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Transcriptome Analysis and Genetic Engineering

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Abstract

Genetic engineering is the most powerful technology of this century which is dramatically revolutionizing the agriculture, health, pharmaceutical, and food industries all over the world. Transcriptomics and genetic engineering go hand in hand from the development of a genetically modified organism (GMO) to its utilization by the humans. Transcriptome analysis is the analysis of messenger RNAs (mRNAs), which are produced by transcription of deoxyribonucleic acid (DNA) in an organism in response to a specific internal/external environment. Transcriptome analysis is not only useful to dig out the potential target genes for genetic modifications but also utilized to study the proper functioning of a genetically engineered gene, evaluation of the GMO for biosafety risks and for monitoring the presence and movement of GMO. Despite huge scope of genetic engineering, these manipulations can upset the natural balance of a genome by insertional, soma clonal, and pleiotropic effects of a foreign gene resulting in unintended alterations along with the targeted changes. The untargeted alterations pose risks to environment and health of animals and plants. In this chapter, the key advancements in the field of biotechnology and the relevant biosafety issues are reviewed. The advantages and limitations of the current methods used for the evaluation, monitoring, and regulation of GMOs are discussed.

Keywords: genetic engineering, gene silencing, genetically modified organisms, unintended modifications, pleiotropic effects, enzyme-linked immunosorbent assay, soma clonal effects, next-generation sequencing

1. Introduction

Genetic engineering is an advanced field of biology that deals with modification of genomic deoxyribonucleic acid (DNA) in the living organisms to introduce desired traits to benefit mankind. Through genetic engineering, a DNA fragment (gene) is isolated from the donor organism

and transferred to the recipient where it can be transcribed into messenger RNA (mRNA) and translated to proteins by utilizing the recipient machinery. The donor protein in the recipient system performs its targeted function to modify the desired character of recipient plant, animal, or microorganism. Genomic DNA manipulations may involve addition of a foreign gene from another genome, deletion of an existing gene, or enhancing the expression of an indigenous gene. RNA interfering (RNAi) technology is used to silence the expression of an unwanted gene by inhibiting the mRNA availability for protein synthesis [1]. The genome-level genetic engineering approaches require an insight in the genome, transcriptome, and metabolome [2] of the organisms under study. Like all other applied fields, genetic engineering requires comprehensive information about the genome structure of the donor and recipient before genetic modification. Decision about the morphological character that needs to be improved, the choice of a particular donor and recipient species, genetic networks, and metabolic pathways involved in the expression of a specific trait need to be explored.

Transcriptome analysis is a robust and cost-efficient method which provides information about the internal biological processes, cellular biosynthesis, and metabolic functions of a cell, tissue, or living organism [3]. This technique can be utilized by the genetic engineering scientists for the identification and quantification of genetic factors which positively or negatively regulate a particular trait of interest [4]. Comparison of gene expression profiles of an organism exhibiting the desired traits with the genetically similar organism lacking that trait can help in the identification of genetic factors involved in the development of that trait [5, 6]. These genetic factors might affect that trait positively or negatively. Enhanced accumulation of a particular transcript in the organism with desired phenotype as compared to the reference organism indicates that overexpression of that transcript is required for the exhibition of that trait. This phenomenon is called as positive regulation. In negative regulation, reduced expression of a gene is responsible for the exhibition of a desired trait [7, 8].

Positively regulated genes serve as genetic engineering tools for overexpression of a gene regulating a particular trait resulting in the introduction of that trait in genetically modified organism (GMO). For example, in transgenic cotton, expression of crystal protein (Cry10Aa) is responsible for resistance against boll weevil [9]. Advances in gene silencing technology through RNAi have led to utilization of genes which are negatively correlated with the desired traits. In cotton plant, seed-oil content increased by 16.7% by silencing GhPEPC1 gene through RNAi technology [8].

