Plant Genetic Engineering and GM Crops: Merits and Demerits

4

Abstract

Genetic transformation in plants agreements a great potential to modify crops for improved agronomic traits including resistance to diseases, pests and good nutritional quality along with enhanced productivity. The transgene could be derived from unrelated plant species and even from non-plant sources leading to a revolution in molecular agriculture. In this chapter, the main approach lies on concept of genetic engineering techniques to improve the plant architect. The concept of GM crops and environmental implications besides their safety assessment is documented in detail and also in the end future perspective for adopting the next generation quantitative genetics is also elaborated.

Keywords

GM crops · Genetic Engineering · Bt crop · Safety assessment · Transformation

4.1 Concept of Plant Genetic Engineering

It may seem like a trivial task today to introduce genes into plants to create new commercially useful varieties. However, in the early 1980s, this was one of the major bottlenecks preventing the completion of an agricultural revolution that began after the discovery and use of restrictive enzymes, followed quickly by the genetic engineering of bacteria for medical and industrial purposes. Since its inception, plant biotechnology has been technologically driven, and the successful establishment of gene transfer technologies for major crops (McCabe et al. 1988; Christou et al. 1991) was a major breakthrough for small biotechnology companies, which led the field in the early 1980s. When it was shown that the soil bacterium Agrobacterium tumefaciens transfers part of the DNA from a resident plasmid to the plant genome, the first model of transgenic plants did not take long (Barton et al. 1983). The first key plant transformation patents on A. tumefaciens and biolistics

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defined the industry and precipitated its transformation and consolidation. While early activities in the field were dominated by start-ups in the US, such as Cetus Madison (Agracetus), Agrigenetics, Calgenetics, Advanced Genetic Systems, Molecular Genetics, and others, as well as Plant Genetic Systems in Belgium and a number of larger, more established agrochemical companies such as Monsanto, DuPont, Lilly, Zeneca, Sandoz, Pioneer, Bayer, and others, the field is now dominated. Insect resistance based on Bacillus thuringiensis (Bt) genes and herbicide tolerance were the first two features to be successfully commercialized. A good example of the broader landscape is the consolidation and turmoil in the Bt industry (Sanahuja et al. 2011).

4.1.1 Evolution of the Commercial Landscape for *Bacillus thuringiensis* (Bt) Crops

The five major companies currently selling Bt seeds have emerged through a series of mergers, acquisitions and spin-offs/demergers as larger companies segregate their agribusiness interests. In its current incarnation, Monsanto Co. was an agribusiness spin-off from Pharmacia in 2002 following the merger with Pharmacia and Upjohn in 2000 of the original Monsanto Co. (established in 1901). In late 2000, Pharmacia established the new Monsanto as an agribusiness subsidiary and in 2002 it became an independent company. Bayer CropScience is an agribusiness subsidiary of Bayer AG, which was formed after Aventis CropScience acquired in 2000. Syngenta was formed in 2000 from the merger of Novartis and AstraZeneca, both of which were spin-offs from agribusiness in previous mergers. Dow AgroSciences is a wholly-owned subsidiary of Dow Chemical Co., which was formed when Dow Chemical Co. purchased Eli Lilly's stake in Dow Elanco (an agribusiness spin-off formed in 1989 by Dow Chemical Co. and Ely Lilly & Co.). Pioneer Hi-Bred International is now DuPont's agribusiness subsidiary, which acquired 20% of the company in 1997 and the remaining 80% in 1999. (Sanahuja et al. 2011) It is interesting that commercial products were first developed and the science behind them came later. It is therefore not surprising that the two original features remain today's most dominant commercial features. Efficiency has improved and the characteristics have been stacked in individual varieties, but the technology remains the same in principle. The academic community's decision to focus on the Arabidopsis thaliana model plant paid beautiful dividends in basic science. In conjunction with advances in DNA sequencing, the genomics field has grown old and it is now considered routine to undertake major sequencing projects for various plant species. Access to major crop gene sequences can now be combined with high-performance transcriptome and proteome analysis, leading to unprecedented advances in gene discovery and functional annotation. Metabolomics and biology of systems now take center stage and generate large amounts of data to create models for the entire plant system. Advances in bioinformatics allow these large data sets to be stored, handled, mined and manipulated, leading to further progress in our understanding of fundamental and more complex plant processes. The impact of this rich stream of

