0.32	0.33	0.32
Histidine	0.23	0.23	0.23	0.22	0.24	0.22	0.23	0.22
Isoleucine	0.30	0.28	0.30	0.28	0.30	0.27	0.30	0.28
Leucine	1.03	0.94	1.05	0.95	1.04	0.93	1.04	0.96
Lysine	0.25	0.25	0.25	0.25	0.26	0.25	0.25	0.25
Methionine	0.19	0.17	0.18	0.17	0.19	0.18	0.18	0.17
Phenylalanine	0.35	0.32	0.35	0.32	0.35	0.32	0.35	0.33
Proline	0.80	0.77	0.79	0.77	0.80	0.75	0.78	0.77
Serine	0.43	0.41	0.44	0.41	0.44	0.41	0.43	0.41
Threonine	0.31	0.29	0.31	0.29	0.31	0.29	0.31	0.29
Tryosine	0.35	0.33	0.36	0.33	0.37	0.33	0.35	0.33
Tryptophan	0.061	0.062	0.062	0.063	0.063	0.061	0.062	0.062
Valine	0.42	0.40	0.42	0.40	0.43	0.40	0.42	0.40
Fatty acids‡								
Palmitic	11.48	11.08	11.26	11.19	11.91	11.29	11.38	10.96
Stearic	1.74	1.84	1.76	1.85	1.71	1.86	1.74	1.82
Oleic	28.83	27.67	28.52	27.78	28.04	27.04	29.66	28.48
Linoleic	55.23	56.61	55.71	56.45	55.63	57.02	54.53	56.04
Linolenic	1.49	1.50	1.49	1.46	1.47	1.52	1.48	1.46
Eicosenoic	0.42	0.46	0.43	0.46	0.45	0.45	0.43	0.43
Eicosadienoic	0.34	0.35	0.33	0.34	0.33	0.34	0.33	0.34
Behenic	0.15	0.14	0.15	0.14	0.16	0.15	0.12	0.14
Tocopherols§								
α-Tocopherol	14.10	11.11	13.74	11.81	12.79	11.74	12.10	10.82
δ-Tocopherol	1.33	0.74	1.34	0.79	1.55	0.98	1.52	0.96
γ-Tocopherol	42.50	31.07	42.01	31.19	46.52	35.96	46.15	35.27
Minerals¶								
Aluminum	41.68	43.43	41.91	43.51	44.87	42.48	43.26	41.52
Calcium	0.0035	0.0040	0.0034	0.0034	0.0034	0.0038	0.0035	0.0034
Iron	15.39	14.90	15.58	14.70	15.50	14.04	15.61	14.30
Magnesium	0.081	0.087	0.081	0.084	0.082	0.085	0.081	0.086
Manganese	4.45	3.60	4.46	3.49	4.74	3.70	4.41	3.46
Phosphorus	0.22	0.22	0.22	0.21	0.22	0.21	0.22	0.22
Potassium	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
Zinc	15.20	15.25	14.58	14.36	15.51	15.02	16.28	14.23

*Least square means determined across all three individual sites and males. Means and standard errors for individual hybrids are shown in Tables S1–S12.

†Expressed as % dwt, starch, protein determined by NIR,

‡Fatty acids expressed as % total FA,

§Expressed as mg/kg dwt.

¶Aluminum, iron, manganese, zinc expressed as mg/kg dwt, calcium, magnesium, phosphorus, potassium expressed as % dwt.

observed differences between two near-isogenic comparators can most realistically be assumed to be due to residual genetic variation. In this context, it is noteworthy that EFSA (2008) states that animal studies are warranted only when compositional differences are seen for the GM effect yet NK603 has been associated with recent controversy related to highly disputed results reported in a formerly retracted (now republished) rat feeding study (Casassus, 2014).

