Gene 515 (2013) 241–248

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Review

Evaluation of designer crops for biosafety—A scientist's perspective

Shweta Mehrotra *, Vinod Goyal [1]

National Research Centre on Plant Biotechnology, Lal Bahadur Shastri Building, Pusa Campus, New Delhi-110012, India

ARTICLE INFO

Article history:
Accepted 4 December 2012
Available online 22 December 2012

Keywords:
Transgenics
Phenomics
Transcriptomics
Proteomics
Metabolomics
Commercialization

ABSTRACT

With the advent of transgenic technology, it has become possible to mobilize and express foreign genes into plants and to design crop varieties with better agronomic attributes and adaptability to challenging environmental conditions. Recent advances in transgenic technology have led to concerns about safety of transgenic crops to human and animal health and environment. Biosafety focuses on preventing, minimizing and eliminating risks associated with the research, production, and use of transgenic crops. Food biosafety involves studies of substantial equivalence related to compositional analysis, toxicity and allergenicity. Environmental biosafety involves glasshouse and field trials and study of unintended effects on non-target organisms. Transgenics are characterized at phenotypic and molecular levels for understanding the location of transgene insertion site, ploidy level, copy number, integrated vector sequences, protein expression and stability of the transgene. Various techniques employed for transgene characterization include flow cytometry, southern, northern and western analyses, real-time (qRT) PCR, competitive PCR, FISH, fiber-FISH, DNA micro-arrays, mRNA profiling, 2DE-MS, iTRAQ, FT-MS, NMR, GC–MS, CE–MS and biosensor-based approaches. Evaluation of transgene expression involves the application of integrated phenomics, transcriptomics, proteomics and metabolomics approaches. However, the relevance and application of these approaches may vary in different cases. The elaborate analysis of transgenic crops will facilitate the safety assessment and commercialization of transgenics and lead to global food security for the future.

© 2012 Elsevier B.V. All rights reserved.

Contents

Abbreviations: PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; FISH, fluorescence in situ hybridization; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; mRNA, messenger RNA; ELISA, enzyme linked immunosorbent assay; TAIL-PCR, thermal asymmetric interlaced PCR; ORF, open reading frame; RFLP, restriction fragment length polymorphism; 2DE/MS, two-dimensional electrophoresis mass spectrometry; FT/MS, Fourier-transform ion cyclotron mass spectrometry; GC/MS, gas chromatography mass spectrometry; CE/MS, capillary electrophoresis mass spectrometry; LC/MS, liquid chromatography mass spectrometry; NMR, nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance; T-DNA, transfer-DNA; AFLP, amplified fragment length polymorphism; iTRAQ, isobaric tags for relative and absolute quantification; PAT, phosphinothricin acetyl transferase; A, adenosine; T, thymine; GEO, genetically engineered organisms; GM, genetically modified.

* Corresponding author. Tel.: +91 87 9062 2714.
E-mail addresses: shwetamehrotra@rediffmail.com (S. Mehrotra), goyal2973@gmail.com (V. Goyal).
[1] Present address: Bioseed Research India Pvt. Ltd., Hyderabad 500033, India.

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.12.029

1. Introduction

With the ever-increasing threat of global population, there has been an urgent need for enhancement of productivity in crop plants by infusion of new genetic variability and improvement of the nutritional and industrial utility of the crop species. Effective utilization of genetic diversity remains a pivotal factor in designing crop varieties with better agronomic attributes and adaptability to challenging environmental conditions. Biotechnology is emerging as one of the most innovative tool in life sciences and is influencing almost every aspect of human life. The feasibility of mobilizing and expressing foreign genes into plants has opened up a new era of genetically engineered (transgenic) crops. With limited natural resources available to improve agricultural production, genetically engineered crops provide a promising alternative for improving and enhancing crop productivity. Last few years have witnessed a remarkable progress in the production and cultivation of transgenic crops.

Organisms whose genomes have been altered by the insertion of a foreign gene or genes from another species or unrelated organism are known as transgenics or genetically engineered organisms (GEOs). They have detectable fragments of expected size and acceptable values of foreign/ transgene protein. They carry the transgene which when integrated and expressed stably and properly, confers either a new trait to the organism or enhances or suppresses an already existing trait. Intensive research over the past few decades has resulted in the development of effective gene transfer procedures with subsequent recovery of genetically modified plants. Today's commercialized transgenic plants have been produced using *Agrobacterium*-mediated transformation or gene gun-mediated transformation/ microprojectile bombardment. Novel techniques allow precise manipulation of transgene incorporation and can help to secure stable expression of the transgenics in a wide range of environmental conditions (Tsaftaris et al., 2000).

Insertion of foreign gene into plants has made them capable of defending against natural stresses (biotic and abiotic) with enhanced survival, persistence and competitive capabilities, producing biofuels, vaccines and antibodies and better quality products and novel compounds of commercial value and improving the nutritional quality of food products. To date, various transgenes have been successfully introduced into the nuclear genomes of various plant species. Major crops where transgenics are commercially available include rice, soybean, maize, cotton, canola, potato, cassava, squash, papaya, groundnut, oilseeds and various vegetables and fruits (Asif et al., 2011; Chakraborty et al., 2010; Hutchison et al., 2010; Llorente et al., 2010; Mendoza et al., 2008; Motoyama et al., 2010). Significant improvements in the commercially available crops including herbicide resistance, insect and virus resistance and nutritional quality enhancement have been achieved (Table 1). The role of genetic engineering in generating transgenic lines/cultivars of different crops with improved nutritional quality, biofuel production, enhanced production of vaccines and antibodies, herbicides, and increased resistance to biotic and abiotic stresses has also received attention (Ahmad et al., 2012). The world's leading producers of transgenic crops are USA, Brazil, Argentina, India, China, Paraguay and South Africa.

Table 1
Commercialized transgenic food crops and the significant improvements achieved.

Transgenic crops	Significant improvements
Corn, soybean, rice, corn and sugar beet	Herbicide resistance
Corn, rice, tomato and potato	Insect Pest resistance
Papaya, squash and potato	Virus resistance
Tomato and melon	Delayed ripening, increased shelf-life
Canola and soybean	Improved oil quality
Canola and corn	Male sterility

2. Biosafety concerns

Rapid advances in the development and commercialization of transgenic crops have led to considerable apprehensions and concerns about the safety of transgenic-derived products for human, animal health and environment. Transgenics are also a subject of a strong hostility especially in Europe as they are perceived as hardly useful, non-natural and risky. Transgenic crops are not considered suitable for agriculture in the European Union due to specific scientific and environmental safety reasons. They are considered to have high potential risks and fewer benefits. They comprise biohazards like creation of virulent microorganisms and other life forms that may endanger human and environment. They also comprise potential dangers and risks for farmers and for biodiversity since the landscape and regional dimension of vertical gene flow make almost impossible the coexistence of transgenic crops with organic farming in Europe. Food derived from transgenic crops also poses human health concerns. However, recently the approval of transgenic 'Amflora' potato by the European Commission in 2010 has signaled a fresh approach to genetically engineered organisms in Europe.