previously untapped data is that targets, such as modulation, were previously considered unattractable such as the modulation of photosynthesis and the ability of plants to fix nitrogen, are now within our reach as shown by the recent substantial investments of time and resources into these areas. Multigene engineering has also helped to develop more complex crops, including extended metabolic pathways that produce valuable compounds such as b-carotene for golden rice (Ye et al. 2000) and three different vitamins for multivitamin corn (Naqvi et al. 2009). The increasingly antagonistic effect of over-zealous regulation was one surprising development that was not planned in the early days of plant biotechnology. A robust regulatory system is required for new technologies, but it should be based on rational principles and evidence rather than political expediency (Farre et al. 2011). The current regulatory environment for genetically modified crops, especially in Europe, is hostile, irrational and inconsistent with the overall effect of seriously impeding scientific progress. The early pioneers of plant genetic engineering foresaw the technology's potential and its ability to increase yields and address our most challenging social problems, such as poverty and food insecurity. While the technology has progressed steadily, the positive impact it could have throughout the world is unnecessarily wasted. My fervent hope is that the change in this situation will take another 30 years (Ramessar et al. 2010).

4.2 Plant Genetic Transformation

Genetic transformation in plants offers a great potential to modify crops for better agronomic characteristics, including disease resistance, pests and good nutritional quality, as well as increased productivity (Vain 2007). Transgenes could be derived from unrelated plant species and even from non-plant sources leading to molecular agriculture revolution. The identification of a wider range of potentially important genes for crop improvement, which are also tailored or redesigned to further improve their properties in specific crops, has subsequently intensified the development of efficient technologies for plant transformation. The combined effort of genetic engineering and conventional breeding programs has enabled the introduction of useful features into commercial crops within an economically viable time frame. In the non-agricultural sector, which includes an alternative source of medicinally important recombinant proteins and vaccines, transgenic plants are more widely used (Fischer et al. 2004). Experiments on genetic transformation of plants began shortly after the discovery of DNA as a transforming genetic material in bacteria (Avery Oswald et al. 1944). However, the development of Agrobacterium-mediated genetic transformation in plants has achieved a successful genetic transformation with reproducibility (Chilton et al. 1977). The limited success of the transformation achieved by Agrobacterium in monocotyledons and other recalcitrant plant species resulted in the discovery of direct DNA delivery methods, including the most commonly used Sanford (1990) method of particle bombardment. Genetic transformation methods can be categorized as indirect and direct DNA delivery systems in general. The indirect method involves the introduction of genes

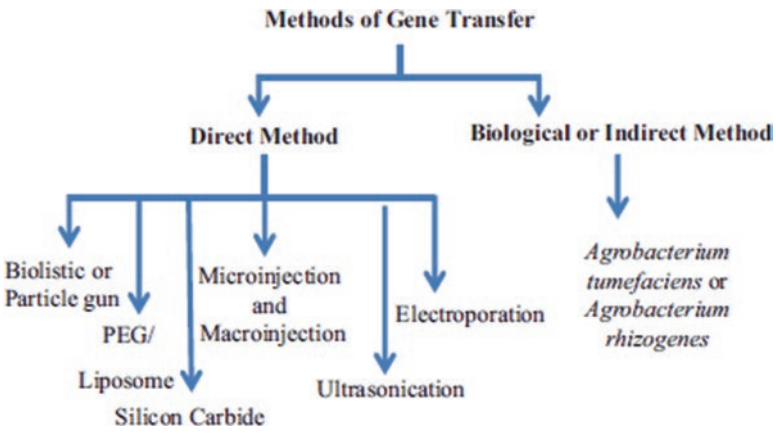

Fig. 4.1 Different methods of genetic transformation

of interest into the target cell by Agrobacterium tumefaciens or Agrobacterium rhizogenes, while no such bacterial cells are used to transfer DNA directly to the target cells (Fig. 4.1).

4.2.1 Methods of Genetic Transformation

4.2.1.1 Indirect/Biological/Agrobacterium- Mediated Genetic Transformation

This method uses the genus Agrobacterium's natural ability to transform plant cells. A. Tumefaciens is a gram-negative bacterium transmitted by oil that causes "crown gall disease," whereas it is called A. The causative agent of hairy root disease is rhizogenes. Other species are A. Rubi causing cane gall disease, A.vitis causing grape galls and avirulent species A. Radio (Otten et al. 1984). The plasmids of A inducing tumor (Ti). Tumefaciens or A plasmids that induce root (Ri). Rhizogenes are pathogenic megaplasmids. These megaplasmids have "T-DNA," also known as "transferred DNA," a region consisting of an oncogenic region, the causative agent. Oncogene is responsible for the production of enzymes involved in the synthesis of auxins and cytokinin, which leads to the development of tumours. The host range of different Agrobacterium strains is the most important factor in the Agrobacterium-mediated transformation. It has been reported that agrobacterium transfers DNA to a relatively large group of organisms, including different dicot and monocot plants (Anderson and Moore 1979) and gymnosperms (McAfee et al. 1993). The transfer of DNA to fungi mediated by Agrobacterium, including yeasts (Bundock and Hooykaas 1996), ascomycetes (Abuodeh et al. 2000) are reported and in recent times, Agrobacterium was reported to transform human cells (Kunik et al. 2001).