Table 4 Number of statistically significant differences (a = 0.05) from the combined-site analysis (three field sites)

Trait	Hybrid A vs B	Control vs A	Control vs B	Total
MON 87460	19*	13*	17*	49 (5.0%)†
NK603	11‡	18‡	9‡	38 (4.52%)§
MON89034	12¶	18¶	12¶	42 (5.83%)**
Total	42/840 (5.00%)	49/840 (5.83%)	38/840 (4.52%)	129/2520 (5.11%)

*From a total of 320 comparisons.
†From a total of 960 comparisons.
‡From a total of 280 comparisons.
§From a total of 840 comparisons.
¶From a total of 240 comparisons.
**From a total of 720 comparisons.

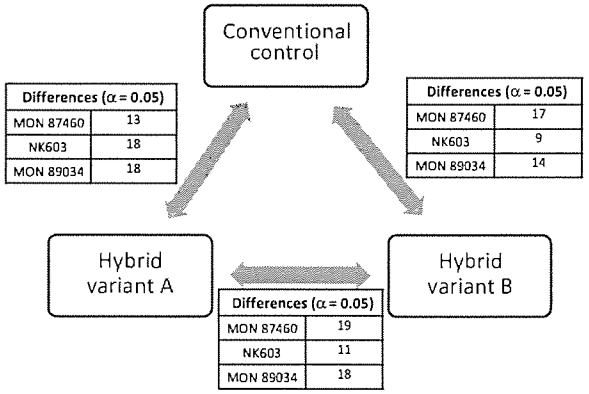

Figure 4 A three-way comparison of the hybrid variants and the corresponding conventional control. Differences in comparisons represented by the diagonal arrows can be attributed to residual genetic variation; differences in comparisons represented by the horizontal arrow can be attributed to residual genetic variation. Components identified as being statistically significantly different are presented in the Supporting Tables.

MON 89034

MON 89034 expresses two Cry insecticidal proteins (Cry1A.105 and Cry2ab2) from *Bacillis thuringiensis* that provide protection against lepidopteran insect pests. The compositional equivalence of MON 89034 to a conventional near-isogenic control has been reported (Drury *et al.*, 2008). In this study, only 42 (5.83%) of 720 comparisons in the combined-site analysis (40 analytes × six hybrid sets × three entries per set) were significantly different ($\alpha = 0.05$). Overall, as for the other traits, no analytes could be consistently associated with the GM trait, or with differences between the variants. In other words, results obtained in this study for the three distinct traits, drought tolerance, herbicide resistance, and insect protection, were near-identical.

With respect to MON 89034, compositional studies on other Cry protein-containing products such as MON 810 have confirmed that differences between GM and non-GM comparators are of similar number and magnitude to that consistent with the residual genetic variation associated with the backcrossing steps used in the generation of conventional and GM products (Zhou *et al.*, 2011b). It can be extrapolated that meaningful compositional changes are unlikely to be induced in any Cry protein-containing products.

Variance component analysis

As mentioned earlier, magnitudes of differences in the comparisons of mean component values from the traited hybrids, A and B, with those of the conventional control hybrid derived from the respective recurrent parent were evaluated. This step involved determining the mean difference between each corresponding comparator at the individual sites and expressing that difference in percentages relative to the combined-site mean for the conventional control. At the same time, the model was used to estimate the means difference between the same conventional control hybrid grown in different sites and provided information on the impact of environment on composition (e.g. Figure 5). To more effectively compare the relative contributions of environment, germplasm, trait, and residual genetic variation, a variance component analysis (VCA) was performed. This analysis confirmed a lack of 'trait' effect; in this context, 'trait' effect could more reasonably be referred to as a 'variant' effect because it refers to differences between A and B variants from the control and therefore includes a contribution from both the GM trait and background genetic effects. The result of VCA combined across all components and all three GM traits is presented in Figure 6. Data presented in Supporting Information show that variation in levels of proximates, amino acids, fatty acids, and tocopherols were generally dominated by germplasm (male–female combination), location, and residual effects. Variation in minerals was dominated, in general, by location and residual effects. The trait effect (or variant effect) was essentially zero for all components. The impact of germplasm and environment on compositional variation is now well established in studies on GM crops (Harrigan *et al.*, 2010; Berman *et al.*, 2011; Zhou *et al.*, 2011a,b; Harrigan and Harrison, 2012; Harrison *et al.*, 2013a,b), and it is increasingly evident that GM is a negligible contributor to that variation.