The major risks perceived because of the introduction of transgenic crops include i) increased weediness, ii) transgene flow into different cultivars/related species/unrelated organisms, iii) possibility of development of new viruses with wider host range, iv) pest resistance, v) unintended delirious effects on non-target species including biological control agents, v) safety of foods derived from transgenics, vii) feed safety, viii) fate of protein in soil, and other unforeseen effects. These risks may not be universal but may vary depending upon the transgenic lines. This has led to debates over the need for rigorous risk assessments including the long-term effects before permitting the release of any transgenic-derived food products. Mulvaney et al. (2011) have reviewed the economic benefits and risks to marketability of transgenics.

The safe application of biotechnology in agriculture for minimizing risks to the environment and human health from the handling and transfer of transgenics is termed as biosafety. The concerns over ecological and food biosafety have escalated beyond scientific rationality. To ensure the biosafety of transgenic crops, there is a need for a comprehensive analysis of transgenic plants and their progenies, and their complex interactions that can influence their performance. It is imperative to build scientific expertise and resources for assessment of possible risks before commercialization of GEOs are contemplated. The Biosafety Laws establish safety standards and enforcement mechanisms on the construction, cultivation, production, handling, transportation, transfer, import, export, storage, research, marketing, consumption, release into the environment and disposal of transgenics and their derivatives for protection of human and animal health and the environment. The following sections provide an overview of the concerns of food and environment safety and the approaches to analyze them, which have been presented in a form of artwork (Fig. 1).

2.1. Food safety

Food biosafety research focuses on the assessment of novel foods produced from transgenic crops which is performed by comparing them with the conventional analog with an established history of safe use in a study called substantial equivalence, which is done

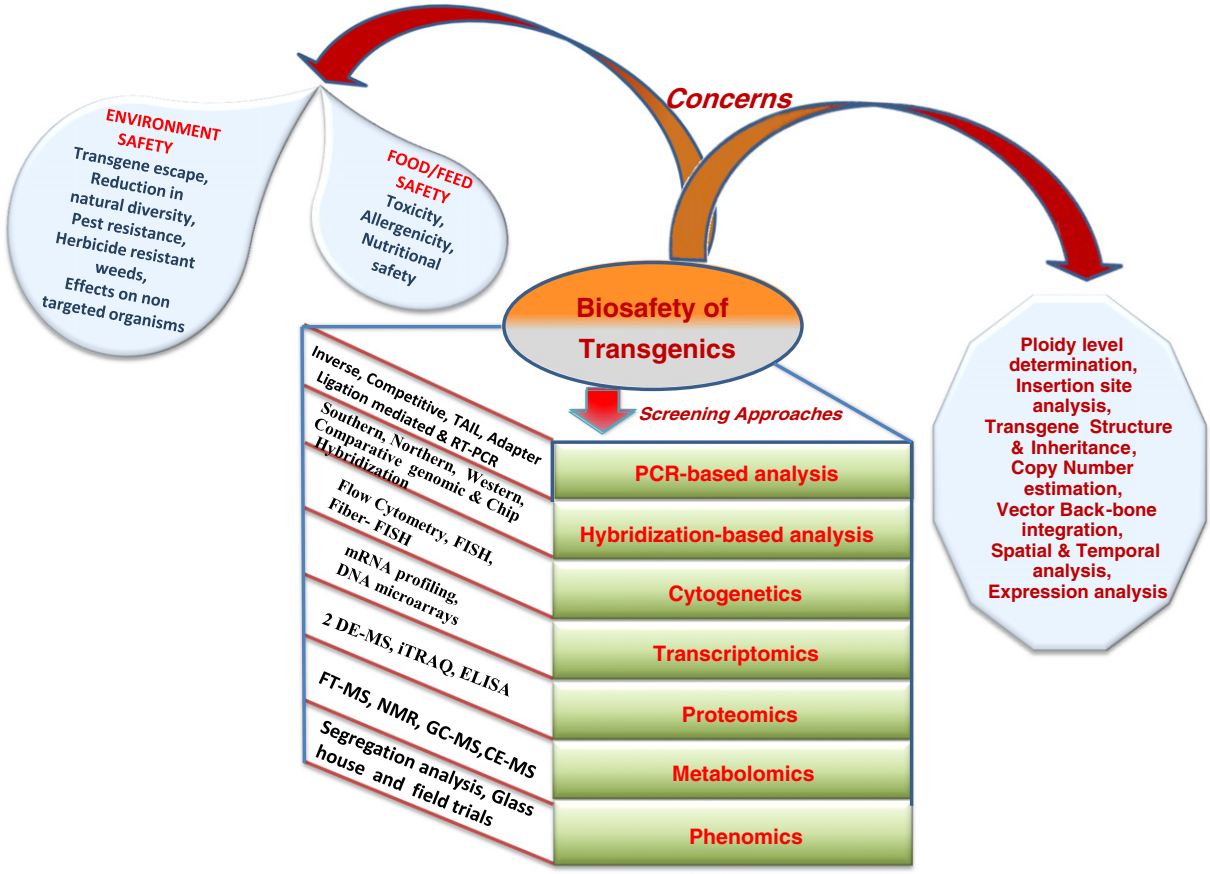

Fig. 1. An overview of different approaches for screening of transgenic crops and concerns for commercialization of transgenic crops.

through evaluation of agronomic, morphological and chemical composition, including macro and micro-nutrients, toxins, and potential changes in the levels of endogenous plant constituents (comparative compositional analysis), allowing the identification of differences between transgenic crops and conventional analogs (Carli et al., 2009; Costa et al., 2011; EFSA, 2004; OECD, 2004; Ridley et al., 2004). The transgenic-derived food should be "substantially equivalent" to its natural counterpart.

The potential hazards of transgenics to human and animal health may be associated with toxicity, allergenicity, intolerance, nutritional quality and microbiological safety of the food, and the possible side effects due to disruption of the metabolic pathways (Costa et al., 2011). There are various assertions that transgenic maize expressing PAT-protein, potato expressing the snowdrop lectin gene, Monsanto's transgenic soya; and the Flavr Savr tomatoes also pose unacceptable health risks but they have not yet been scientifically proved (Pusztai, 2001).

A case-by-case and a tiered approach, and the toxotest approach are employed to study toxicity and allergenicity in the transgenic derived foods or feed. The feeding trials, reproductive, developmental, and transgenerational studies and characterization of dose response are performed on mammalian species. However, studies of Kuntz and Ricroch (2012) do not provide any evidence that long term or multigenerational feeding tests are needed on a routine basis. In fact, a 90-day feeding study performed on rodents can be considered sufficient (apart from specific cases). The traceability of products from animals fed on transgenic derived food is also a crucial aspect to be considered. Moreover, before releasing for human consumption, post-marketing monitoring should be done to monitor the adverse effects or other health outcomes related to the consumption of transgenic-derived foods (Séralini et al., 2011).

