Chapter 3

Transgenic Plants: Gene Constructs, Vector and Transformation Method

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http://dx.doi.org/10.5772/intechopen.79369

Abstract

The human population has reached 7 billion by 2015 and is estimated to exceed 10 billion by the end of 2050. As such, crops which are the main food source must be produced at a higher pace in order to cater in tandem with the food demand. In the past, traditional plant breeders practice classical breeding techniques to propagate plants with desirable traits. However, traditional breeding technique lies in that only individuals of the same or closely related species can be crossbred. Moreover, traditional breeders will not be able to obtain traits which are not inherent within the gene pool of their target plants through classical breeding. With recent advancements in the field of genetic engineering, it is now possible to insert beneficial genes from a completely different species or even kingdom into a target plant, yielding transgenic plants with multiple ideal traits. To develop a transgenic plant, parameters such as vector constructions, transformation methods, transgene integration, and inheritance of transgene need to be carefully considered to ensure the success of the transformation event. Hence, this chapter aimed to provide an overview of transgenic plants' development, its advantages and disadvantages, as well as its application for the betterment of mankind.

Keywords: genetic engineering, genetic-modified organism (GMO), selectable marker, traits' improvement

1. Introduction

Transgenic plants are plants that have had their genomes modified through genetic engineering techniques either by the addition of a foreign gene or removal of a certain detrimental gene [1]. A foreign gene inserted into a plant can be of a different species or even kingdom. The first transgenic plant was developed through the insertion of *nptII* bacterial antibiotic resistance gene into tobacco [2]. Since then, with the rapid development in plant molecular biology and genetic engineering technology, a wide variety of transgenic plants with important agronomic traits such as pest resistance and drought tolerance have been developed, ranging from dicots to monocots that are amenable to genetic modifications. The main purpose in the production of transgenic plants is to produce crops, which have ideal traits, quality, and high yield. Besides being beneficial to the agriculture sector, the plants are found to be able to act as the factory for pharmaceutical protein production [3].

2. Application of transgenic plants

2.1. Resistance to biotic or abiotic stresses

Biotic stresses occur naturally as a result of stress exerted from other living organism within the same ecosystem. These include bacteria, viruses, herbivores, or native plants [4]. Crop plants are incorporated with disease resistance gene to confer resistance toward these pathogenic diseases that are caused by pest, bacteria, and viruses; this includes tolerance to herbicides. The introduction of genetic modification technology could reduce the usage of expensive pesticides and herbicides in agriculture. The removal of natural pests will lead to a greater yield and better quality of crops. As such, insecticidal toxin genes from a bacterium can be introduced into the plant of interest's genome, thus providing protection to the plant against insect pests [5, 6]. *Bacillus thuringiensis* (*Bt*) crops are an example of transgenic plant produced through this method. In addition, virus-resistant plants can be achieved through the introduction of viral coat proteins into plants [7, 8].

Development of transgenic plants resistant to abiotic stresses is important in this "Global Warming's Terrifying Era". The world climate in the past few decades has changed tremendously culminating in changes to soil composition, humidity, water, sunlight availability, and many other agricultural problems that led to reduction in the crop yield [9, 10]. Hence, genetic engineering technology is needed as a tool to solve these problems by providing the plants with enhanced stress tolerant ability or protection. The manipulation of transcription factors (TFs), late embryogenesis abundant (LEA) proteins, and antioxidant proteins had successfully produced plants tolerant to drought and salinity [11, 12]. Overexpression of the proline biosynthesis enzyme (P5C), which allows the accumulation of osmoprotectant during drought season provides transgenic plants with osmotic stress resistance [13, 14].

2.2. Improving crop yield and nutritional value

Malnutrition is a major health concern that is prevalent especially in the underdeveloped and developing countries due to limited access to nutritious food [15]. Genetic engineering of staple crops has become one of the more effective solutions in addressing this problem. To date, a variety of crops had been successfully modified for better yield as well as for higher nutritional value. Biofortification is a technique used in agriculture to increase the nutritional value of crops. A well-known example would be the golden rice, a variety of *Oryza sativa*, produced to biosynthesize beta-carotene through genetic modification. The golden rice was developed by adding two beta-carotene synthesis genes: phytoene synthase (*psy*) and lycopene β-cyclase (β-*lcy*) (originated from *Narcissus pseudonarcissus*). These genes were driven under the control of the endosperm-specific glutelin promoter together with a bacterial phytoene desaturase (*crt*I, from *Erwinia uredovora*) [16].