Conclusion

There are numerous rigorous selection and quality control steps that ensure a lack of unintended effects in newly developed GM crops (Privalle *et al.*, 2012). Moreover, the development of new GM maize hybrids is, at its core, a conventional breeding venture involving extensive backcrossing steps to ensure a high degree of genetic similarity to elite, high performing commercial germplasm. By generating GM variants that are as near-isogenic as practically possible to the respective conventional control, as well as to each other, our study design allowed us to distinguish potential GM effects on composition from residual genetic variation associated with backcrossing. Results showed that differences that would be observed in comparisons between any near-isogenic comparators, conventional or GM, greatly exceed that of GM effects and provide a strong underpinning as to why the impact of GM technology on composition has been consistently found to be negligible. This observation extended not only to physiologically simple single gene traits such as herbicide tolerance or insect protection but to traits that enhance plant physiology to better enable yield or stress tolerance, traits that have also been sought through conventional breeding selection.

It is also evident that the term *genetic modification* is a misnomer when referring to crops developed through modern biotechnology (Herring, 2008). GM traits can be introgressed into a wide range of conventional germplasm without impacting

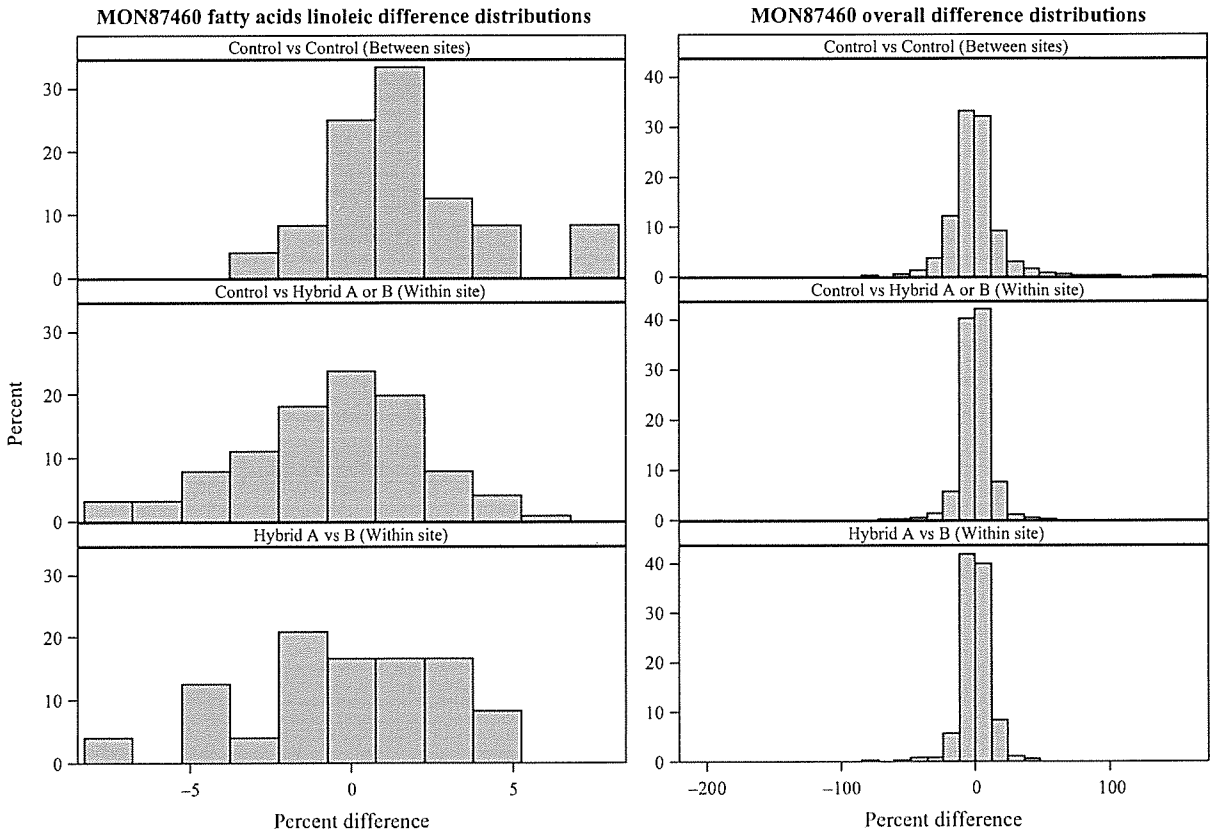

Figure 5 The plots on the left show (bottom caption) magnitudes of difference observed for all corresponding A versus B comparisons expressed as percentage difference, (middle caption) magnitudes of differences observed of A and B hybrids versus control and (top caption) differences between control hybrids grown at different sites. The plots on the right are similar plots combined across all components.

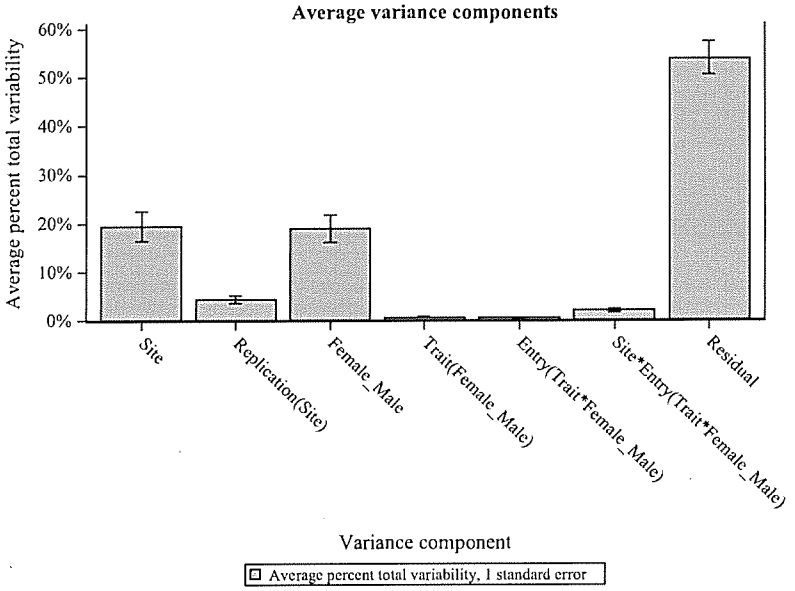