Various exploratory techniques like transcriptomics, proteomics and metabolomics have also been used to assess the variations associated with transgenics (Cellini et al., 2004; Gong et al., 2012; König et al., 2004; Ruis, 2010). However, despite the recent reports of numerous omics in assessment of transgenic crops, it does not yet seem feasible to propose large-scale methods that can be internationally certified and accepted. At present, published profiling studies of transgenic crops represent merely a compilation of data, and mandatory use of these techniques in food safety assessment would be pointless. Basic research should be carried out to improve methods and evaluate the reliability of the results. Gürel et al. (2011) discussed the strategies for analysis of transgenic products by DNA based methods such as PCR, DNA hybridization methods, real-time PCR and biosensors. Omics technologies involving nutrigenomics and nutriproteomics allow visualization of nutritional and compositional changes that take place in the transgenics and can add more value to food quality and safety assessment studies (Benkeblia, 2012). Transcriptome studies including microarray analysis in transgenic and non-transgenic *Arabidopsis* extend an understanding of the principle of substantial equivalence (Abdeen et al., 2010; Ouakfaoui and Miki, 2005). Detailed studies of substantial equivalence of transgenic crops at metabolite level were conducted using a hierarchical metabolomics approach employing proton nuclear magnetic resonance (^{1}H NMR) (Baker et al., 2006). Salt et al. (2008) and Baxter (2010) discussed a technique, 'Ionomics', the study of the ionome (mineral nutrient and trace element composition of an organism), which involves the quantitative and simultaneous measurement of the elemental composition of living organisms and changes in this composition in response to physiological stimuli, developmental state, and genetic modifications using high-throughput elemental profiling

and their integration with bioinformatic and genetic tools. Recently, a new MS-based technology, 'Foodomics' has been introduced which includes the genomic, transcriptomic, proteomic, and metabolomic studies of transgenic-derived foods for compound profiling, authenticity, and/or biomarker-detection related to food quality or safety; the development of new transgenic foods, food contaminants, and whole toxicity studies; and new investigations on food bioactivity, and food effects on human health (Herrero et al., 2012). In fact, molecular characterization using omics platforms along with compositional analysis and studies of toxicity and allergenicity will provide a holistic approach for risk assessment of transgenic products.

2.2. Environment safety

Ecological biosafety research has identified potential risks associated with certain crop/transgene combinations, such as intra- and interspecific transgene flow, persistence and the consequences of transgenes in unintended hosts. Concerns have been raised that transgenic crops themselves could become weeds and invade agricultural or natural ecosystems, and the engineered traits could be introduced into non-transgenic counterparts and wild relatives leading to further undesired consequences (Lu, 2008; Raybould and Gray, 1993). Concerns have also been raised that the wide spread of transgenic crops could adversely affect the levels of natural diversity and genetic variability through replacement of traditional varieties (land races), hybridizations between transgenic crops and land races or wild relatives and interactions with non-target organisms. The widespread cultivation of pest-resistant transgenic crops might lead to resistance developing in the targeted pests (Tabashnik et al., 2008). Glyphosate resistant weeds have also been reported in some countries (Kruger et al., 2009).

The risks of transgenic crops may be direct/indirect/immediate or delayed (Conner et al., 2003; Hilbeck et al., 2011). 'Direct effects' are primary effects on human health and the environment which are the result of the transgenic crop itself. Direct effects can be due to toxicity through ingestion by the non-target organisms of a toxin produced by the transgenic plant. 'Indirect effects' occur through a causal chain of events like multitrophic food chains, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management of the crop. 'Immediate effects' refer to the effects which are observed during the period of the release of the transgenic crops. 'Delayed effects' may not be observed during the period of the release of the transgenic but become apparent as a direct or indirect effect either at a later stage or after termination of the release (Hilbeck et al., 2011). Extensive and detailed studies of the long-term effects of the transgenics to the environment in addition to regular glasshouse and field trials are essential for regulatory approval and public acceptance. Transgene confinement and mitigation strategies can provide an effective tool for minimizing any environmental consequences created by transgenics.

3. Characterization of transgenics

The biology of transgenics needs to be fully understood, so that the maximum degree of predictability of transgene effect, both phenotypic and genotypic, can be ensured. Uniformity and stability of T-DNA integration in transgenic plants are a pre-requisite for commercialization. Transgene stability implies stable physical integration and maintenance of the foreign gene in the genome of the host species. Stability of transgene expression depends on the position where the transgene has landed in the genome, the number of copies incorporated, the promoter used, and the presence of repeated elements or cryptic remnants of transposable elements. The following section highlights the biosafety concerns with regard to phenotypic and molecular aspects of transgenic crops and approaches to characterize transgenics to enable a biosafe commercialization.

3.1. Phenotypic analysis

Analysis of phenotype is one of the true measures of safety. The functional transgene leads to phenotypic changes, which allow the selection of transgenic plants. Transgenic crop plants are of value only if their phenotype is stable in the field conditions and is transmitted faithfully in the subsequent generations. The silent genomic changes occurring due to insertion of foreign genes like gene silencing, may lead to undesirable phenotypic consequences. Phenomics studies can help to define and analyze the phenotypic variation in transgenic crops as a whole. The commercialization of transgenic crops requires extensive phenotypic testing which includes segregation analysis, extensive mRNA profiling, metabolic profiling and specific analysis of nutrients and plant toxins, as well as extensive greenhouse trials and field trials (Freese and Schubert, 2004; Kuiper et al., 2001; Roessner et al., 2001). Whole plant phenomics approaches would facilitate the analysis of segregating transgenic plant populations in which trait-specific mutation-linked changes may be missed if plant phenotypes are studied individually. High-throughput phenomics and precision phenotyping can be exploited to study the performance of transgenic plants at cellular and organismic levels as well as to monitor the changes in phenotypic characteristics of transgenic plants expressing the foreign genes.

3.2. Genetic and molecular analysis

The complete characterization of transgenes in plants is a holistic process relying on a wide variety of experimental approaches. Molecular characterization is an essential step in safety assessment of transgenics. After the initial laboratory analysis where transformants are selected and their transgenic status is verified, the transgenics are subjected to detailed genetic, molecular and biochemical analysis including the determination of ploidy level of transgenics, identification of insertion site, structure of transgene loci, estimation of copy number, vector backbone contamination, expression analysis of transgene including RNA and protein expression, and spatial and temporal analysis of transformed plant (Bhat and Srinivasan, 2002; Gase et al., 2011; Zhang et al., 2008). The confirmed transgenic plants with desired levels of gene expression are then shifted to a containment facility for further genotypic and phenotypic analyses (Fig. 1).