4.2.2 Structure of Ti Plasmid and Ri Plasmid

The size of Ti plasmids is between 200 and 800 kbp (Wood et al. 2001). Most plasmids in Ti have four common regions, in other words. (B) gene cluster required for DNA replication, (c) gene cluster required for conjugation and (d) "vir" region also known as "virulence" r (a) T-DNA region, which is so-called due to the transfer and integration of its homologous counterpart into the nuclear genome of host plant cells (this region has the potential to alter the morphology of the host plant by inducing galls (shooty or rooty mutant galls) (B) the gene cluster required for DNA replication, (c) the genecluster required for conjugation and (d) the "vir" region, also known as the "virulence" region, which consists of a gene cluster solely responsible for the encoding of a set of proteins involved in the excision, transfer and finally integration of T-DNA into the nuclear genome of host plant cells. Any mutation in this area leads to a loss of virulence. The components of these natural plasmids were therefore used as a basis for the development of vectors with a high efficiency of plant transformation. The T-region varies from 10 to 30 kbp and generally accounts for less than 10% of the Ti plasmid (Zambryski et al. 1980). Some Ti plasmids consist of only one T-region, while other plasmids have had several T-regions (Suzuki et al. 2000). The T-DNA is divided into "oncogenic" or "onc" and "os" regions and is bordered by a repeat of 25 bp on both sides. The oncogenic region consists of three genes, in other words. Tms1, tms2 and tmr represent' shooty loci with tms1 and tms2 representing' rooty locus.' These oncogenes are primarily responsible for the encoding of two phytohormone biosynthesis enzymes, i.e. Auxin (acetic acid indole) and a cytokinin (isopentyladenosine 5′-monophosphate). The inclusion of oncogenes in the host plant's nuclear genome stimulates the synthesis of phytohormones in the host plant. Phytohormones induced uncontrollable growth of host plant cells, leading to the development of tumors of the crown gall. The 'os' region consists of genes that encode enzymes necessary for the synthesis of specialized chemicals called opines metabolized by the bacteria. Opines are amino acid and sugar derivatives and provide the bacteria with carbon and energy. Ti plasmids are named after the type of opines encoded by their genes, such as oc topine, nopaline, succinamopine and leucinopine. The two most frequently produced opines are octopine and nopaline. The T-DNA contains genes for enzymes octopine synthase and nopaline synthase, which are required for the production of corresponding opines, octopine and nopaline. In addition, the T-DNA region is bordered by 25 base pairs of left (LB) and right border (RB), arranged in a directly repeated orientation (Veluthambi et al. 1988). These bordered sequences serve as a signal for the successful transmission of T-DNA to host plants (Zupan et al. 2000). The presence of polarity between the borders of T-DNA has been observed, as right borders are more important than left borders (Sen et al. 1989). Many right borders of T-DNA have shown the presence of sequences of T-DNA 'overdrive' near them, while such sequences are absent from left borders. The function of enhanced T-strand transmission to plants was attributed to overdrive sequences, but the molecular mechanism of this process is not clear (Hansen et al. 1992). However, it has been suggested that

the protein of Vir C1 binds to the overdrive sequence and may improve the cleavage of T-DNA by endonuclease VirD1/D2 (Toro et al. 1989). The virulence region of pTi is external to the T-DNA region, and genes (called "vir" genes) are grouped into ABCDEFGH operons. These operons are responsible for encoding enzymes responsible for carrying out conjugative transfer of T-DNA to host plant cellular genome. Other operons that facilitate the transfer of T-DNA are the chromosomes containing chv genes (chvA, chvB, chvF). A's smid Ri pla (pRi). The pTi is functionally homologous with rhizogenes. The pRi-like pTi consists of the T-DNA region, the vir region that is primarily responsible for transformat ion (White et al. 1982). The structural analysis of Ri plasmids of the agropine type revealed the presence of two T-DNA regions separated from each other by non-transferred 15 Kb DNA. The RB sequences fl anchored to the T-DNA (T R) contain genes homologous to the T-D NA (Tms1 and Tms2) of pTi (Willmitzer et al. 1982). The loss of virulence in the T-R region of pRi results in y mutation (White et al. 1985). T R -DNA region has been reported to contain genes involved in agropine biosynthesis (ags), but the precise number of genes required for agropine production is not yet recognized (Huffman et al. 1984). The transcripts homologous to the Ri tms loci in A. rhizogenes mediated transformed tissues of *Nicotiana glauca* were of same size as that of tms region derived transcripts of pTi (Willmitzer et al. 1983). The agropine Ri plasmid A4b has shown to possess 20 Kb T L -DNA but is related to any other characterized Ti plasmid, unlike the T R - DN A (Huffman et al. 1984).

