Molecular Approaches to Address Intended and Unintended Effects and Substantial Equivalence of Genetically Modified Crops

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Abstract

The release of GM organisms into the environment and marketing of GM crops have resulted in public debate in many parts of the world. This debate is likely to continue, probably in the broader context of plant biotechnology and consequences for human societies. The general issues under debate include cost–benefit analysis and safety issues, but might exhibit regional differences and crop-specific nuances. This chapter addresses an in-depth understanding of events involved in transgene insertion, but also the unintended effects of transformation following the production of genetically enhanced plants. In order to dissect this topic, a foundational overview is given on biolistic- and Agrobacterium-based techniques. Background information of possible transformation-induced unintended alterations to transgenic plant genomes is reviewed and aspects that collectively constitute possible unintended transformation - and post-transformation events are described. This is followed by an overview of molecular techniques to study gene insertion and – expression with special focus on differential gene expression analysis techniques to investigate unintended effects of genetic transformation. Historical and current safety assessment guidelines and requirements are also briefly discussed.

Keywords: intended effects, molecular analysis, transgenic plants, substantial equivalence, unintended effects

1. Introduction

The modern biotechnology era as applied to crop production was initiated by molecular marker-assisted selection to select agriculturally important traits [1]. Other developments included recombinant DNA technology which helped breeders by providing a diverse gene pool for trait selection, targeted deletion or insertions of genes into genomes, and site-directed mutagenesis to modify gene functions [2]. GM crops have been developed over the years for improvement of desired traits for enhanced agricultural production, as well as to facilitate reduced use of agricultural pesticides [3]. The technology employed to produce GM crops has been described as advantageous when compared to conventional plant breeding, since the desired traits can be obtained in a relatively shorter period of time. In addition, the technology may enable the introduction of desired characteristics that cannot be accomplished solely through conventional plant breeding.

In view of the global population increases, factors that have been considered important to cope with the increasing food demand include the development of crop varieties with improved nutrition and high yield in different climatic conditions, development of varieties that require the use of less water and fertilizers, and the production of varieties with enhanced resistance against abiotic and biotic stresses [4]. Moreover, new varieties should exhibit high storage quality and appropriate features for processing and market consumption. Specific traits that have been used to improve crops include herbicide - and insect resistance, salt and drought tolerance, increased yield, high protein content and vitamin A enrichment.

Pest-resistant and herbicide tolerant varieties were the first products of GM technologies and they were commercialized in the mid-1990s. In general, farmers have widely accepted GM technologies and the use of GM crops has expanded rapidly in developing countries [5]. The expected expiration of patents on earlier varieties of GM crops will serve as an opportunity for other companies to produce alternative varieties that may compete within the GMO market, thus challenging existing GM varieties. In addition, it will elicit innovative competition in terms of traits to be investigated which were previously not considered. It is therefore important to ensure that existing and future GM crops and - products created through recombinant DNA technology are assessed with regards to any potential risk they may have on human, animal and environmental health.

2. Benefits and limitations of genetically modified crops

One of the highlighted advantages of GM crops, among others, is their ability of these to enhance food security, particularly to small-scale and resource-poor farmers in developing countries [6]. Some of the noted benefits include increased crop yield in a relatively shorter period of time, reduction in the utilization and cost of plant protection chemicals, crops with enhanced tolerance to environmental stresses, reduction in labor input, and production of foods that are affordable with enhanced nutritional contents [6]. These benefits have been said to, overall, improve agricultural production and plant breeding in developing countries.

However, the documented benefits have been countered by shortcomings and concerns. Some of the issues brought forward include potential toxicity, the assumption that the products may contain allergens and the possible development of antibiotic resistance from the utilization of GM products [7]. Genetically, concerns about GM crops include the possible unintended transfer of genes from GM to non-GM crops or to their wild type relatives, the evolution of GM crops into becoming weeds, the direct and indirect impact of GM crops on non-target species, environmental risks associated with GM crops, and the impact of GM crops on genetic diversity [8]. Directed at the small-scale and resource-poor farmers, the highlighted concerns also include hindrances that may result in limited access to seeds created by patents on the GM crops [9]. Furthermore, contrary to the highlighted benefits of GM crops, crops with enhanced resistance have been viewed as having a one-sided benefit to commercial farmers that grow the crops and companies that own the production technologies [8, 10].