The routine generation of transgenic plants involves analysis of transgene integration into the host genome as well as evaluation of the transgene expression. PCR amplification of transgene is considered an indication of successful transgenic, which is further confirmed by southern analysis. Later, reverse transcription PCR (RT-PCR), real-time (qRT) PCR, northern and western assays and enzyme linked immunosorbent assay (ELISA) are employed to assess the expression of the transgene (Fig. 1). DNA micro-arrays, mRNA profiling, proteomics, and metabolomics profiling serve as complementary tools for the safety assessment of transgenic crops (Zolla et al., 2008). Recently, biosensor-based approaches have also become an important part of transgenic analysis (Gürel et al., 2011). Biosensors represent advanced assays with high detection sensitivity provided by the physico-chemical transducers, which sense DNA hybridization events. Target DNA is captured by a complementary specific probe, which is attached to the surface of sensors. Hybridization event is translated to a value by the transducer, which is associated with the probe. Various electrochemical, piezoelectric and optical biosensors with high sensitivity are being used for testing GM crop derivatives (Bai et al., 2010; Passamano and Pighini, 2006; Stobiecka et al., 2007; Zhang et al., 2009).

3.2.1. Determination of ploidy

Ploidy level determination is an essential component in the characterization of successful transgenics. Determination of ploidy level of the first and second transgenic generation (T0, T1) is considered to be the first step in selecting the transgenic lines (Gase et al., 2011). The transgenics should have the same ploidy as the plant that has

been transformed since the variations in ploidy levels can alter the expression of native genes and transgenes (Johnson, 2001; Schwachtje and Baldwin, 2008). Nuclear DNA-flow cytometry is the most efficient method of rapid detection of ploidy levels in plants (Freese and Schubert, 2004).

3.2.2. Analysis of insertion site

The stability of transgenics depends upon the genomic location of transgene insertion. The transgene cannot be targeted to specific/non-functional regions of the genome and since it integrates randomly into the genome (Gelvin and Kim, 2007; Kim et al., 2007) by non-homologous end-joining, the endogenous sequences flanking the integration site are not often known. Insights into the process of T-DNA integration and sequence information regarding target sites are important to avoid transgene loss. Information of flanking sequences of transgene supports the risk assessment substantially (Cullen et al., 2011).

Physical mapping combined with genetic mapping gives a clear picture of the location and flanking sequences of the transgene. Cytogenetic techniques like fluorescent in situ hybridization (FISH) and fiber FISH have been used to localize the exact transgene insertion site on metaphase and interphase chromosomes, respectively (Svitashev and Somers, 2001; Svitashev and Somers, 2002).

Chromosome localization of inserted T-DNA that includes the transgene insertion site, the fragment including transgene terminus and the flanking chromosomal region can be determined by inverse PCR (Liang et al., 2008), random primed PCR or TAIL-PCR (thermal asymmetric interlaced PCR) (Swensen, 1996; Trueba and Johnson, 1996) and adaptor ligation-mediated PCR (Zhou et al., 1997). By determining the genomic regions flanking the transgene insertion, it is possible to determine the position of the transgene in the genome and to determine whether any functional or regulatory genes have been disrupted and whether any new open reading frames (ORFs) have been created, resulting in the possible synthesis of novel chimeric proteins (Cullen et al., 2011). Sequence information of the flanking regions can be used further to identify the homozygous transgenic plants.

The insertion of a transgene into the plant genome inevitably disrupts the sequence of the endogenous plant DNA and may be accompanied by modification or silencing of genes, and other mutations, and pleotropic effects. Therefore, determination of insertion site of transgene by analysis of short stretches of DNA flanking the transgene insert may under-estimate the number of insertion events, which disrupt the functional plant DNA. Accurate characterization of insertion-site mutations requires sequencing the complete transgene insertion event (including the full DNA sequence of the transgene and any superfluous DNA and/or rearranged flanking plant DNA) and comparison with pre-insertion target sequences from the non-transgenic parent plant. Precise insertion events without genomic disruptions can create loss-of-function mutations due to transgene insertion into gene coding or regulatory sequences, such as promoters or enhancers, which result in potentially harmful phenotypes (Wilson et al., 2006). Transgenic plants, which have multiple transgene insertion sites exhibit transformation-induced mutations leading to unintended consequences. Transformed plants may also have heritable unintended genome-wide mutations throughout the plant genome that are not linked to the transgene (Sala et al., 2000), which can be visualized by restriction fragment length polymorphism (RFLP) and PCR-based techniques as DNA polymorphisms (band differences) between transgenic and non-transgenic plants. Chandel et al. (2010) reported the use of AFLP analysis for quantitative comparison of the extent of genomic changes occurring during the development of transgenic rice plants, so that genetic distortion in the genomes of transgenic populations can be rigorously examined and transgenic research can be given a stronger basis of field evaluation and commercialization.

3.2.3. Structure and inheritance of transgene loci

The transgene locus structure has a major influence on the level and stability of transgene expression. The transgenic plant should ideally carry a complete functional T-DNA at a single locus. Transgene locus formation involves the concerted action of several DNA strand followed by truncations, deletions, inversions and rearrangements in T-DNA may result into non-functional transgenes (Gambino et al., 2009; Gase et al., 2011; Lange et al., 2006; Latham et al., 2006; Muller et al., 2007; Svitashev et al., 2002). Complete sequencing of the transgene and the flanking regions is essential to characterize the transgene loci. Transgene fragment is analyzed for the presence of internal structure like direct repeats, inverted repeats, tandem repeats, AT rich sequences, satellite sequences, palindromes, stem loop structures and the sequence difference between the locus and the flanking regions of wild type and transgenic.

Characterization of the transgene locus/loci at the molecular level and the segregation analysis of transgene-encoded phenotype (expression of the transgene) in the subsequent progenies provide insights into the nature of transgene inheritance. Non-independent transgene loci and complex insertion events at one locus are identified by southern analysis (Forsbach et al., 2003). Transgenic plants with more than one independent transgene loci can be identified by inheritance studies (Vain and Thole, 2009). Inheritance and molecular structure of transgene loci are studied by comparing the hybridization pattern of primary transformants and subsequent progenies of transgenic, and then backcrossing each generation with the wild type to see stability of transgene through sexual transmission and to analyze whether the inheritance follows Mendelian or non- Mendelian principles. Yin et al. (2004) reviewed the patterns of transgene inheritance in plants and explained that the nature of recipient genome, nature of transgene and the interactions between them contribute to non-Mendelian segregation of the transgene. Transgene inheritance has been studied in various plant species like alfalfa (Samis et al., 2002), barley (Ritala et al., 1995), maize (Zhang et al., 1996), rice (Gahakwa et al., 2000; Park et al., 1996), soybean (Choffnes et al., 2001) and tobacco (Matzke et al., 1994).