2.3. Transgenic plants as bioreactors for recombinant proteins

Plants had been used as a biofactory in the production of the first recombinant human protein in 1989. Product yields from recombinant proteins using mammalian expression systems are low and expensive [17], while bacteria system is incapable of post-translational modification in complex protein formation. Due to this, the production methods had shifted to plant cell systems, which provide cheaper and better alternative sources for recombinant proteins production [18, 19]. The recombinant proteins produced in transgenic plants include antibodies, metabolites or catabolites, proteins, and vaccines [20, 21]. Antibodies and vaccines against gastrointestinal tract diseases, cholera, and malaria are known to be produced in transgenic plants such as potato, banana, algae, and tobacco [22, 23]. An anticancer antibody that recognizes the cells of lung, breast, and colon cancer had also been successfully expressed in rice and wheat seed [24]. However, despite a lot of successful plant-produced antibodies and vaccines, it is difficult to commercialize them and to date, the only plant-produced Newcastle disease vaccine had been approved by the United States Department of Agriculture for poultry farming with several vaccines in clinical trials [25].

3. Gene constructs

A simple functional gene construct consists of a promoter region, gene coding region, and terminator/stop region. In addition, certain gene constructs may contain special sequences such as an enhancer, silencer, or reporter sequences depending on the nature of study. Plant transformation always starts with the transgene construction. Transgene construct generally has similar elements other than the inclusion of the gene of interest and selectable markers. A proper gene construct is crucial for the success of producing ideal transgenic line.

3.1. A typical plant gene

A typical plant gene consists of the regulatory and structural genes [26]. Regulatory genes are usually located at the 5′ upstream of a gene, with its own promoter, enhancer, or silencer region. Structural genes, on the other hand, begin with a catabolite activator protein (cap) site, followed by a leader sequence, start codon, exons, introns, terminator, and a polyadenylation site (poly-A tail). These elements are responsible for DNA transcription. The transcribed pre-mRNA, then undergoes RNA splicing, producing mature mRNA without the introns (non-coding region). This mature mRNA is delivered to the cytoplasm for translation initiated by the binding of ribosomal subunits to the promoter. Translation then begins at the start codon (ATG), with the ribosome moving downstream to the next codon creating a peptide chain with the help of tRNAs and ends once it reaches the chain terminator (stop codon, TAA/TAG).

3.2. Promoters/enhancers

The promoter region is typically located at the 5′ upstream of a gene. Promoters are known for their function in governing gene expression, likened to an on/off switch. In DNA transcription, the promoter sequence is recognized by transcription factors. These transcription factors bind to the consensus region of the promoter and recruit the RNA polymerase. Formation of the RNA polymerase transcription complex marks the beginning of DNA transcription.

The promoters can be categorized into three main groups: constitutive promoters, tissue-specific promoters, and inducible promoters [27]. The constitutive promoters are active at most of the developmental stages, and they directly participate in maintaining moderate and constant level of gene expression. Tissue-specific promoters provide restricted gene expression to certain tissues or gene expression involves in developmental-specific stages. Gene expressions associated with the inducible promoters are greatly affected by environmental stimuli, which allow for the regulation of genes through external factors. **Table 1** shows selected promoters used in plant transformation.

Enhancers are short (50–1500 bp) regions in a gene that can be recognized and bound by activator proteins. These proteins, also referred to as transcription factors, bind to the enhancer, forming an enhancer-bound transcription factor complex, which will later on interact with the mediator complex (TFIID) ultimately aiding in the recruitment of RNA polymerase II. The enhancer-bound transcription factor complex forms a loop and toward the intervening sequence and comes in contact with the promoter region, thus increasing the accessibility of the promoter to the transcription proteins [39]. In contrast, silencers function as the

Promoter	Source	Activity	References
CaMV35S	*Cauliflower mosaic virus*	Constitutive	[28, 29]
Ubiquitin RUBQ1, RUBQ2 and rubi3	Rice	Constitutive	[30]
Ubiquitin Gmubi3	Soybean	Constitutive	[31, 32]
SCR, SRK	*Brassica rapa*	Pollen and stigma specific	[33]
Exo70C2	*Arabidopsis*	Pollen and root specific	[34]
LMW Glu, HMW Glu-1D1	Wheat	Seed specific	[35]
Expansin PcExp2	Sour cherry	Ripened fruits	[36]
Potato class I patatin	Potato	Tuber/storage organ specific	[37]
NtHSP3A	Tobacco	Stress inducible	[38]

Source: Adapted from Hernandez-Garcia et al. [27].