Figure 6 Variance component analysis averaged across all traits and all hybrids. These results highlight the lack of any trait effect; the term Trait (Female_Male) represents variation due to trait (Control, MON87460, MON89034 and NK603) within a female and male combination but does include a contribution from residual genetic variation. The term Entry (Trait × Female_Male) represents variation due to differences between the A and B variants (i.e. residual genetic variation).

extant levels of genetic quality and diversity; the binary classification of GM and non-GMO crops is an artificial dichotomy from the perspective of plant composition and genetics.

Our results have implications for current practices and principles of current safety assessments. The results of previous studies which have assigned differences between near-isogenic conventional and GM comparators to the GM trait may have to be reconsidered. As has been pointed (Herman and Price, 2013), the lack of unintended compositional consequences observed after decades of studies on a range of GM crops support the

safety of the GM process. Compositional studies can therefore only have merit if there is a specific hypothesis on effects that can be directly associated with insertion of a given GM trait. In other words, issues related to safety and nutrition can be more effectively addressed through targeted hypothesis-driven evaluations than by large-scale prescriptive studies that fail to distinguish residual genetic variation between near-isogenic comparators. This is especially true when considering hypothesis-free nontargeted profiling approaches that can neither associate observed differences with the GM trait nor a clear safety or nutritional endpoint. The lack of any compositional effect comprehensively established here for a range of diverse GM traits suggests that citing the so-called precautionary principle to limit the development of modern agricultural biotechnology is misplaced and that the expense and resources associated with precautionary approaches are preventing beneficial traits being developed by smaller organizations that lack the resources to generate required regulatory data.