3.2.4. Estimation of copy number

Transgene copy number greatly influences the genetic stability of the target gene. The ideal transgenic plant for most research and breeding purposes should contain a single intact copy of the desired transgene inserted into a single non-functional region of the plant genome, without further alteration of the host plant DNA. Transgenics may carry several insertions of the transgene at single or multiple loci within the genome of the host plant, which may cause co-suppression leading to transgene silencing that may manifest early or only over a period of time (Bhalla and Singh, 2008; Yang et al., 2012). *Agrobacterium*-mediated gene transfer is known to be the preferable transformation method mainly because of the increased chance for single copy transfer of target genes to transgenic plants (Bhalla and Singh, 2008; De Buck et al., 2004, 2009; Meza et al., 2002; O'Malley and Ecker, 2010; Sallaud et al., 2003). Southern blotting, comparative genomic hybridization (Kallioniemi et al., 1994), multiplex probe amplification and hybridization, and chip hybridization (Lucito et al., 2000) are commonly used methods for copy number analysis. But these methods are laborious and time consuming and may lead to inaccurate estimation of the foreign gene copy number when tandem integration or rearrangement of the foreign gene occurs associated with these methods (Mason et al., 2002).

In recent years, a high-throughput quantitative real-time PCR (qRT-PCR) method with high specificity and high signal-to-noise ratio has been used to determine gene copy number in transgenic corn, rapeseed, rice, and cotton plants (Bubner and Baldwin, 2004; Song et al., 2002; Weng et al., 2004; Yang et al., 2005, 2012). Various methods like TaqMan qRT-PCR detection system, modified 2-△△CT method and competitive PCR have been developed for rapid, precise

and high-throughput estimation of copy number and zygosity (Callaway et al., 2002; Tesson et al., 2010; Williams et al., 2008; Yang et al., 2012; Yi et al., 2008).

3.2.5. Analysis of vector backbone integration

Vector backbone contamination is another important aspect, which has substantial significance for both applied and basic research. For many years T-DNA transfer was assumed to involve only DNA sequences between the left and the right border and with a highly efficient T-DNA transfer initiation at the right border and termination at the left border. However, studies have identified vector backbone contamination in the transgenics of *Arabidopsis* and tobacco (Meza et al., 2002), rice (Afolabi et al., 2004), maize (Shou et al., 2004), barley (Lange et al., 2006), and cotton (Zhang et al., 2008) illustrating that vector backbone integration appears to be a regular feature of *Agrobacterium* transformation. The primary determinant for integration of vector backbone appears to be inefficient recognitions of the left and right border as initiation and termination sites for DNA transfer resulting in read-through at both borders (De Buck et al., 2000).

The transfer of vector backbone sequences into plants also has a number of practical implications. The vector backbone may contain bacterial genes and bacterial origin of replications (Tzfira et al., 2004) which might have unpredictable effects such as causing mutations or the silencing of endogenous or non-endogenous gene(s). Vector backbone has been inferred to alter the gene expression and even induce silencing. Regulatory agencies that govern the release of transgenic organisms demand that the transgenic plants should be free of superfluous, foreign DNA and the accompanying antibiotic resistance genes. Therefore, screening for the possible transfer of vector backbone sequences must be carried out as a requirement for gaining regulatory approval for the release of genetically modified crops.

The backbone contamination has been analyzed by southern hybridization and dot-blot with vector specific probes (Lange et al., 2006) and also by AFLP based cluster analysis (Chandel et al., 2010).

3.2.6. Spatial and temporal analysis

Successfully integrated transgene should remain stable in space and time which means that the relative transgene content within a specific uniformly transformed plant should remain constant throughout all tissues of the plant, and at different times. Spatial and temporal regulation of induction of transgene expression is an important tool for genetic analysis of the transgenic plants. The tissues transformed with foreign genes could harbor both transformed and non-transformed cells. As a result, the regenerated plant will be a chimera for the transgenes. Chimeras of transgenic crops should be identified and discarded to leave fully transgenic plants. The qRT-PCR analysis helps to investigate the effect of transgene on different tissues and developmental stages of the transgenic plant and allows the identification of chimeras (Faize et al., 2010; Maizel and Weigel, 2004).

3.2.7. Expression analysis of the transgene

The production of transgenic plants with stable and high level expression of transgene is important for the success of transgenics. The expression of a stably integrated transgene in plants is highly variable and heterogenous because of various genetic and epigenetic changes. Recent years have seen huge improvement in the techniques for analysis of transgene expression in plants. Omics platforms can be used to make a snapshot of the transcript, protein and metabolite composition of a transgenic plant. High-throughput transcriptome, proteome and metabolome analysis of transgenic crops is essential to gain insights into the functioning of transgene in transgenic crops (Kogel et al., 2010; Ricroch et al., 2011). Transcript profiling, protein profiling, metabolome profiling and metabolic fingerprinting have been used in parallel to assess the effects of transgene expression in transgenic plants (Ricroch et al., 2011).

The function of transgene is initially confirmed at the RNA level. Common methods of transcript analysis are northern and dot-blot hybridizations. The transcript abundance in transgenic is quantified by qRT-PCR (Page and Minocha, 2005). However, the differences revealed by transcriptome analysis may not be equally reflected in differences in the proteome and/or metabolome and as such in plants' physiology. Therefore, proteome and metabolome screening are considered more reliable for expression studies. Protein-based methods like ELISA, western blot and protein arrays, and proteomics profiling with two-dimensional electrophoresis combined with mass spectrometry (2DE/MS) have been widely used approaches to study and compare differentially expressed proteins in transgenic and non-transgenic counterparts, and to detect novel proteins generated during insertion of foreign genes (Gong et al., 2012; Zolla et al., 2008). Currently, a new proteomics technique, iTRAQ (isobaric tags for relative and absolute quantification) is being applied to plants to understand changes in protein profiles during growth or due to environmental stimuli and to discover the molecular network responsible for altered phenotypes (Fukao et al., 2011; Lan et al., 2011; Lücker et al., 2009; Pang et al., 2011). This approach can also be applied to analyze the protein profiles of transgenic plants.

Metabolomics is a highly reliable, sensitive and the most prevalent approach for characterizing transgenic plants (Ricroch et al., 2011). Metabolome profiling can help to analyze the overall metabolite composition (metabolite fingerprint) to make broader and deeper assessments of transgenic crops (Hoekenga, 2008; Rischer and Oksman-Caldentey, 2006). Metabolite profiling can also distinguish between changes induced by natural variability and by genetic modification in crops. Metabolome analysis of transgene with FT-MS (Fourier-transform ion cyclotron mass spectrometry), NMR (nuclear magnetic resonance), GC/MS (gas chromatography mass spectrometry) CE/MS (capillary electrophoresis mass spectrometry) analysis has been used to detect intracellular metabolites and to analyze differences in plant metabolism of transgenic and wild plants (Takahashi et al., 2005, 2006). Multiplatform metabolomics which combines GC/MS, LC/MS and CE/MS is a sensitive and robust approach to profile metabolome of transgenic plants (Kusano et al., 2011). However, these profiling techniques are highly heterogeneous. Variable patterns in transcriptome, proteome, or metabolome are reported depending on growth conditions, geography, season, or variety. Therefore, it seems premature to infer precise conclusions from variations assigned to transgenic crops based on any specific technology (Ricroch et al., 2011).