3. Transformation techniques used for the production GM crops

Transgenic technology deals with the integration of exogenous DNA into the plant genome using gene transfer technologies [11]. While newer methods such as nanoparticle-mediated delivery are in development, two methods are predominantly used for exogenous DNA transfer into plants; Agrobacterium-mediated transformation and particle bombardment. The first is an indirect or vector-based transformation method, and utilizes the ability of *Agrobacterium tumefaciens* bacteria to copy and transfer a specific portion of DNA (T-DNA) present on a tumor-inducing (Ti) plasmid into the nucleus of the plant cell. This allows for the integration of the DNA into chromosomes and subsequently leading to the integration of the T-DNA into the plant genome. This type of transformation involves three stages [12]. The initiation stage entails the insertion of the gene of interest into a suitable functional construct. The construct includes the gene expression promoter, gene of interest, selectable marker as well as codon modification. The initiation stage then continues to the insertion of the transgene into the Ti-plasmid. The final step of the initiation stage involves the insertion of the T-DNA, which contains the transgene, into Agrobacterium. The next stage is the bacterium-to-plant transfer during which the transformed bacteria are mixed with plant cells to facilitate the transfer of T-DNA into the plant genome. The final stage is nucleus targeting where the transgene is randomly integrated into the plant chromosome. Following nucleus targeting, non-homologous end-joining processes [13] enables the integration of T-DNA into the plant genome in the absence of any homology between the T-DNA and plant DNA sequences [14]. The possible need for tissue culture steps on selective artificial media associated with Agrobacterium transformation may lead to somoclonal variations, which in itself may lead to genetic changes in the host genome.

In contrast, biolistic transformation is commonly used to transform plants that are not susceptible to Agrobacterium transformation [15]. The integration of transgenes into a host plant genome, following particle bombardment, generally occurs non-randomly at AT-rich regions carrying nuclear matrix attachment region (MAR) motifs [16]. These elements have been postulated to be target sites for transgene integration into the host plant genome [16, 17]. Their function has been explained as creating open chromatin to make the host plant genome accessible to transgenes.

Both Agrobacterium and biolistic methods may be used for chloroplast/plastid transformation [18], but is applicable to only a relatively small number of crops. Chloroplast transformation is attractive because of its maternal inheritance, ensuring is a strong level of biological containment [18].

Newer techniques for genome editing include zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and very importantly, the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein) system. The latter shows much promise for genetic modification and its versatility to modify the genome contributed to the current genome editing revolution [19].

Transformation methodology that include viral delivery systems is consistently being improved and recent advances in nanotechnology may overcome some of the limitations of the conventional methods in regards to species-independent passive delivery of transgenes [20].

4. Integration of transgenes into plant genomes: Aspects of possible unintended effects in transgenics

Single copy or repeated or multiple insertions of exogenous DNA may occur during genetic engineering transformation [21]. In addition, multiple insertions can take place into linked or unlinked sites [22, 23]. Moreover, following transformation, the transgene may be unstable within the host genome, and the insertion site may also be unstable owing to the transgene instability [24, 25].

Directed and inverted repeats are some of the complex integration patterns which have been found to result from Agrobacterium-mediated transformation [26]. Inversion [23] and translocations [27] have been found to be some of the types of chromosomal rearrangements linked to T-DNA insertion occurring at the insertion site in the plant genome. Vector-based filler DNA (non-T-DNA sequence from the transformation vector backbone) has also been observed following the integration of exogenous DNA into the plant genome. Plant-based filler DNA has been found between T-DNA repeats [25, 28], whereas vector-based filler DNA sequences were found outside the left and right borders of the T-DNA [29]. The plant-based filler DNA is regarded as an important facilitator of the integration of T-DNA into plant chromosomes [25].

Agrobacterium-based integration occasionally causes the recurrent integration of T-DNA vector backbone sequences into the transgenic plant genome [30]. It is possible to have vector backbone flanking the right border (RB) integrated into the host plant genome following transgene insertion [31]. This event has been hypothesized to be the result of T-DNA processing that occurred where, instead of the insertion initiated at from the RB, this initiation site is skipped and T-DNA insertion occurs from the LB.