Materials and methods

Maize samples, nursery and field production

In summary, there were a total of four males (Manufacturer Codes: T3653Z, T5373Z T5927Z and A8389Z) and two females (Manufacturer Codes: A7196Z and V0064Z) (Table 1). These males and females provided the foundation for a range of different traited and conventional control hybrids in this study. The three GM traits chosen for the study included stress tolerance, (MON 87460) (Castiglioni *et al.*, 2008), herbicide tolerance, (NK603) (Ridley *et al.*, 2002), and insect protection, (MON 89034) (Drury *et al.*, 2008). For each GM trait, sets of hybrid variant (A and B) were generated from respective base inbred (male) or tester (female) variants (A and B). Multiple hybrids were generated for each GM trait and each variant, in three to four different genetic backgrounds depending on the GM trait. Control hybrids used for comparison were generated in same genetic background but without the GM trait. A total of 51 hybrids were generated for analysis (Table 1).

Production of transgenic conversions

The conversions used in this experiment were produced by standard backcross breeding supplemented with molecular markers (Eathington *et al.*, 2007). Tissue samples for each individual seedling growing in the conversion nursery were shipped to the Monsanto marker laboratory in Ankeny, Iowa, where they were assayed with PCR using 100 polymorphic markers. Individuals most closely related to the recurrent parent for each conversion were pollinated and advanced to the next generation. This was continued for three to five generations depending on the inbred. After the last backcross, plants were self-pollinated to produce homozygous lines and further increased to produce F4 seed bulks to be used in hybrid production.

The A and B versions were developed by selfing two different plants in the last backcross generation BC3 generation and continuing two separate lineages from that point.

Genetic fingerprint analysis

A bulked seed sample from each of the finished conversions was shipped to the genotyping laboratory at Monsanto St Louis for fingerprinting. The conversions were fingerprinted using the Illumina Infinium™ platform. The Infinium™ microarrays used for genotyping consisted of 35 000 SNPs markers. The SNPs are proprietary Monsanto genetic markers, mostly discovered from the sequencing of two public lines: MO17 and B73 and two elite Monsanto inbreds. By comparing or overlaying genotypes of GM conversions with parental line on the genetic map, we can identify genomic regions where they differ. For example, Figure 2 compares a MON 89034 conversion with its recurrent parent, colouring SNP markers (vertical tics) on genetic map grey if their genotypes are the same, and either red or green if their genotypes are different; if recurrent parent is homozygous and conversion is homozygous with the opposite allele, marker is coloured red; if recurrent parent is homozygous and conversion is heterozygous, marker is coloured green. As expected, and as exemplified in Figure 2, mismatched SNPs tend to cluster, with the clusters defining continuous genomic segments where the lines differ. In Figure 2, the region on chromosome 4 is near the event insertion site, corresponding to genomic segment from donor line that was selected with the GM transgene in backcross process. A large unconverted genomic region on chromosome 1 is also apparent.

To delimit contiguous regions on the genetic map where the near isolines differ, we used a clustering algorithm that identified groupings of improbably neighbouring mismatched markers, accounting for the probability that some SNPs within a region of difference will match (i.e. identical by state but not identical by descent), variability in marker map positions, as well as fingerprint error rate. Delimiting genomic regions is complicated by the large number of matching SNPs within a region. In a straightforward backcross conversion with a single donor and a single recurrent parent, this complication can be avoided by first identifying markers whose genotypes differ between the donor and recurrent parent line; these are the so-called informative markers. When the clustering algorithm is applied using informative markers only, the genomic segments can be more precisely defined. In related work, Frisch and Melchinger (2006) used low-density genetic fingerprints of donor, recurrent parent and offspring to attribute genomic regions in offspring to donor or recurrent parent. Their application required knowledge of informative markers, and extensive interpolation due to low marker density.