Table 1. Examples of promoter used in plant transformation.

direct opposite of enhancers. Silencers are binding sites for transcription factors known as the repressors. These repressors are known to downregulate the transcription of a gene. In plant genetic engineering, suitable promoter and enhancer are chosen based on the intended regulation of gene expression. Gene expression is kept at basal level when the transgene exerts mild toxicity to the target plant. On the other hand, higher gene expression levels facilitate the detection and monitoring of a transgene which may usually be under expressed in nature.

3.3. Reporter genes

Reporter genes are genes attached to the regulatory sequences or to gene of interest to allow for detection of the transgene expression as well as the localization of expressed proteins [40]. Reporter gene sequences encode proteins or products of the protein after being catalyzed for detection through instruments or simple assays. In contrast, selectable marker genes such as antibiotic genes, herbicidal-resistant genes, and anti-metabolic genes confer resistance toward certain chemical agents, which inhibit nontransgenic plant development [41]. The common reporter genes used to monitor plant transgene expression include green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), beta-galactosidase (LacZ), luciferase (Luc), and beta-glucuronidase (GUS). These reporter genes allow differentiation between transformed and nontransformed cells and enable detection of transgene localization and regulation of the expressed and tagged protein. Dual reporter systems such as Luc/Luc and GUS/Luc are also available for better detection in distinguishing proteins [42, 43]. Ideal reporter genes should be highly sensitive, stable, and reliable for large-scale measurements within a wide range of cells and tissues [44]. However, the ideal reporter genes encompassing all the desired properties are still unavailable despite current reporter systems being extensively studied. Each reporter system manifests its own beneficial and detrimental traits. Therefore, due consideration would have to be given when contemplating a suitable reporter gene based on the nature of the study.

3.4. Problem posed by antibiotic resistance reporter genes

Plant transformation techniques available currently are rather efficient but not perfect yet. There are no techniques that are able to provide 100% transformation efficiency. In order to distinguish the transformed and nontransformed plant cells, markers are needed. Antibiotic or herbicide resistance genes act as the primary selective markers in transformant selection to efficiently eliminate the nontransformants [45]. The effectiveness of an antibiotic resistance system is dependent on three criteria: (1) selective agent used should completely inhibit the growth of nontransformed cells, (2) resistance gene is expressed in transformed cells, and (3) explant used for transformation. **Table 2** shows some of the antibiotics used in transgenic plant screening.

Antibiotic screening has provided the initial identification of successful transgenic plant. However, the use of antibiotics always leads to issues on environmental problems and genetic modified (GM) food safety. This is mainly due to the concern of gene pollution when antibiotic gene escapes from the GM plant into the environment through microorganism. Bacteria are known to be able to uptake and integrate foreign DNA pieces into their genome [51]. The microbes surrounding the GM plant might uptake the DNA fragments from the transgenic plant and hence developed resistance to the antibiotics. Besides, gene escape may occur as the

Antibiotics	Mechanism of action	General working concentration (μg/ml)	Selection	References
Kanamycin	Inhibiting ribosomal translocation and eliciting miscoding	50	*nptII*	[46]
Hygromycin B	Inhibit protein synthesis	20–200	*hph*	[47]
Streptomycin	Inhibit protein synthesis	100	*spt*	[48]
Spectinomycin	Inhibit protein synthesis	100	*aadA*	[48]
Phleomycin	DNA breakage	10	*ble*	[49]
Bleomycin	DNA breakage	10	*ble*	[50]

Table 2. Selective antibiotics used for transgenic plants screening.

antibiotic resistance gene can be transferred to the neighboring plants through pollen dispersal [52]. There is also the possibility that consuming the transgenic plant with antibiotic resistance gene may result in the transfer of the genes to the probacteria present in the guts. Hence, the antibiotic resistance marker genes are normally avoided in the transgenic whole plant screening.

Efforts had been made by replacing antibiotic marker gene with another potential marker gene such as reporter genes. Reporter genes such as GFP are not reported as toxic to the environment, instead they are widely used as biosensors. Engineering plants with these reporter genes could prevent the unnecessary buildup of antibiotic resistance in the environment. On the other hand, gene escape can also be avoided through the removal of antibiotic resistance gene from the transgenic plant. The latest genome editing tools such as TALEN and CRISPR/Cas9 may be good tools in the removal of gene markers from the transgenic plants. However, these advanced tools have yet to be shaped.