Transformation methods directed at the chloroplast has the advantage of minimizing the insertion of unnecessary DNA that may accompany nuclear genome transformation. Furthermore, gene insertion into the chloroplast genome is not associated with inadvertent inactivation of a host gene due to transgene integration and, due to a less compact chromatin structure, does not exhibit positional effects [18].

5. Distribution of transgene integration sites

Predictions into the fate and integration site of a transgene into the plant genome are not possible, based on the genome's nucleotide sequence of the host genome [32]. Several authors have used various genetic mapping techniques to demonstrate that, in several plants species, transgenes integrate throughout the entire plant genome without any preference for a specific chromosome [33]. However, T-DNA containing transgenes have been found to show preference toward gene-rich regions [22, 34]. This preference has been found to be responsible for disruptions to endogenous gene functions.

Several cytological methods have been employed to detect transgene chromosomal location and structure, and these include genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) [35, 36]. These methods have assisted some researchers in identifying the transgene integration site/s at the sub-telomeric and telomeric regions of individual chromosomes [37].

In addition to the cytological methods, identification of the transgene insertion site has been done through direct sequencing of flanking DNA followed by the rescue of clones carrying transgene/genomic DNA junctions [24, 33, 38]. A high correlation was found between complex integration patterns and transgenic loci with unstable gene expression [23, 24, 39]. As a result, it was concluded that the determining factors of the stability of an expressed gene are the site as well as the structure of the integration site. In addition, it was found that the locus of transgene integration and the regions surrounding the insertion site are crucial for the stable expression of a transgene [15, 35].

Studies of transgenic tobacco indicated that chromosome telomeres are preferred by stable inserts where no binary vector sequence is present [35]. On the other hand, the integration of transgenes was found to have preference for the distal part of chromosome arms which are gene-rich regions [34, 40]. This preferred integration was found to be true in monocot species [37] and petunia [41].

During the integration of a transgene into the plant genome, a disruption may occur within the DNA and it is important to establish whether the disruption is contrary to an event that may occur during natural recombination mechanisms. Furthermore, the transgene site of integration must be clearly analyzed to investigate whether this site is not an active gene-rich region, thus causing changes to biochemical pathways within the plant. Sequence data of the regions flanking the transgene following the T-DNA insertion into the tobacco genome revealed the frequent presence of motifs, and include microsatellite sequences, AT-rich sequences characteristic of matrix-attached regions, retro-elements and tandem repeats [39]. MARs are important for the expression of integrated reporter genes, the protection of transgenes from position effects, serve as the replication origin, as well as targeting transgene integration into the host genome [13]. Several authors evaluated the junction regions in transgene loci and found genomic sequences that contained AT-rich MARs elements [13].

In contrast to the random insertion of the Agrobacterium – and biolistic methods targeted at the nuclear genome, chloroplast transformation involves homologous recombination with sequences flanking the insertion site and transgene integration is therefore more specific and predictable [18].

6. General views on the safety of GM crops

Some consumer concerns brought forward were regarding the safety of GM crops, food and feed [42]. As a result, the production of GM crops led to increased investigations within government regulatory boards in terms of research required to determine the safety of these products [43]. Activists, particularly in Europe, have intensified their opposition to the introduction and production of GM crops, food and feed [44]. To date, no international consensus has been reached for evaluation of the safety of GM plants for consumption. Over the last decade, the safety of GM crops has been routinely tested in some countries and protein- and DNA-based methods have been developed for testing of GM crops. Worldwide, legislation now faces questions on the use and labeling requirements of GM crops and their derivatives. However, there are still concerns about the safety of GM crops [9, 45]. Also, GM crops could contain toxic substances produced as by-products of the expression of the transgene. Moreover, the GM crops might not be substantially equivalent at the genome-, proteome- and metabolome levels to traditional untransformed counterparts due to possible disruption of the expression of endogenous genes.

Extensive safety assessments are an important component of the production of GM crops [46]. There are factors that are essential to interrogate to understand the impact of the expression of the "foreign" transgene on the expression of endogenous genes and on the host plant as a whole [32, 47].

7. Safety assessment of GM crops, food and feed

The current techniques utilized for the safety assessment of derivatives of genetically modified crops, particularly food- and feed-based derivatives, evolved from collaborations between international agencies, which include the United Nations' World Health Organization (WHO) and Food and Agricultural Organization [48] and the Organization for Economic Co-ordination and Development [49]. The techniques entail a comparative assessment between the characteristics of the modified crop and an existing crop, which is usually the parent crop from which the genetic modification was developed.