Hybrid production

The hybrid seed was produced at the Monsanto research farm in Kihei, Hawaii. The parents (male and female inbreds) for each hybrid were planted in three rows called 'triplets' where the male parent was planted in the centre row flanked by two rows of the same female parent. This configuration was used for each hybrid in the study. To produce the seed, pollen from the male parent in the centre row was collected in standard paper pollinating bags and transferred to the silks of the female parents in the adjoining rows by holding the pollinating bag over the silks and shaking the bag, so the cloud of pollen would settle on all of the exposed silks. To prevent other pollen from contaminating the sample, a 'shoot bag' was kept over the ear shoot until pollination and the pollinating bag was left over the ear after pollination until harvest. The ears were harvested after maturity, dried to approximately 12% moisture in the seed and shipped to the Monsanto research facility in Huxley Iowa for processing and packaging for planting.

Field trials

The hybrids were planted in the field in 2012 growing season in a randomized complete block design at three different locations in United States (Boone County, Iowa and Sangamon and McClean Counties, Illinois). At each location, the plots comprised four rows (7 m long and 0.7 m between rows). Each hybrid material was planted in three replications at each location. Standard agro-

nomic practices for each geographic region were followed. At harvest, six ears were harvested by hand and were dried down to 14% moisture before shelling the seed form the cobs. The seed for the six ears was bulked and shipped to St Louis for compositional analysis.

Analytical methods

Grain samples were analysed for protein, oil, starch, amino acids, fatty acids, minerals and tocopherols. Moisture levels were measured for the re-expression of fresh weight values on a dry weight basis. Values for protein, oil and starch were determined using NIRS (near-infrared spectroscopy) following ISO (International Standard Organization) certified methods. Approximately 250-g sample of whole-kernel was used in a Foss Infratec 1221 near-infrared transmittance (NIRT) instrument. The NIRT was calibrated using reference wet chemistry methods. %RSD (relative standard deviation) calculated from control samples for protein, oil and starch were 1.49%, 3.02% and 0.71%, respectively. Each test sample was measured in duplicate to verify repeatability and average of the two repeats was reported. Where replicate analyses were more than 3 × standard deviation, a third replicate was run. When there were more than two replicate values, a Q-Test was performed to determine whether there is an outlier. If Q (observed) > Q (tabulated), the outlier is discarded and average of the only the two remaining results were reported. All NIRT results were reported on a dry basis percentage (percentage of nonwater material).

Methods for determination of amino acids were as described previously in Harrigan *et al.* (2007). Fatty acids were determined using AOCS (American Oil Chemists' Society) methods as described in Harrigan *et al.* (2007). Minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) levels were estimated using inductively coupled plasma emission (ICP) mass spectrometry-based AOAC methods as described in Drury *et al.* (2008). Tocopherol analysis was based on a reversed-phase HPLC method using fluoresence detection with excitation at 290 nm and emission at 336 nm (Hogarty *et al.*, 1989). Tocopherols were extracted from ground lyophilized seed with 0.1% pyrogallol in ethanol. The reversed-phase HPLC system comprised a Keystone Aquasil C_{18} column at 40 °C, and methanol as mobile phase. Flow rate was 1 mL/min.

Statistical analysis

Statistical analyses were performed using SAS Software (SAS Institute, Cary, NC, USA) Release 9.4. All compositional components were statistically analysed using a mixed model analysis of variance. The three replicated sites were statistically assessed individually (individual site analysis) and as a combination of the all three sites (combined-site analysis).

Combined-site analysis was performed using the following model:

$$Y_{ijklm} = U + S_i + R(S)_{ij} + H_k + T(H)_{kl} + E(\mathrm{TH})_{klm} + \mathrm{SE}(\mathrm{TH})_{iklm} + e_{ijklm}$$

where Y_{ijklm} is the unique individual observation, U is the overall mean, S_i is the random site effect, $R(S)_{ij}$ is the random replicate within site effect, H_k is the hybrid or male × female effect, $T(H)_{kl}$ is the trait within hybrid effect, $E(\mathrm{TH})_{klm}$ is the entry within trait and hybrid combination effect, $\mathrm{SE}(\mathrm{TH})_{iklm}$ is the random site by entry within trait and hybrid combination interaction effect, and e_{ijklm} is the residual error.