4. Vectors for the production of transgenic plants

A vector acts as a vehicle that transports the gene of interest into a target cell for replication and expression. Common vector consists of three components: an origin of replication, multicloning site or recombination site, and selectable marker. The origin of replication is an AT-rich region on the vector that initiates the replication of the vector itself by binding to a protein complex, unwinding the vector and thus replicating it with the help of polymerases. The multicloning site is a region that contains multiple unique sequences otherwise known as restriction site that can be cut by specific restriction enzyme, allowing the insertion of the gene of interest. The recombination site allows site-specific recombination to occur between two plasmids. The selectable markers are genetic markers that functions as mentioned in the gene construct section, serving its purpose in validating the insertion of the vector into the *Agrobacterium sp*. In plant transformation, vectors commonly used are Ti plasmid-based vector and plant viral-based vector.

4.1. Plasmid vectors

4.1.1. Ti plasmid

The Ti plasmid is the most commonly used vector in the production of a transgenic plant. The Ti plasmid has an estimated size ranging between 200 and 800 kbp depending on the classes of the Ti plasmid. The Ti plasmid is divided into three main regions: the transfer DNA (T-DNA) region, virulence region, and opine catabolism region. The T-DNA region that is transferred into the plant genome is about 24 kbp in size [53]. This region is bordered by repeat sequences on each end commonly known as the left border and right border. The right border is the critical part essential for the transfer of DNA-causing tumorigenesis. The virulence region, however, is responsible for encoding the *vir* genes, which aids in the transfer of the T-DNA. The T-DNA sequence also codes for opine and phytohormones (auxin and cytokinin) biosynthesis. The three oncogenes (opine, cytokinin, and auxin biosynthesis gene) within the T-DNA are the main causes of tumor formation in plant, leading to the crown gall disease [54]. The growth hormones synthesized are responsible in causing uncontrolled plant cells' proliferation and worsen the situation by enhancing crown gall formation. Opines are the main carbon source utilized by the *A. tumefaciens* that are not naturally synthesized from plant metabolism. Therefore, *A. tumefaciens* will develop its own biosynthetic machinery for production of nutrients by genetically modifying the host cells. The opine catabolism region encodes the genes for proteins involved in opines catabolism. The origin of DNA replication allows stable maintenance of the Ti plasmid in the bacterium. For plant transformation, the Ti plasmid is usually disarmed, with the tumor-inducing genes removed and replaced with the reporter genes together with the gene of interest [55].

The Ti plasmid is large and would become larger with the genes of interest and selectable markers. Large-sized plasmids are cumbersome to handle and have low copy numbers in nature. However, this drawback eventually led to the development of a co-integrative system in combination with the binary vector system which solved the problem for large-sized plasmids.

4.1.2. The co-integrative vector

The co-integrative vector is developed through homologous recombination between an intermediate vector and disarmed Ti plasmid. The intermediate vector is normally the *E. coli* plasmid harboring the gene of interest. Both the intermediate vector and disarmed Ti vector consist of some common sequences, which allow the homologous recombination of the two plasmids to occur. The recombination will result with a large co-integrative vector containing the merged *E. coli* plasmid and disarmed Ti plasmid. This co-integrative vector will later be introduced back into the *Agrobacterium* for transgenic plant transformation. However, the enormous size of the plasmid as a result from the recombination may prove an ominous challenge to be manipulated. Thus, the use of this vector had been discontinued since the binary vector system was introduced.

4.1.3. The binary vector

A two-plasmid system called the binary vector system was developed when researchers found that T-DNA functioned independently without the needs to attach to the Ti plasmid. The binary system involved two plasmids which are the helper vector and mini vector. The mini vector refers to a smaller size plasmid consisting of the T-DNA and the origin of replication of both *E. coli* and *A. tumefaciens*, which allow the plasmid to be cloned in *E. coli* and *A. tumefaciens*. The helper vector refers to a wild-type Ti plasmid without the T-DNA region. The wild-type Ti plasmid is also known as a helper plasmid as it provides the template for all the genes necessary for gene transferring and integration. Both of these helper and mini vectors are introduced together into the *Agrobacterium* and the transformed *Agrobacterium* will be used in plant transformation.

4.2. Plant virus vectors

Viruses are intracellular obligate parasites that require molecular machinery from a specific host to replicate. Viruses have not been found to infect plants through the use of transmission vectors such as aphids, insects, nematodes, and fungi. These viruses have been modified and are used as alternative sources for plant transformation [56]; common plant viruses used in transgenic plant production include the *Cauliflower mosaic virus* (CaMV), *Tobacco mosaic virus* (TMV), *Alfafa mosaic virus* (AMV), *Potato virus X* (PVX), and *Cowpea mosaic virus* (CPMV). The wild-type plant viral vectors have been improved and modified to accommodate their use with *Agrobacteria* as well as the plant host for an increased efficiency level through two approaches. The first approach would be designing virus vectors that are similar to wild types carrying the gene of interest, which are capable of infecting plants.