4. Conclusions

The article provides an overview on the food and environmental safety assessment measures of transgenic crops and highlights the phenotypic and molecular profiling approaches for comprehensive characterization of transgenic plants. Integration of molecular 'omics' and phenomics would be useful to analyze the performance of transgenic plants to ensure their safe release and commercialization to meet the demands of the ever-growing population and lead to global food security for the future.

Acknowledgment

We are thankful to Dr. Srinivasan (National Research Centre for Plant Biotechnology, New Delhi, India) for his suggestions, critical readings and improving the quality of manuscript.

References

Abdeen, A., Schnell, J., Miki, B., 2010. Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants overexpressing the transcription factor ABF3. BMC Genomics 11, 69.

Afolabi, A.S., Worland, B., Snape, J.W., Vain, P., 2004. A large-scale study of rice plants transformed with different T-DNAs provides new insight into locus composition and T-DNA linkage configurations. Theor. Appl. Genet. 109, 815–826.

Ahmad, P., et al., 2012. Role of transgenic plants in agriculture and biopharming. Biotechnol. Adv. 30 (3), 524–540.

Asif, M.A., et al., 2011. Enhanced expression of AtNHX1, in transgenic groundnut (*Arachis hypogaea* L.) improves salt and drought tolerance. Mol. Biotechnol. 49 (3), 250–256.