4.2.3 Biology of Tumour Formation by Agrobacterium

The colonization and establishment of the virulence system by bacteria include various steps involved in the genetic transformation mediated by Agrobacterium. The next step is the formation of a T-DNA transfer complex, which helps to transfer T-DNA into the nuclear genome of host plant tissues and subsequently incorporate it. The entire T-DNA transfer mechanism begins with the production of phenolic compounds as a result of plant wound, which leads to a cascade of sensory signal transduction. First, the signal is received by virA, which acts together with ChvE, a monosaccharide transporter that senses the presence of a particular phenolic compound, as a periplasm antenna (Doty et al. 1996). In addition to an autophosphorylating, VirA also transphosphorylates the VirG protein, which is activated by phosphorylation, leading to the increase in levels of transcription of other genes of viral protein machinery (Jin et al. 1990a, b). The proteins VirD1 and VirD2 then nick both LB and RB sequences at the bottom of the T-DNA. In conjunction with VirD2, another T-DNA strand is coated by VirE2, which results in the formation of a T-complex, which is actually transported to the host plant genome. Different workers have demonstrated the ability of VirE2 to transfer to the plant cell in the absence of a T-strand (Vergunst et al. 2000), and it may be possible that T-strand is compounded with VirE2 protein either in the bacterial export passage or in the host plant cell. This single-stranded DNA-binding Agrobacterium protein VirE2, which protects the T-DNA from degradation when transported to the plant cell, has been

assigned a protective function. The VirD4 protein and the 11 VirB proteins form a membrane channel to support the successful transport of the T-complex to the host plant cells in which the Vir protein is linked, i.e. VirD4, makes processed T-complex is facilitated through the combined effort of dynein-like Arabidopsis protein DLC3 and VirE2 interacting protein2 (VIP1) (Tzfira et al. 2002). Another recently discovered protein of Arabidopsis, VIP2 (VirE2 interacting protein2), was also reported to be involved in the successful incorporation of T-DNA into the host plant cell's nuclear genome (Anand et al. 2007). The T-DNA is integrated in the host genome at random positions by the non-homologous recombination process.

4.2.4 Vectors Based on Ti and Ri Plasmid

The integration of the gene of interest into the T-DNA region for its transfer into the host plant involved the tedious task of genetic recombination of the gene of interest into the T-DNA region (Zambryski et al. 1983). The Agrobacterium Ti/Ri plasmid wild type cannot be used as gene cloning vectors due to its large size, presence of oncogenes and lack of unique sites of endonuclease restrictions and marker sites in T-DNA. Other problems with these plasmids include difficulty in isolating them, a low number of copies in bacteria, recalcitrant in vitro manipulation and the inability to replicate genetically transformed host within the preferred host. i.e. E. coli. The difficulty in using wild-type Ti plasmid for genetic transformation was overcomed with the development of binary and cointegrates vectors.

4.2.5 Binary Vector

The binary vector was introduced when the virulence region and the T-DNA region of pTi could be divided into different replicates (de Framond et al. 1983). The transfer of T-DNA is mediated by Vir proteins encoded in the vir region located on separate replicates, but present in the same cell of Agrobacterium. The binary vector therefore has two components: The first component is a disarmed (lack of oncogenes) Ti plasmid consisting of T-DNA, the origin (s) of E replication. Tumefaciens coli and agrobacterium, as well as antibiotic-resistant genes used to select binary vector bacteria. The second component of the binary vector is the helper Ti plasmid, which contains the viral genes that mediate the transfer of T-DNA in the other replica. To facilitate genetic manipulation studies, a large number of more sophisticated T-DNA bi nary vectors and vir helper plasmids have been developed over the last 25 years. The examples of some commonly used T-DNA binary vector series include pBINPLUS (van Engelen et al. 1995), B IBAC (Hamilton 1997), pGreen (Hellens et al. 2000), pGD (Goodin et al. 2002), pS ITE (Chakrabarty et al. 2007), pMSP (Lee et al. 2007) and many more. Moreover, some of the frequently used disarmed Agrobacterium vir helper strains are C58-Z707 (Hepburn et al. 1985), AGL-1 (Lazo et al. 1991), EHA 105 (Hood et al. 1993), NT1 (pKPSF2) (Palanichelvam et al. 2000), etc.