The second approach would be the development of a 'deconstruct' virus, which occurs through the removal of the undesired viral genes, for example, the coat protein-expressing gene, and to replace them with functional gene such as reporter genes or antibiotic resistance gene, which facilitates transgenic screening.

5. Transformation techniques

Plant transformation refers to the process of altering the genetic constituents in a plant of interest by introducing DNA segments into the plant genome to achieve desired gene expression. Numerous types of plant transformation techniques have now been made accessible to the public. These plant transformation techniques can be categorized under two groups: indirect or direct gene transfer. Indirect gene transfer (also known as vector-mediated gene transfer) involves the introduction of exogenous DNA into the plant genome via biological vectors, whereas direct gene transfer methods involve the introduction of exogenous DNA directly into plant genome through physical or chemical reactions. Different gene transfer methods and their salient features are tabulated in **Table 3**.

Method	Features
Vector-mediated gene transfer	
a. *Agrobacterium*-mediated gene transfer	Efficient to wide range of plants.
b. Plant virus vectors	Efficient and high expression of transgenes.
Direct gene transfer	
a. Physical Methods	
i. Electroporation	Confined to protoplasts that can be regenerated to produce complete and viable plants.
ii. Microinjection	Requires highly skillful technical personnel and limited to one cell per microinjection.
iii. Particle bombardment/microprojectile	Special instrumentation required. High risk of gene rearrangement. May be used for a wide range of plant tissues.
iv. Silicon carbide fibers	Requires careful handling. Requires regenerable cell suspensions.
b. Chemical methods	
v. Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Problems encountered when regenerating these cells into viable plants.
vi. Liposome fusion	Confined to protoplasts which may be regenerated into a viable plant.
vii. Diethylaminoethyl (DEAE) dextran mediated	Does not result in stable transformation.

Table 3. Gene transfer methods in plants and their features.

5.1. *Agrobacterium*-mediated gene transfer

Agrobacterium-mediated transformation is the most common technique used in plant transformation as it is efficient and effective in a wide range of plants. *Agrobacteria* are indigenous to the soil ecosystem. They are pathogenic Gram-negative bacteria that cause crown gall or hairy root disease in plants. The genetic information for tumor growth is encoded on a tumor-inducing plasmid (Ti plasmid) or hairy root-inducing plasmid (Ri plasmid) in the genome of these bacteria. There are generally two types of *Agrobacterium* species that are commonly used in plant transformation; *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *A. tumefaciens* contains the Ti plasmid which causes crown gall disease, whereas *A. rhizogenes* contains the Ri plasmid that causes hairy root disease. The discovery of these two species provides efficient vector systems for the development of transgenic plants when the detrimental genes in *Agrobacteria* are removed. This method had successfully transformed a broad variety of plants such as rice, maize, barley, and tobacco.

A. tumefaciens used for plant transformation are modified *Agrobacteria* which has no tumor-promoting and opine-synthesis genes in their genome. These genes are removed (disarmed) from the bacterial plasmid and replaced with the desired foreign gene or selective markers, making them useful vectors that enables the incorporation of foreign genes into plant's genome, transiently or stably. In order to achieve stable incorporation of genes, the *Agrobacteria* function to transport and integrate the T-DNA into the host's genome through these steps: (1) chemical signal recognition of host, (2) activation of the *vir* gene in *Agrobacterium*, (3) attachment of *Agrobacterium* to plant cells, (4) activation and transportation of virulence proteins, (5) production of T-DNA strand, (6) transfer of T-DNA and virulence protein out of *Agrobacterium*, (7) transfer of T-DNA into plant nuclear, and (8) integration of T-DNA into plant genome. The steps involve in transient transformation are postulated to be identical to the stable transformation with the exception of steps (7) and (8).

Ever since the *Agrobacterium*-mediated transformation protocol had first been introduced, various refinement of the protocol had been ongoing to improve its efficiency. Although traditional *Agrobacterium*-mediated transformation works efficiently in dicotyledonous plants such as potatoes, tomatoes, and tobacco, it is less successful in recalcitrant crops such as wheat and maize due to the lack of wounding response system [57]. This has remained a critical obstacle until the development of plant tissue culture and the introduction of in-planta transformation protocol that has improved the transformation efficiency on these plants at many folds [58].