Research conducted by the International Council for Science (ICSU) and the FAO, showed no evidence of adverse effects of GM crops on the environment as well as no toxicity presented by the consumption of foods derived from GM crops [50, 51]. The results further showed that gene transfer that occurred from GM crops to the wild-type relative was similar to the occurrence obtained from traditional crops. Further research conducted on the environmental impact of GM crops found no evidence of negative effects [52].

Concerns that have been raised in terms of the safety of GM crops, environmental risks, protection of biodiversity and impact on human and animal health have been investigated through the Cartagena Protocol on Biosafety (Secretariat of the Convention on Biological Diversity 2000). This protocol has been used by countries to develop national GMO regulatory frameworks. Details required for application of the release of GMOs include a description of the GM plant, the GM trait, as well as the country of origin of the GM plant. Furthermore, requirements include general information on the release of the GM plant, description of GM-derived products and uses, and description of field trials undertaken for the GM plant. In addition, details required for the release of the GM plant include description of the pollen spreading characteristics of the GM plant, handling of seeds and the vegetative reproduction methods of the plant. Moreover, information is required on transgenes and their respective products, which include information such as transgene expression levels, declaration on whether the expression is constitutive or induced and expression site on the plant. Additionally, information on the potential resistance to environmental or biological conditions, potential risks to human and animal health, potential long-term impact of the GM plant on biotic and abiotic components of the environment, and socio-economic impact of the GM plant on communities in the proposed release region. The release also requires information on how the GM plant will be monitored, how possible pathogenic and ecologically disruptive impacts will be evaluated, how unused parts of the GM plant will be disposed of and measures that will be used for risk management [42].

8. Outcomes of safety assessment, substantial equivalence, intended and unintended effects

As defined by the European Commission, three possible outcomes exist following safety assessment studies. Firstly, the modified food can be similar to the traditional food or ingredient, thus eliminating the need for further testing. Secondly, the modified food can be homologous to the traditional food, with some distinctly characterized differences, in which case safety assessments targeted at the differences must be performed. Thirdly, the modified food can stand apart from the traditional counterpart in numerous and complicated aspects, or no traditional counterpart is available. In this instance, the modified food will require a comprehensive assessment similar to that discussed by König et al. [47]. This may be due to the fact that the endogenous genes and their functions will possibly be disrupted through the random integration of the transgene in the plant DNA. These effects of transformation are termed ‘unintended’ or ‘non-target’ effects as they occur secondary to the primary aim of crop improvement [46].

Prior to studying the possible unintended effects of recombinant DNA techniques, it is important to understand the definitions of these effects. There are intended effects of genetic engineering and these are changes that occur following genetic modifications which are aimed to take place as a result of the introduction of the transgene and will consequently result in the accomplishment of the original objective of the genetic engineering process [32]. Unintended effects are those changes that occur following genetic engineering where significant differences are found in the response, phenotype and composition of the GM plant when compared with the traditional plant from which it is derived.

Unintended effects have further been divided into 'predicable' and 'unpredictable' unintended effects [32]. Predictable unintended effects are changes that exceed the primary expected effects of the introduction of the transgene, but are, however, applicable through the aid of the current knowledge of plant biology and metabolic pathways. On the other hand, unpredictable unintended effects are changes that are currently undefined and not clearly understood. Methods that can be exploited to determine the presence of unintended outcomes of transformation include, among others, determining the transgene integration site/s, the events that occur during the integration of the transgene into the host plant, as well as gene expression analysis of the transgenic genome compared to the traditional counterpart, thus showing the impact of transformation on the expression of endogenous genes.

9. The need for molecular characterization of GM crops

Guidelines have been set for the molecular characterization of GM crops prior to market and commercial release and these were placed in six categories [53]. These categories are (i) description of the genetic material used for the transformation, including the origin of the donor organism and how the gene was isolated, (ii) description of the transformation method, (iii) description of the transgene loci, (iv) transcript and protein characterization, (v) inheritance and stability of the transgene and (vi) detection and identification of the transgene.