Transcriptome analysis and genetic engineering go hand in hand in the modern era of genetic improvements. Comparative transcriptional studies using single gene approaches or high-throughput approaches are used to identify the differentially expressed genes in a specific condition/organism as compared to reference. In single gene approaches, the expression of a gene of interest is quantified in different sets of conditions/tissues using northern blotting or reverse transcriptase polymerase chain reaction (RT-PCR). Northern blotting technique utilizes the gene-specific probes for comparative quantification of mRNAs of the target gene, whereas RT-PCR uses gene-specific primers to amplify and subsequently quantify the mRNA molecules. High-throughput technologies have the power to measure and analyze the expression of all the genes in a set of conditions. Differential display reverse

transcriptase PCR (DDRT-PCR), gene expression microarray, and next-generation sequencing (NGS) techniques are high-throughput techniques which are currently used. DDRT-PCR can study the expression of hundreds of genes at the same time, whereas microarray and NGS can study the whole transcriptome in a single experiment. Expression microarrays can give insight of the comparative transcriptomics, whereas NGS can provide absolute quantification of each transcript. All these techniques help in the identification of genes which give differential expression under different conditions.

These identified genes serve as targets to be used in different genetic engineering events. These genes are manipulated in the living organisms to produce GMOs. The modified organism is tested for the proper functioning of the transgene by single gene transcriptional analysis. Then the GMO is tested for the potential risks to the environment and human/animal health using targeted approaches which are biased and require preexisting knowledge of the risk. The comprehensive and unbiased assessment of the GMO should be done using global transcriptome analysis of the GMO with the commercial safe variety. After biosafety testing, GMO is released for commercialization and human/animal utilization. There is great deal of resentment and resistance against utilization of genetically altered organisms. Many governments have designed policies to properly monitor the presence and movement of GMOs. Transcriptional analysis is widely being utilized for the monitoring of various newly developed organisms.

2. Genetic engineering for human benefit

Genetic engineering is the field of science which is revolutionizing the world by manipulating the genome and transcriptome of living organisms to introduce desired traits in them. Since the commercialization of “Flavr Savr” tomato in 1994 [10], 357 GM crops belonging to 27 species all over the world have been commercialized [11], and this number is increasing day by day. Genetic engineering is widely being used for the improvement of crops, animals, fungi [12], bacteria [13], and other organisms to benefit mankind. Insect resistance, herbicide resistance, disease resistance, and abiotic resistance are being incorporated in the industrially important crops to make them tolerant to stresses. Yield and nutritional content of food crops are being modified to improve the feed for humans and animals. Scientists [14] produced transgenic maize with overexpressing *Oryza sativa* myeloblastosis 55 (OsMYB55) gene and found that the transgenic maize became more tolerant to heat and drought stress through activating the expression of stress-responsive genes. Microorganisms (bacteria and fungi) are being genetically engineered for the production of useful enzymes [13], secondary metabolites, beneficial oils [12], and antibiotics on commercial scale to be utilized in the pharmaceutical, food, and medical industry.

In 2010, 29 countries were growing genetically modified crops, and 31 countries had the approval to import GM crops. In USA, more than 94% of the cultivated soybean and cotton while 92% of corn is genetically modified [15]. The commercialization of the first genetically modified animal “AquAdvantage Salmon” for food was approved recently in 2015 [16].

RNA-based genetic engineering technology is becoming more attractive after the approval of white button mushroom for commercialization without stringent testing by the USDA, as this technology does not involve the introduction of foreign DNA [17].

3. GMOs and biosafety issues

Due to the advancements in the field of biotechnology and genetic engineering, new varieties are extensively prevailing in the society. Despite their huge potential for human welfare, their commercialization is controversial. Many people perceive all the GMOs to be bad for their health and environment. People who are aware of the mechanism of genetic engineering are concerned about the unintended modifications and their effect on the soil microorganisms [18], plant-microbe interaction [19], and imbalances in the natural biosystems. GMO's controversy mainly revolves around environmental safety [20], human and animal health [21], concerns over interfering with nature [22], and patent issues [23].