Agrobacterium-mediated in-planta transformation is a method that does not involve plant tissue culture; transformation is done directly onto a developed plant. This technique includes agroinoculation and agroinfiltration. In agro inoculation, the transformed *Agrobacteria* with the gene of interest is inoculated onto the surface of the plant tissue of a whole plant. It is generally done using either by toothpick, wire loop, or direct organ immersion (floral dip method). Agroinfiltration, on the other hand, can be carried out using syringe or vacuum. Syringe infiltration is simple and cost effective as it injects the transformed *Agrobacteria* onto the underside of the leaf while concurrently ensuring the application of counter pressure on the other side of the leaf. However, it is time consuming and only suitable for small-scale expression.

Vacuum infiltration, however, is rapid and more efficient, thus enabling large-scale production intended commercialization. In this approach, the whole plant is submerged into the transformed *Agrobacteria* suspension with application of a vacuum environment that forces the *Agrobacteria* to penetrate throughout the whole plant. These alternative methods have become popular in plant transformation especially in monocots.

Heat and hydrolysis treatment on target tissues prior to transformation have been reported to enhance transformation efficiency when heat treatment is used, enhancing the efficiency of transformation in different plant tissue such as switchgrass, ryegrass, and rice [59–61]. Similar enhancement had also been obtained in hydrolysis treatment via hydrolytic enzyme such as cellulase, macerase, and pectinase, which provides milder disruption that improved the recovery and regeneration rate of transformed cells [62]. In addition, sonication-assisted plant transformation applied to the target plant tissues prior to *Agrobacteria* immersion or agroinfiltration resulted in effective transformation of recalcitrant plants by creating microwound on explants, which provides better access to *Agrobacterium* [63].

5.2. Direct gene transfers

Direct gene transfer, as the name suggests, involves the direct introduction of exogenous DNA (naked DNA) into the plant nucleus. In order to introduce foreign DNA into the plant cell, the outer membrane of the cell is first disrupted, permeating it for foreign DNA to enter. Most of the methods under direct gene transfer are simple and effective. However, gene expression in these transgenic plants can be transiently or stably transformed.

Direct gene transfer can be categorized into two main groups: physical gene transfer and chemical gene transfer. Physical gene transfer disrupts the cell wall and cell membrane via mechanical means. Among these methods, particle bombardment biolistic is the most common one used in plant transformation since it was first introduced by Sanford et al. [64]. The DNA coated with gold or tungsten particles are shot into the target plant cell under high pressure using a "Gene Gun" (Helios® Bio-Rad). The fast-moving particles allow for the penetration of coated DNA through the thick plant cell wall, directing the foreign DNA into its nucleus. The coated DNA will then separate from the metal particles and integrate itself into the chromosomes within the nucleus of the plant cell. This method had been found to be effective in transforming both dicots and monocots which compensates for the less successful *Agrobacterium*-mediated transformation process. Furthermore, it is also less toxic and applicable to almost all plant cells [65]. The major setbacks of this method, however, lie in the availability of special instruments as well as the delivery efficiency of DNA fragments to the plant nucleus instead of other organelles [66]. In traditional biolistic method, microprojectiles (gold or tungsten) are normally coated with DNA in the presence of calcium chloride and spermidine [67]. The spermidine helps to stabilize the DNA structure and enhances the binding of DNA to the microprojectiles [68]. In the effort of improving this tool, other cationic polyamine such as protamine provides better results when compared with spermidine, as this ensures by protecting the coated DNA from DNase degradation. Biolistic transformation via protamine had been performed in rice and peanut, and the results were shown to be threefolds better when compared to spermidine [69, 70]. Other methods of improving the efficiency of transformation via biolistic guns involved reduction of the amount of DNA coated on the microcarriers [70, 71].

Other physical gene transfer methods include electroporation that uses electrical impulses to facilitate the transfer of foreign DNA into the plant cells. Plant cells are first incubated in a buffer solution containing foreign DNA, followed by the application of electrical impulses into the buffer, resulting in the formation of temporary transient pores on the cell membrane of the plant to allow the foreign DNA to enter. This method is relatively easy and time saving but is only applicable to protoplasts (cell without cell wall). Hence, this method is not commonly practiced in plant transformation.