Bai, S., et al., 2010. Detection of six genetically modified maize lines using optical thin-film biosensor chips. J. Agric. Food Chem. 58 (15), 8490–8494.
Baker, J.M., et al., 2006. A metabolomic study of substantial equivalence of field-grown genetically modified wheat. Plant Biotechnol. J. 4, 381–392.
Baxter, I., 2010. Ionomics: the functional genomics of elements. Brief. Funct. Genomics 9 (2), 149–156.
Benkeblia, N., 2012. Omics Technologies: Tools for Food Science. CRC Press, USA.
Bhalla, P.L., Singh, M.B., 2008. *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. Nat. Protoc. 3, 181–189.
Bhat, S.R., Srinivasan, S., 2002. Molecular and genetic analyses of transgenic plants: considerations and approaches. Plant Sci. 163, 673–681.
Bubner, B., Baldwin, I.T., 2004. Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep. 23 (5), 263–271.
Callaway, A.S., Abranches, R., Scroggs, J., Allen, G.C., Thompson, W.F., 2002. High-throughput transgene copy number estimation by competitive PCR. Plant Mol. Biol. Rep. 20, 265–277.
Carli, M., et al., 2009. Leaf proteome analysis of transgenic plants expressing antiviral antibodies. J. Proteome Res. 8 (2), 838–848.
Cellini, F., et al., 2004. Unintended effects and their detection in genetically modified crops. Food Chem. Toxicol. 42, 1089–1125.
Chakraborty, S., et al., 2010. Next-generation protein-rich potato expressing the seed protein gene AmA1 is a result of proteome rebalancing in transgenic tuber. PNAS 107 (41), 17533–17538.
Chandel, G., Datta, K., Datta, S.K., 2010. Detection of genomic changes in transgenic Bt rice populations through genetic fingerprinting using amplified fragment length polymorphism (AFLP). GM Crops 1 (5), 327–336.
Choffnes, D.S., Philip, R., Vodkin, L.O., 2001. A transgenic locus in soybean exhibits a high level of recombination. In Vitro Cell. Dev. Biol. Plant 37 (6), 756–762.
Conner, A.J., Glare, T.R., Nap, J.P., 2003. The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. Plant J. 33 (1), 19–46.
Costa, T.E., Dias, A.P., Scheidegger, E.M., Marin, V.A., 2011. Risk assessment of genetically modified organisms. Cien. Saude Colet. 16 (1), 327–336.
Cullen, D., Harwood, W., Smedley, M., Davies, H., Taylor, M., 2011. Comparison of DNA walking methods for isolation of transgene-flanking regions in GM potato. Mol. Biotechnol. 49, 19–31.
De Buck, S., Wilde, D.E., Montagu, M.V., Depicker, A., 2000. T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Mol. Breed. 6, 459–468.
De Buck, S., Windels, P., De Loose, M., Depicker, A., 2004. Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable beta-glucuronidase accumulation levels. Cell. Mol. Life Sci. 61, 2632–2645.
De Buck, S., Podevin, N., Nolf, J., Jacobs, A., Depicker, A., 2009. The T-DNA integration pattern in *Arabidopsis* transformants is highly determined by the transformed target cell. Plant J. 60, 134–145.
EFSA, 2004. Draft guidance document for the risk assessment of genetically modified plants and derived food and feed (April 2004). Scientific Panel on Genetically Modified Organisms. European Food Safety Authority, Brussels.
Faize, M., Faize, L., Burgos, L., 2010. Using quantitative real-time PCR to detect chimeras in transgenic tobacco and apricot and to monitor their dissociation. BMC Biotechnol. 10, 53.
Forsbach, A., Schubert, D., Lechtenberg, B., Gils, M., Schmidt, R., 2003. A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant Mol. Biol. 52 (1), 161–176.
Freese, W., Schubert, D., 2004. Safety testing and regulation of genetically engineered foods. Biotechnol. Genet. Eng. Rev. 21, 299–324.
Fukao, Y., et al., 2011. iTRAQ analysis reveals mechanism of growth defects due to excess zinc in *Arabidopsis*. Plant Physiol. 155, 1893–1907.
Gahakwa, A.D., Maqbool, S.B., FU, X., Sudhakar, D., Christou, P., Kohli, A., 2000. Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes show similar behaviour in diverse genetic backgrounds. Theor. Appl. Genet. 101, 388–399.
Gambino, G., et al., 2009. Characterization of T-DNA insertions in transgenic grapevines obtained by *Agrobacterium*-mediated transformation. Mol. Breed. 24 (3), 305–320.
Gase, K., Weinhold, A., Bozorov, T., Schuck, S., Baldwin, I.T., 2011. Efficient screening of transgenic plant lines for ecological research. Mol. Ecol. Resour. 11 (5), 890–902.
Gelvin, S.B., Kim, S.-I., 2007. Effect of chromatin upon *Agrobacterium* T-DNA integration and transgene expression. Biochim. Biophys. Acta 1769, 410–421.
Gong, C.Y., Li, Q., Yu, H.T., Wang, Z., Wang, T., 2012. Proteomics insight into the biological safety of transgenic modification of rice as compared with conventional genetic breeding and spontaneous genotypic variation. Proteome Res. 11 (5), 3019–3029.
Gürel, F., Arican, E., Gözükirmizi, N., Ari, Ş., 2011. Recent molecular tools for detecting transgenic events in genetically modified (GM) crop products. Sci. Res. Essays 6 (24), 5091–5099.
Herrero, M., Simó, C., García-Cañas, V., Ibáñez, E., Cifuentes, A., 2012. Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrom. Rev. 31 (1), 49–69.
Hilbeck, A., Meier, M., Römbke, J., Jänsch, S., Teichmann, H., Tappeser, B., 2011. Environmental risk assessment of genetically modified plants — concepts and controversies. Environ. Sci. Eur. 23, 1–13.
Hoekenga, A.O., 2008. Using metabolomics to estimate unintended effects in transgenic crop plants: problems, promises, and opportunities. Biomol. Tech. 19 (3), 159–166 (July).
Hutchison, W.D., et al., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330 (6001), 222–225.
Johnson, A.A.T., 2001. Effect of ploidy elevation, copy number and parent-of-origin on transgene expression in potato. Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Horticulture.
Kallioniemi, O.P., et al., 1994. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. Genes Chromosomes Cancer 10 (4), 231–243.
Kim, S.I., Veena, Gelvin, S.B., 2007. Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. Plant J. 51, 779–791.
Kogel, K.H., et al., 2010. Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variances. Proc. Natl. Acad. Sci. U. S. A. 107, 6198–6203.
König, A., et al., 2004. Assessment of the safety of food derived from genetically modified GM crops. Food Chem. Toxicol. 42, 1047–1088.
Kruger, R.G., et al., 2009. U.S. grower views on problematic weeds and changes in weed pressure in glyphosate-resistant corn, cotton, and soybean cropping. Systems. (http://news.uns.purdue.edu/x/2009a/090414JohnsonSurvey.html).
Kuiper, H.A., Kleter, G.A., Noteborn, H.P.J.M., Kok, E.J., 2001. Assessment of food safety issues related to genetically modified foods. Plant J. 27 (6), 503–528.
Kuntz, M., Ricroch, A.E., 2012. Is it Time to Adjust the Current Regulatory Risk Assessment for GM Food and Feed? ISB NEWS REPORT. 1–4.
Kusano, M., et al., 2011. PLoS One 6 (2), e16989. http://dx.doi.org/10.1371/journal.pone.0016989.
Lan, P., et al., 2011. iTRAQ protein profile analysis of *Arabidopsis* roots reveals new aspects critical for iron homeostasis. Plant Physiol. 155, 821–834.
Lange, M., Vincze, E., Møller, M.G., Holm, P.B., 2006. Molecular analysis of transgene and vector backbone integration into the barley genome following *Agrobacterium*-mediated transformation. Plant Cell Rep. 25, 815–820.
Latham, J.R., Wilson, A.K., Steinbrecher, R.A., 2006. The mutational consequences of plant transformation. J. Biomed. Biotechnol. 2, 1–7 (Article id: 25376).
Liang, Z., Breman, A.M., Grimes, B.R., Rosen, E.D., 2008. Identifying and genotyping transgene integration loci. Transgenic Res. 17, 979–983.
Llorente, B., et al., 2010. Improvement of aroma in transgenic potato as a consequence of impairing tuber browning. PLoS One 5 (11), e14030. http://dx.doi.org/10.1371/journal.pone.0014030.
Lu, B.-R., 2008. Transgene escape from GM crops and potential biosafety consequences: an environmental perspective. Report to Ministry of Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, Fudan University, Shanghai, China.
Lucito, R., et al., 2000. Detecting gene copy number fluctuation in tumor cells by microarray analysis of genomic representations. Genome Res. 10 (11), 1726–1736.
Lücker, J., Laszczak, M., Smith, D., Lund, S.T., 2009. Generation of a predicted protein database from EST data and application to iTRAQ analyses in grape (*Vitis vinifera* cv. Cabernet Sauvignon) berries at ripening initiation. BMC Genomics 10, 50.
Maizel, A., Weigel, D., 2004. Temporally and spacially controlled induction of gene expression in *Arabidopsis thaliana*. Plant J. 38 (1), 164–171.
Mason, G., Provero, P., Vaira, A.M., Accotto, G.P., 2002. Estimating the number of integrations in transformed plants by quantitative real-time PCR. BMC Biotechnol. 2, 20.
Matzke, M.A., et al., 1994. Inheritance and expression of a transgene insert in an aneuploid tobacco line. Mol. Gen. Genet. 245, 471–485.
Mendoza, E.M.T., Laurena1, A.C., Ramo´ n Botella, J., 2008. Recent advances in the development of transgenic papaya technology. Biotechnol. Annu. Rev. 14, 423–462.
Meza, T.J., et al., 2002. Analyses of single-copy *Arabidopsis* T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. Nucleic Acids Res. 30, 4556–4566.
Motoyama, T., et al., 2010. Development of transgenic rice containing a mutated beta subunit of soybean beta-conglycinin for enhanced phagocytosis-stimulating activity. Peptides 31 (7), 1245–1250.
Muller, A.E., Atkinson, R.G., Sandoval, R.B., Jorgensen, R.A., 2007. Microhomologies between T-DNA ends and target sites often occur in inverted orientation and may be responsible for the high frequency of T-DNA-associated inversions. Plant Cell Rep. 26, 617–630.
Mulvaney, D.R., Krupnik, T.J., Koffler, K.B., 2011. Transgenic rice evaluated for risks to marketability. Calif. Agric. 65 (3), 161–168.
O'Malley, R.C., Ecker, J.R., 2010. Linking genotype to phenotype using the *Arabidopsis* unimutant collection. Plant J. 61 (6), 928–940 (Special Issue: SPECIAL ISSUE Arabidopsis: A rich harvest 10 years after completion of the genome sequence).
OECD, 2004. Consensus documents for the work on the safety of novel foods and feed. Task Force for the Safety of Novel Foods and Feed, Organization for Economic Cooperation and Development, Paris. http://www.oecd.org/document/9/0,2340,en_2649_34391_1812041_1_1_1_1,00.html.
Ouakfaoui, S.E., Miki, B., 2005. The stability of the *Arabidopsis* transcriptome in transgenic plants expressing the marker genes nptII and uidA. Plant J. 41 (6), 791–800.
Page, A.F., Minocha, S.C., 2005. Analysis of gene expression in transgenic plants. Methods Mol Biol. 286, 291–312.
Pang, Q., Chen, S., Dai, S., Chen, Y., Wang, Y., Yan, X., 2011. Comparative proteomics of salt tolerance in *Arabidopsis thaliana* and *Thellungiella halophila*. J. Proteome Res. 9, 2584–2599.
Park, S.H., Pinson, S.R.M., Smith, R.H., 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol. 32, 1135–1148.
Passamano, M., Pighini, M., 2006. QCM DNA-sensor for GMOs detection. Sens. Actuators, B 118, 177–181.
Pusztai, A., 2001. Genetically modified foods: are they a risk to human/animal health? An ActionBioscience.org, original article.
Raybould, A.F., Gray, A.J., 1993. Genetically modified crops and hybridisation with wild relatives: a UK perspective. J. Appl. Ecol. 30, 199–219.
Ricroch, A.E., Berge´, J.B., Kuntz, M., 2011. Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiol. 155 (4), 1752–1761.