Genetically modified organisms produced by genetic engineering or conventional plant breeding are targeted to enhance the desired commercial traits, but GMOs might exhibit unintended traits as well. In the international meeting on "Genetic Basis of Unintended Effects in Modified Plants," biotechnology industry, government, and academia emphasized that no genetic modification is without unintended effects whether conventional breeding or genetic engineering [24]. The source of unintended modifications could be attributed to gene insertions or deletions involving deletion or disruption of endogenous genes and chimeric protein production which perform abnormal function. Genetic engineering approaches involving tissue culturing and in vitro culturing pose the risk of soma clonal modifications arising from the genetic and epigenetic effects of in vitro cultures [25]. Pleiotropic effects may contribute to the unintended modifications if the transgene plays multiple roles or is the part of multiple pathways in an organism leading to the production of potentially harmful secondary metabolites [26].

Biosafety policies involve principles, procedures, and rules devised and adapted for protecting the environment and health of the individuals against potentially harmful metabolites and toxins. Biosafety involves containment of harmful material to avoid unintentional exposure to toxic agents produced by genetically modified organisms [27].

4. Monitoring of GMOs

Due to the resentment of the consumers in utilizing GMOs for food and animal feed purposes, many governments have devised policies to give its people freedom over utilization of GMOs. Policies mainly revolve around detection, proper labeling, isolation of propagation area, and tracking of GMOs. International trading requires standardization of procedures and policies related to GMO monitoring and marketing among trading countries. Moreover, in order to

limit the entry of approved varieties across the borders of a country, proper monitoring of GMOs is required.

The first step in the monitoring of GMO is the detection of transgene in an organism under question. Many methods are being used to detect the genetically modified varieties. GMOs produced by insertion of DNA fragments can be detected by protein-based assays [Enzyme-linked immunosorbent assay (ELISA), Western blotting, etc.] or nucleotide-based assays including PCR. PCR-based detection is the most sensitive method which makes use of sequence-specific primers [28]. Due to the abundance of GMOs in the market, it has become very difficult to keep the sequence information of all the transgenes. The advanced high-throughput technologies for GMO detection/monitoring are developed to detect multiple transgenes or related nucleotide components (promoter, enhancer, and terminator) of the cassette [29, 30] in a single experiment. For rapid PCR at atmospheric temperature, various methods have been developed [31]. DNA microarray chips are being developed which contain the probes against all the transgenes present in the commercial varieties [32]. Sampling and hybridization of DNA of a variety under question can detect the presence of any transgene. More efficient, sensitive, and robust methods are required for proper monitoring.

All the above methods are used for the detection of DNA insertion in the transgenic organisms. However, in the RNA-based GMOs, the detection of transgene requires transcriptomic approaches. Transcriptional methods including RT-PCR, gene expression microarray, and RNA-seq can detect all types of GMOs produced through RNA- or DNA-based methods. In transcriptomic approaches, RNA is isolated from the sample and reverse transcribed to produce complementary DNA (cDNA). Due to resemblance in the biochemical properties of RNA and DNA, DNA is often present in the RNA preparations which is eliminated by treating the sample with DNase enzyme. By avoiding this step of DNase treatment, we get both RNA and DNA in the sample. This crude RNA is transcribed and RT-PCR is used for the detection of RNA or DNA of the transgene.

5. Validation of genetically modified organisms

The developers of GMOs are required to assess the phenotypic and molecular characteristics of modified organisms. Many countries have adopted regulations for commercialization of GMOs which mainly include the comprehensive risk assessment of the new organism before field trials, to be used as feed/food or before release to the environment. These risk assessment methods mainly involve the comparison of the agronomic traits, composition, animal nutrition, and production of toxins of the new product with commercially available for multiple years and at multiple sites. But these assessments are targeted and require the prior information about the risk. The untargeted risks can be left without evaluation with the potential to harm the environment and health.