Chemical gene transfer approaches involves the use of chemical to disrupt cell membrane enabling the entry of foreign DNA. This particular method is not preferable in plant transformation as it is only effective when applied to protoplasts. One of the most prominent chemicals used in this approach is polyethylene glycol (PEG) that is used for destabilizing the cell membrane in the presence of a divalent cation, thus increasing the permeability of the cell membrane, allowing for the uptake of foreign DNA. The exact mechanism for chemical gene transfer is not fully understood, but it was postulated that PEG increases the osmotic pressure and causes contraction in the protoplast; this facilitates endocytosis of the divalent cation/DNA complex [72]. Besides those, liposome is another chemical method that is used in the transformation of plant's protoplast cells. Liposomes act as vehicles to encapsulate and deliver foreign genetic materials into the protoplast. The lipophilic attribute of liposomes provide easy access into the protoplast in transforming the cell [73].

6. Integration and inheritance of the transgenes

6.1. Integration of transgenes

Integration of transgenes into plant cells can be carried out either stably or transiently. In stable transformation, the process normally begins with the introduction of transgenes into the nucleus of plant cells. Stable transformation is achieved when some of the transgenes integrate successfully into the genome of the cell. These transgenes then become a part of the genome and are replicated together, enabling the next generation to inherit and express the transgene. In contrast, transient transformant expressed the transgene transiently, and the transgene is not integrated into the plant genome. In the transiently transformed plant, the copy numbers of transgene inserted remain as they are not replicated. These transgenes are expressed for a limited period of time, and the genes will be lost after several days through cell division. The way how the transgene is expressed in the cell is dependent on the transgene construct design and the method of transformation used. Currently, transient and stable transformation can be achieved through the *Agrobacterium*-mediated method [74] and biolistic method [75]. In the *Agrobacterium*-mediated method, the T-DNA region is inserted into the plant genome forming a stable transformant, whereas the non-integrated T-DNA plasmid expresses the transgene transiently. In the biolistic method or other direct gene transfer methods such as electroporation, transient and stable expression of the transgenes are usually dependent on the plasmid or transgene constructs. Virus-mediated vectors are generally nonintegrative vectors for which transient transformants are frequently produced.

6.2. Inheritance of transgenes

Inheritance of plant genetic information usually obeys the Mendelian law of inheritance in nature. Mendel's first law, the principle of segregation, states that a pair of alleles for each gene will segregate during the formation of gametes, resulting in each gamete harboring only one allele of the gene. Mendel also discovered that the genes of different traits assort independently of each other in the formation of gametes; these genes are passed down to the subsequent progeny generation according to the rules of probability. In addition, the third Mendelian's law states that one allele is dominant to the other allele, which finally determines the corresponding phenotypic attribute of the offspring. However, there are certain cases in which inheritance of a gene does not comply with Mendel's law (non-Mendelian law). These instances include incomplete dominance, codominance, gene controls by multiple alleles, and polytraits.

	Factors
Nature of recipient genome	Genetic background
	Gamete viability
	Chromosome abnormality
	Transformation method
Nature of transgene	Transgene silencing
	Unstable integration of transgene
Interactions between the recipient genome and the transgene	Homozygous lethality
	Poor transmission of transgene
	Mitotic crossover
	Meiotic instability

Source: Adapted from Yin et al. [77].

Table 4. Factors leading to non-Mendelian inheritance of transgene.

Similarly, transgene inheritance may or may not obey the Mendelian law. The rule for transgene inheritance, however, varies due to the location of transgene integration and the copy number of transgenes integrated [76]. Transgene inheritance not obeying the Mendelian law includes deletion of the transgene locus, rearrangement of the inserted transgene, and silencing of the transgene. The factors leading to non-Mendelian inheritance are listed in **Table 4** that had been reviewed by Yin et al. [77]. Overall, the pattern of transgene inheritance is usually analyzed through molecular characterization of the transgene transmission and the segregation analysis of the transgene phenotypic expression pattern.

7. Analysis and confirmation of transgene integration

Analysis and confirmation of transgene integration has to be done through an appropriate method based on the transgene constructs, selectable marker, and reporter gene used. Transgenic plant cells incorporated with antiherbicidal or antibiotic resistance genes are screened by the addition of herbicides or antibiotics to the growing media to distinguish transformed plant cells from the nontransformed plant cells. However, this method requires a large quantity of antibiotics and herbicides that are expensive and worsen by the risk of horizontal gene transfer to other bacteria. Thus, other screening methods such as polymerase chain reaction (PCR) and reporter gene expression screening are used for better accuracy as an alternative screening method for transgenic plants.