Individual site analysis was performed using the following model:

$$Y_{ijkl} = U + R_i + H_j + T(H)_{jk} + E(\mathrm{TH})_{jkl} + e_{ijkl}$$

where Y_{ijkl} is the unique individual observation, U is the overall mean, R_i is the random replicate effect, H_j is the hybrid or male × female effect, $T(H)_{jk}$ is the trait within hybrid effect, $E(\mathrm{TH})_{jkl}$ is the entry within trait and hybrid combination effect, and e_{ijkl} is the residual error.

The SAS procedure PROC MIXED was employed to run the analysis. A residual is the difference between the observed value and its predicted value from a statistical model. A studentized residual is scaled so that the residual values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ±3. Extreme data points that are also outside of the ±6 studentized residual range are considered for exclusion, as outliers, from the final analyses. A total of 18 observations of 18 360 had studentized residuals outside of the ±6 range (one cysteine, one glycine, three eicosadienoic, five iron, one magnesium, one manganese, one zinc, three γ-tocopherol and two δ-tocopherol). These observations were identified as outliers and removed from analysis.

For each compositional component, the mean of the control hybrid was compared to the mean of the respective A and B inbred variants of each trait. Likewise, for each compositional component and trait, the mean of the respective A and B inbred variants was compared to each other. Statistically significant differences between the mean values were declared at $\alpha = 0.05$.

Assessment of magnitudes of difference was performed using the combined-site ANOVA model with the effects of site and the interaction between site and entry within trait and hybrid combination as fixed effects. The model was used to estimate the within site means difference between each of the traited hybrids, A and B, (Table 1) and the conventional control hybrid derived from the respective recurrent parent for each compositional component. At the same time, the model was used to estimate the means difference between the same conventional control hybrids grown in different sites. All differences were expressed as percentages relative to the combined-site mean for the conventional control. This step therefore allowed a direct comparison of the range and distribution of component that could be associated with GM trait effects or with residual genetic variation.

Variance components analysis (VCA) was also conducted to estimate the relative contribution of the experimental factors to the total variance in the study. In this application, all effects from the combined-site ANOVA model were set as random effects. The SAS procedure PROC MIXED was employed to run the analysis. The output table of covariance parameter estimates from SAS PROC MIXED procedure gives estimates of the variance component parameters for each of model components. The variance component parameters of each model component were divided by the total variance to obtain the variance proportions for each component.

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Conflict of interest

There is no direct conflict of interest, but the authors are employed by the agricultural biotechnology industry.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Genetic distance and similarities of the conventional inbreds represented in this study.

Table S1 Starch protein and amino acid combined site mean values for grain from control hybrids.

Table S2 Starch protein and amino acid combined site mean values for grain from MON87460 hybrids.

Table S3 Starch protein and amino acid combined site mean values for grain from MON89034 hybrids.

Table S4 Starch protein and amino acid combined site mean values for grain from NK603 hybrids.

Table S5 Oil fatty acid and tocopherol combined site mean values for grain from control hybrids.

Table S6 Oil fatty acid and tocopherol combined site mean values for grain from MON87460 hybrids.

Table S7 Oil fatty acid and tocopherol combined site mean values for grain from MON89034 hybrids.
Table S8 Oil fatty acid and tocopherol combined site mean values for grain from NK603 hybrids.
Table S9 Mineral combined site mean values for grain from control hybrids.
Table S10 Mineral combined site mean values for grain from MON87460 hybrids.
Table S11 Mineral combined site mean values for grain from MON89034 hybrids.
Table S12 Mineral combined site mean values for grain from NK603 hybrids.
File S1 *Supporting_File1_Ind SiteData* provides individual site data in xml format.
File S2 *Supporting_File2_Histogram of Differences* provides graphical plots showing distribution of magnitude differences between near-isogenic comparators.
File S3 *Supporting_File3_Variance Component Plots* provides graphical plots showing the results of variance component analysis for all assessed crop compositional analytes.