During the screening and selection of a GMO, the emphasis is given to the insertion of the transgene as a single copy without disruption of an endogenous gene, preserving the gene

cassette and the absence of vector backbone. Safety of the GMO is tested on a very limited scale only when the GMO is ready to be commercialized. The main focus of the biosafety studies is limited to the assessment of the effect of the GMO on the consumer health and safety. The phenotypic and agronomic traits of the newly produced plant and a genetically similar organism are compared [33], but thorough profiling of the genetically modified organism is lacking.

Newly produced plants by genetic engineering and other genetic methods should not only be assessed by target-based approaches as these assessments are biased and cannot recognize the unintended risks thoroughly [34]. Genome-wide approaches like transcriptome analysis, proteome analysis, or metabolome analysis have the advantage of being unbiased and robust [35–37] and provide a lot of information about the new plant variety. Scientists compare the protein profiles of genetically modified organisms with their wild types to identify the aberrant proteins. Proteome of a commercial variety of maize was compared with the isogenic transgenic line which was resistant to European corn borer by expressing Cry1Ab gene [38]. The results spotted unwanted/unintended protein expression in the transgenic lines and suggested for the untargeted evaluation of the new transgenic organisms. Other studies using proteomic or transcriptomic approaches to compare the GMO with the wild type found only intended alterations [7], while no unintended changes were found.

Unintended changes arising as a result of pleiotropic effects of genetic modification are not always harmful. A group of scientists has performed transcriptome analysis in GMO lines developed for enhanced insect attraction in *Arabidopsis* and compared it with naturally occurring non-GMO lines to identify transcriptional distance between the two groups [39]. They identified that the pleiotropic effects of gene insertion are equivalent to the gene expression changes naturally occurring in *Arabidopsis* indicating that the specific modified lines of *Arabidopsis* were equally safe as naturally occurring lines. Thus unbiased and untargeted risk assessment of GMOs through newly developed “omic” techniques is necessary [40] before its release in the environment or trials for human and animal use.

6. Transcriptome analysis for GMO validation

Unbiased detection of unintended effects of transgene in a genetically modified organism requires comparison of transcriptome [41], proteome [38] and metabolome [40] of the modified organism with the isogenic unmodified organism. The thorough profiling helps in the identification of genes, proteins, and metabolites modified in the newly developed organism. By digging the gene networks, protein functions, and metabolic processes of the altered biomolecule, scientists can depict the effects of GMO on the environment, health, and nutrition of the consumer. The absence of unintended aberrations in the biomolecules declares the new variety as safe, whereas the presence of unintended aberrations does not declare it to be unsafe but indicates that the variety requires more targeted validation before commercialization [7].

Transcriptome analysis stands out of the other omic-based approaches due to its comparative simplicity and cost efficiency. Latest technologies of gene expression microarray and NGS are commonly used for global transcriptional profiling of GMO and wild-type ecotype for transcriptional equivalence. Gene expression microarray involves the use of chips containing probes which represent the complete genome of an organism under study. Hybridization of these chips with fluorescently labeled cDNA can identify the genes which are differentially expressed between GMO and wild type. NGS technologies involve sequencing and quantification of nucleotides at the same time. RNA-seq is the type of NGS which specifically deals with the transcriptional studies. Gene expression microarray and RNA-seq have proved themselves equally for the detection of intended and unintended effects. However, both approaches have some advantages and disadvantages. Microarray experiments are comparatively cheaper and easier than RNA-seq. But the chips are commercially available only for a limited number of organisms, and custom printed chips require the genome sequence information of the specific organism. The full power of this technology can only be utilized for sequenced genomes. While RNA-seq is the only technology which can sequence as well as quantify the mRNA libraries of unsequenced genomes. Moreover, RNA-seq provides us the absolute quantification as compared to microarray which give comparative quantification. **Table 1** shows some examples where scientists have utilized these transcriptomic approaches for GMO validation.