The specified requirements under (i) include information on the plasmid used in the production of the recombinant, detailing genetic elements such as the orientation and position of the transgene expression cassette within the vector, the restriction endonuclease sites of the transformation construct and clearly marked T-DNA borders and promoters. In order to comply, the number of insertion events of the transgene must also be supplied, as well as the transgene insertion site(s). Insertion site detection is expected to be presented as the transgene sequence accompanied by approximately 500 bp of plant DNA in both flanking regions. Possible novel chimeric open reading frames (ORF) should be described and their functionality evaluated. If the flanking sequence contains part of the chimeric ORF, it is expected that more sequencing must be performed beyond the 500 bp radius until a putative ORF is obtained.

Requirements regarding the expression of the transgene entail, among others, details on the translation of the transcript to protein, tissue specificity of the transcript and protein expression, as well as levels of expression. Furthermore, information on the biochemical, molecular and physiological properties of the transgene product is required as well as the stability of the protein(s) in the cell and in the surrounding environment.

It is quite evident that extensive molecular analyses are required for safety assessments, the main objective being the need to demonstrate that GM crops are equivalent to their traditional counterparts, (*i.e.* substantial equivalence), and that there are no introductions of any additional or new risks to consumer health [32]. These assessments are put in place to quantitatively detect or identify the GM crops, food and feed that are being introduced into the market [54].

10. Molecular comparison of transgenic plants: Genome and transcriptome approaches

Several molecular marker techniques that have successfully been used for various research applications, such as cultivar identification, identification of genes for important agricultural traits and marker-assisted selection, can also be applied toward transgenic crops [55]. Molecular marker technologies may therefore serve as rapid and cost-effective methods for genome comparison and as such may be used as an initial screen of recombinant plants.

Simple Sequence Repeats (SSRs), also known as microsatellites, are tandem short oligonucleotide repeat sequences flanked by conserved DNA sequences that can be used to obtain a DNA-based fingerprint of the plant under investigation and are reliable and efficient [56, 57]. Microsatellites are regarded as advantageous as they are simple to perform, low amounts of DNA are required, highly reproducible and the ability to detect high levels of polymorphism [56]. A related marker technique that has been introduced in transgenic crop research is retrotransposon-based markers. The novelty of this technique stems from its ability to reveal extensive chromosomal distribution, as well as randomized genome distribution [58, 59]. Random Amplified Polymorphic DNA (RAPD) techniques are suitable for studies focused on the identification of specific and desired traits and the identification of clonal variants [56], while mutations, insertions and deletions to specific chromosomes or chromosomal regions can be studied through the Restriction Fragment Length Polymorphism (RFLP) technique [60]. For the determination of the insertion site of a transgene and filler DNA, gene-walking methods from known into unknown sequences can be applied [61].

An older technique for gene expression analysis was Northern (mRNA) blotting that only allowed the analysis of a single gene per study. However, developments have facilitated analysis of differential gene expression, or transcript profiling, where the expression of a multitude of genes can be simultaneously analyzed. Differential gene expression has been divided into two categories, namely closed and open architecture systems [62]. A closed system is one where the genes of interest are known and the genome from which the genes are derived has been well characterized [62]. On the other hand, open systems are those that do not require prior knowledge of the transcriptome, as well as the genome of origin.

Several methods, alone or in combination, might be appropriate for optimal gene expression profiling in transgenic plants. Some examples include (not exclusively): Serial Analysis of Gene Expression (SAGE), a gene expression method which allows for quantification and analysis of genes with unknown sequences [63]. This method employs two processes which entail the production of short sequence tags (STTs) from cDNA followed by linking and cloning of these tags for sequencing. LongSAGE enables transcriptome analysis of increased lengths which in turn improves the accuracy of annotating genes [64].

Microarrays provide a global view of gene expression and are found in two forms; DNA-fragment-based and oligonucleotide-based microarrays [65] with the source of array fragments being either anonymous genomic clones, EST clones or ORF amplified DNA fragments. The advantage of this technique is that a range of both weak and strong signals can be monitored on the same microarray, enabling the simultaneous analysis of a large number of genes. In addition, the technique allows for a pair-wise comparison of samples [66]. However, a major disadvantage of this technique is that an accurate sequence database must be available to facilitate the construction of the microarrays, as well as a large amount of mRNA as starting material to perform the gene expression analysis [65].