Some reporter genes such as the GFP, GUS, and Luc expression are fluorometric or colorimetric, where the expression of these genes could be observed visually or directly under microscopy [78]. Quantifications of the reporter expression are possible with the use of a spectrophotometer. The GUS expression can also be detected through histochemical assay in which the localization of the transgene can be observed. In addition, some of the reporter gene expressions such as CAT and LacZ activity are screened through enzyme assays.

Southern blotting is a molecular method used for the detection of specific DNA sequences within DNA samples. Southern blotting is generally used to identify the number of transgenes inserted into the host genome as well as for the detection of transgene integrity and transgene rearrangement [79, 80]. It is done by cutting the DNA into fragments with endonuclease restriction enzymes, separation by size through electrophoresis, and subsequently transferred onto a nitrocellulose or nylon membrane. Membranes with bound DNA will be incubated in a solution consisting labeled probes, and the pattern of hybridization is detected through autoradiography or via chromogenic detection. The transgene copy number is proportional to the number of bands observed.

The polymerase chain reaction (PCR) method is one of the most sensitive and easiest methods among all the molecular techniques employed for the verification of the transgene. The PCR is generally done with primers specific to the site of plasmid constructs and gene of interest used for development of the transgenic plants. Successful amplification of the DNA fragment with expected band indicates the possible presence of transgene, and this DNA fragment is further confirmed through DNA sequencing. A real-time PCR provides fast, sensitive, and high-throughput molecular PCR–based analysis compared to the traditional Southern blot analysis especially in the area of transgene copy number and zygosity detection in transgenic plants [81]. Real-time PCR is convenient wherein it allows for quantitative, semi-quantitative (qPCR), or qualitative (RT-qPCR) monitoring of target DNA in real time.

In recent years, the emergence of next-generation sequencing (NGS) technologies allows massive parallel generation of sequences from whole genome in a relatively short time with a lower cost. The PCR-based techniques in transgenic analysis often limits by the generation of non-specific products and failure to amplify large exogenous DNA insertion in highly repetitive genomes, multiple insertion, truncated transgene sequences and hinders precise transgene identification [82]. The availability of NGS tools and bioinformatic resources facilitate the study of genome and molecular characterization of complex traits. Besides, the analyses of NGS data allow the identification of precise genomic locations of transgene insertion especially in highly repetitive genome sequence and transposable elements which was not able to be done through the traditional PCR-based method [83]. Hence, NGS approach provides an alternative high-resolution analysis tool for transgenes insertion in GM crops [84].

8. Future directions

GM crops will be a valuable alternative in solving food security problem that happens in a world of growing human population and drastic climate change. However, transgenicity remains a major controversy in the view of biosafety issues spurred by public misconceptions and perceptions to GM plants [85]. In addition, GM crops require years of risk assessments that is time and cost consuming. On the other hand, unintended effects arise could be one of the issues in GM plant production. This is generally due to the transgene integration through illegitimate recombination in plant as the consequences of random transgene integration, gene disruptions, sequence changes, and the production of new proteins [86]. Thus, unintended effects of gene transfer in GM crops should be examined thoroughly through metabolic profiling methods to avoid production of GM plant with significant difference in chemical composition from non-GM plant grown under the same condition.

Recently, the development of engineered site-specific endonucleases such as TFN, TALEN, and CRISPR/Cas9 allows the genetic engineering of plant to be carried out more efficiently and precisely [87]. Problems such as heterozygosity that is commonly faced in agro and gene gun-mediated approaches can be avoided. Hence, the future of transgenic technology is shifting toward the engineered endonuclease genome editing technology. This endonucleases genome editing involves the introduction of a targeted double-stranded DNA breakage (DSB) in genome and consequently stimulating the cellular DNA repair mechanisms. In addition, different genome modification can be done dependent on the types of DSB repair pathways used: (1) non-homologous end joining (NHEJ) and (2) homologous recombination (HR). In NHEJ-mediated genome editing, the target cell self-edits its genome without the addition of foreign gene that may lead to mutation and gene knockout. Since this genome editing is performed without introducing a foreign gene, nontransgenic crops could be obtained. Hence, effort needs to be concerted toward improving the genome editing technology to genetic engineered crops with better agronomic traits and public acceptance.

Acknowledgements

The authors thank Science Fund (02-05-19-SF0026) from the Ministry of Science, Technology and Innovation and Malaysia and Fundamental Research Grant Scheme (FRGS/1/2014/SG05/MOSTI/1) from the Ministry of Higher Education, Malaysia for the support.

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