Ridley, W.P., et al., 2004. Development of the International Life Sciences Institute Crop Composition Database. J. Food Compos. Anal. 17, 423–438.
Rischer, H., Oksman-Caldentey, K.M., 2006. Unintended effects in genetically modified crops: revealed by metabolomics? Trends Biotechnol. 24 (3), 102–104.
Ritala, A., et al., 1995. Transgenic barley by particle bombardment. Inheritance of the transferred gene and characteristics of transgenic barley plants. Euphytica 85 (1–3), 81–88.
Roessner, U., et al., 2001. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell 13, 11–29.
Ruis, A.C., 2010. Variability associated to the insertion and expression of transgenes in plants. PhD thesis, Universitat de Giorna.
Sala, F., et al., 2000. Somaclonal variation in transgenic plants. Acta Hortic. 530, 411–419.
Sallaud, C., et al., 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L) functional genomics. Theor. Appl. Genet. 106, 1396–1408.
Salt, D.E., Baxter, I., Lahner, B., 2008. Ionomics and the study of the plant ionome. Annu. Rev. Plant Biol. 59, 709–733.
Samis, K., Bowley, S., McKersie, B., 2002. Pyramiding Mn-superoxide dismutase transgenes to improve persistence and biomass production in alfalfa. J. Exp. Bot. 53 (372), 1343–1350.
Schwachtje, J., Baldwin, I.T., 2008. Why does herbivore attack reconfigure primary metabolism? Plant Physiol. 146, 845–851.
Séralini, G.-E., Mesnage, R., Clair, E., Gress, S., deendômois, J.S., Cellier, D., 2011. Genetically modified crops safety assessments: present limits and possible improvements. Environ. Sci. Eur. 10.
Shou, H., Frame, B.R., Whitman, S.A., Wang, K., 2004. Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Mol. Breed. 13, 201–208.
Song, P., Cai, C.Q., Skokut, M., 2002. Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS (TM)-derived transgenic maize. Plant Cell Rep. 20, 948–954.
Stobiecka, M., Ciesla, J.M., Janowska, B., Tudek, B., Radecka, H., 2007. Piezoelectric sensor for determination of genetically modified soybean roundup ready in samples not amplified by PCR. Sensors 7, 1462–1479.
Svitashev, S.K., Somers, D.A., 2001. Genomic interspersions determine the size and complexity of transgene loci in transgenic plants produced by micro projectile bombardment. Genome 44 (4), 691–697.
Svitashev, S.K., Somers, D.A., 2002. Characterization of transgene loci in plants using FISH: a picture is worth a thousand words. Plant Cell Tissue Org. Culture 69 (3), 205–214.
Svitashev, S.K., Pawlowskiy, W.P., Makarevitch, I., Plank, D.W., Somers, D.A., 2002. Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. Plant J. 32, 433–445.
Swensen, J., 1996. PCR with random primers to obtain sequence from yeast artificial chromosome insert ends or plasmids. Biotechniques 20, 486–491.
Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., Carriére, Y., 2008. Insect resistance to *Bt* crops: evidence versus theory. Nat. Biotechnol. 26, 199–202.
Takahashi, H., Hotta, Y., Hayashi, M., Kawai-Yamada, M., Komatsu, S., Uchimiya, H., 2005. High throughput metabolome and proteome analysis of transgenic rice plants (*Oryza sativa* L.). Plant Biotechnol. 22, 47–50.
Takahashi, H., et al., 2006. Evaluation of metabolic alteration in transgenic rice overexpressing dihydroflavonol-4-reductase. Ann. Bot. 98, 819–825.
Tesson, L., Rémy, S., Ménoret, S., Usal, C., Anegon, I., 2010. Analysis by quantitative PCR of zygosity in genetically modified organisms. Methods Mol. Biol. 597, 277–285.
Trueba, G.A., Johnson, R.C., 1996. Random primed gene walking PCR: a simple procedure to retrieve nucleotide fragments adjacent to known DNA sequences. Biotechniques 21 (1), 20.
Tsaftaris, A.S., Polidoris, A.N., Karavangeli, M., Nianiou-Obeidat, I., Madesis, P., 2000. Goudoula C transgenic crops: recent developments and prospects. In: Balazs, E., Galante, E., Lynch, J.M., Schepers, J.S., Toutant, J.P., Werner, D., Werry, P.A.T.J. (Eds.), Biological Resource Management. Springer, Berlin Heidelberg New York, pp. 187–203.
Tzfira, T., Jianxiong, L., Lacroix, B., Citovsky, V., 2004. *Agrobacterium* T-DNA integration: molecules and models. Trends Genet. 20 (8), 375–378.
Vain, P., Thole, V., 2009. Insertion patterns and sites. Methods in Molecular Biology, Transgenic Wheat, Barley and Oats, p. 478.
Weng, H.B., Pan, A.H., Yang, L.T., 2004. Estimating number of transgene copies in transgenic rapeseed by real-time PCR assay with HMG I/Y as an endogenous reference gene. Plant Mol. Biol. Rep. 22, 289–300.
Williams, N.M., et al., 2008. Analysis of copy number variation using quantitative inter species competitive PCR. Nucleic Acids Res. 36 (17).
Wilson, A.K., Latham, J.R., Steinbrecher, R.A., 2006. Transformation-induced mutations in transgenic plants: analysis and biosafety implications. Biotechnol. Genet. Eng. Rev. 23, 209–237.
Yang, L., et al., 2005. Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. Plant Cell Rep. 23, 759–763.
Yang, X., et al., 2012. Analysis of the copy number of exogenous genes in transgenic cotton using real-time quantitative PCR and the 2-△△CT method. Afr. J. Biotechnol. 11 (23), 6226–6233.
Yi, C.X., Zhang, J., Chan, K.M., Liu, X.K., Hong, Y., 2008. Quantitative real-time PCR assay to detect transgene copy number in cotton (*Gossypium hirsutum*). Anal. Biochem. 375, 150–152.
Yin, Z., Plyder, W., Malepszy, S., 2004. Transgene inheritance in plants. J. Appl. Genet. 45 (2), 127–144.
Zhang, S., Warkeentin, D., Sun, B., Zhong, H., Sticklen, M., 1996. Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected (*bar*) transgenes in maize (*Zea mays* L.). Theor. Appl. Genet. 92, 752–761.
Zhang, J., et al., 2008. Transgene integration and organization in cotton (*Gossypium hirsutum L.*) genome. Transgenic Res. 17, 293–306.
Zhang, Y., et al., 2009. Electrochemical DNA biosensor based on the proximity-dependent surface hybridization assay. Anal. Chem. 81, 1982–1987.
Zhou, Y., Newton, R.J., Gould, J.H., 1997. A simple method for identifying plant/T-DNA junction sequences resulting from *Agrobacterium*-mediated DNA transformation. Plant Mol. Biol. Rep. 15, 246–254.
Zolla, L., Rinalducci, S., Antonioli, P., Righetti, P.G., 2008. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. J. Proteome Res. 7 (5), 1850–1861.