Gene expression microarray and RNA-seq methods not only identify the unintended effects of genetic engineering but are also useful in elucidating the mechanism of action of a transgene. Pathway analysis and gene ontology analysis of modified genes lead to the evaluation of molecular basis of phenotypic changes in the newly produced organisms [48]. Transgenic variety of papaya (*Carica papaya* L.) fruit which was resistant to papaya ring spot virus (PRSV) was evaluated against its progenitor variety through RNA-seq analysis. The transcriptional profiles revealed the transcription factors, signaling pathways which were responsible for the stress tolerance and pathogen resistance [43].

Biotic and abiotic stress tolerance is a complex mechanism involving many gene networks and pathways causing changes in the morphology and physiology. Stress-related transcription factors which can bind to the promoters of multiple genes are largely used as transgenes to produce stress-tolerant GMOs. Genetically engineered crops for tolerance against stresses are difficult to get approval for commercialization due to increased risk of pleiotropic effects. Global transcriptome analysis can identify all the pathways affected by any kind of genetic modification and targets for risk assessment.

Transcriptomic approaches have an added benefit of detection of gene silencing in the GMOs produced by gene silencing technology. RNAi-based technologies where double-stranded RNA targeting a specific gene is introduced in an organism. This RNA after being processed in the recipient organism is converted into smaller piece of nearly 21–22 nucleotides. These RNAs reach their targets and inhibit the translation of specific messenger RNA into respective proteins, thus functionally silencing the genes post-transcriptionally. The increasing popularity of this technology is due to its ability to not affect the genome of the GMO [49].

| Organism | Altered trait | Gene | Method of evaluation | References |
|--------------------|--|--|-----------------------|-----------------------|
| Wheat | Drought and salt tolerance | Glycine max drought-responsive element-binding factor (GmDREB1) | RNA-seq | Jiang et al. [7] |
| <i>Arabidopsis</i> | Drought tolerance | Absciscic acid-responsive element binding factor 3 (ABF3) | Expression microarray | Abdeen et al. [42] |
| <i>Arabidopsis</i> | Insect attraction | Farnesyl diphosphate synthase 1 long isoform (FPS1L), nerolidol synthase 1 from <i>Fragaria ananassa</i> (FaNES1), short (cytosolic) isoform of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMGRIS) | Expression microarray | Houshyani et al. [39] |
| Papaya | Resistance against papaya ring spot virus | Coat protein (CP) of PRSV | RNA-seq | Fang et al. [43] |
| Maize | Insect resistance | Cry1Ab | Expression microarray | Coll et al. [44] |
| Rice | Antifungal protein | Antifungal protein (AFP) | Expression microarray | Montero [45] |
| Barley | Defense against stresses | Endochitinase | Expression microarray | Kogela [46] |
| Soybean | Human and viral protein production in plants | Human myelin basic protein (hMBP), human thyroglobulin protein (hTG), mutant nontoxic staphylococcal enterotoxin B gene (mSEB) | RNA-seq | Lambirth et al. [47] |

Table 1. Evaluation of GMOs by transcriptome analysis.

7. Conclusion

The newly produced GMOs could be very harmful for the environment, microbial life, and human and animal health, but they are not always harmful. The producers of genetically modified organisms should analyze the global transcriptional profiles of the GMO in comparison with the safe commercial variety to assess the presence or absence of unintended modifications. This data would also provide comprehensive and unbiased information about the metabolic pathways altered in the new organism that can be helpful in designing the strategy for biosafety risk assessment of GMOs.

Transcriptome analysis is very useful for detection and evaluation of transgenics produced by RNAi technology or transcription factor transformations. However, evaluation of gene expression is a very sensitive phenomenon and variable in different tissues and changing conditions. So, for transcriptional analysis, the selection of suitable sample and experimental conditions is critical for reliable results.

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