With the advent of next-generation sequencing, RNA sequencing (RNASeq or whole transcriptome shotgun sequencing), was developed. RNA-Seq is used to analyze changes in the different RNA species comprising the cellular transcriptome and can inform on the presence and quantity of RNAs in plant samples [67]. Specifically, RNA-Seq facilitates the ability to look at genetic alterations, mutations and changes in gene expression, or differences in gene expression in different groups or treatments such as transgenic – *vs*. conventional plants.

However, all of the above techniques require substantial amount of sequence information of the genome under investigation. Moreover, availability of funding is another factor for consideration. As a result, alternative gene expression techniques can also be investigated for suitability of intended use [61]. These include mRNA Differential Display (DD), Representational Difference Analysis (RDA), Amplified Fragment Length Polymorphism (AFLP) and quantitative reverse transcriptase real time PCR (qRT-PCR).

Differential gene expression analysis was first performed using mRNA Differential Display (DD) [68]. During DD, cDNA is synthesized from mRNA of each sample of interest, followed by amplification using a combination of anchored oligo-dT and random oligonucleotides. The obtained differentially amplified fragments each represent a transcript or an expressed sequence tag (EST). The advantage of this technique is that it requires a small amount of bioinformatics application during data analysis. Improvements of the technique generated the second generation annealing control primer (ACP)-differential display RT-PCR [69]. Under optimal conditions of use, mRNA DD is a relatively inexpensive but powerful tool, used to identify and isolate differentially expressed transcripts, as well as for comparative studies between several mRNA populations [70].

Subtractive hybridization of mRNA is another method that has been employed to differentially identify mRNAs associated with a cell- or tissue type or cellular responses [71]. A reduction in the number of genes in need of analysis in a comparative transgenic study is an important advantage [72]. Another advantage of the technique is its ability to reveal lower abundance transcripts [73], but the technique is also time consuming and labor intensive.

Representational Difference Analysis (RDA) is a subtractive DNA enrichment technique designed to identify differences between two genomes without quantifying expression levels [74]. The technique was later modified by using cDNA as template to facilitate the detection of rare transcripts. cDNA-RDA utilizes subtractive DNA enrichment in association with PCR amplification, where two cDNA populations are hybridized to analyze genes that are differentially expressed under set and differing conditions [61]. The obtained difference products are sequenced and analyzed to determine the difference in gene expression levels between the two genomes. A noted disadvantage of this technique is the high levels of labor it requires.

Amplified Fragment Length Polymorphism (AFLP) is a PCR-based technique that has been widely used for its advantages since it utilizes PCR analysis on a small amount of DNA for the identification of various polymorphisms [61]. Several applications have been reported for AFLPs and these include identification of the relatedness of cultivars [56] and the relatedness between transgenic offspring and parental plants [61]. Moreover, the use of mRNA expression analysis through cDNA-AFLPs allows for the evaluation of a large pool of genes differentially expressed between the transgenic and the traditional counterpart. Since it affords the researcher the ability to target coding regions, it facilitates gene expression analysis that leads to the identification of genes involved in different biological processes [61, 75].

Once candidate genes have been identified, qRT-PCR is generally used for quantitative gene expression analysis [76]. This sensitive, highly specific and broad range technique offers researchers the ability to investigate rare transcripts, as well as to analyze multigene families. qRT-PCR is also the technique of choice to measure and quantify expression levels of the inserted transgene(s). However, researchers can only benefit from the effectiveness of this technique if proper internal controls are included. These controls, also known as reference genes, normalize the expression analysis, since they are consistently expressed in tissues of interest under varying experimental treatments [77].

11. Conclusion

Due to the non-selective nature of traditional methods of genetic modification, the possibility exists that endogenous genes and their functions will be disrupted through the random integration of the transgene into the plant genome. This phenomenon is linked to unintended effects of genetic modification. Gene expression analysis is thus a crucial part of investigations into the effect of transgene insertion on endogenous gene expression. An understanding of the dynamics of the various available techniques is thus important in selecting the most appropriate technique(s) for the realization of the set objectives. Each method described above has its advantages and limitations. Furthermore, the choice of technique would depend on whether prior knowledge of the host genome is available or not. Using more than one technique in complement would ensure optimum results for investigating comparative / differential gene expression analysis in transgenic crops.

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Conflict of interest

The authors declare no conflict of interest.

Notes

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