4.2.6 Co-integrate Vectors

These vectors are also called hybrid Ti plasmid, in which the same vector contains both T-DNA and virulence regions. For the construction of co-integrated vectors, two component vectors are required, the disarmed pTi vector and the intermediate vector. The oncogenic region of T-DNA was exchanged with the gene of interest in the disarmed Agrobacterium pTi. The two examples of these vectors include: (a) SEV series in which the RB sequences and the oncogenic region of T-DNA have been replaced by the bacterial gene resistant to antibiotic kanamycin. The LB sequences and the adjacent minor part of the left segment (T L) of novel T-DNA called as left inside homology (LIH) are left intact. (b) pGV series in which a part of pBR322 vector is used to replace oncogenic region of pTi. The conserved regions of these vectors include LB and RB sequences as well as the nopaline synthase gene of the pTi.

4.2.7 Intermediate Vectors

This consists of E. Coli plasmids (small plasmids based on pBR322) with border sequences of T-DNA and vir area. Replicate the intermediate vectors in E. Coli, but cannot replicate ate in Agrobacterium and are transmitted by conjugation to Agrobacterium. They carry DNA segments with disarmed T-DNA homology. Therefore, both the intermediate and disarmed pTi plasmids have some common sequences (pBR322), which help to recombine and integrate two plasmids in a homologous way. The newly formed cointegrate vector therefore has both the disarmed T-DNA with the desired gene and virulence area, e.g. pGV2260.

4.2.8 Direct Delivery Methods

4.2.8.1 Biolistics or Microprojectiles or Gene Gun or Particle Bombardment Method

This method was developed to achieve success in the genetic transformation of monocots or other plants recalcitrant to Agrobacterium-mediated transformation, and the technique was used in a number of plant species to produce transgenic lines (Breitler et al. 2002). The use of this gene transfer method overcomes the limitations on transgenic size, cell type, species or genotype. Approximately 149 hits between 1987 and 1995, 500 hits between 1995 and 2002, 200 hits between 2002 and 2004, 945 hits between 2005 and 2010 and nearly 1225 hits between 2011 and 2015 were recorded on the basis of the literature database available on the Web of Science for citations of microprojectile method in plants. Sanford and coworkers developed the first particle delivery method using PDS-1000/He machine. In this technique gold or tungsten particles of about 0.6–1.0 mm diameter known as microcarriers are coated with the DNA of interest followed by their acceleration at an elevated speed so as to get integrated inside the target cell.

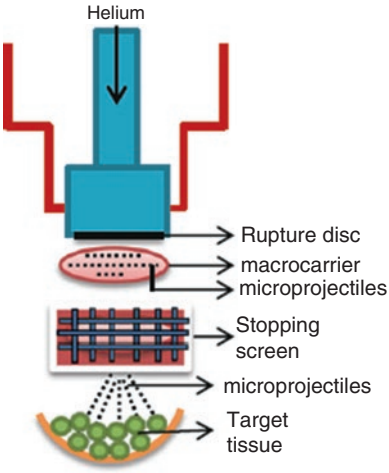

Fig. 4.2 Diagrammatic representation of particle bombardment method

These microcarriers are evenly dispersed on the macro-carrier consisting of circular plastic film, and the whole unit is then kept in the main vacuum chamber in the machine under the rupture disk. Underneath the macrocarrier is placed a wire mesh called a stop screen to retain the macrocarrier and allow the microcarrier to pass through it, as well as to place the target tissue underneath the entire system. The various types of rupture disks that burst at different pressures ranging from 450 to 2200 psi are now available. The microprojectile is fitted red under a partial vacuum and the gas acceleration tube is fitted with helium gas (He), which increases the necessary pressure. Macrocarriers are retained by the wire mesh during this process while microcarriers pass through it and hit the target cell at high speed. Microcarriers penetrate through the cell wall into the host cell and release DNA leading to the formation of transformed cells (Fig. 4.2). The advantages of this method include the absence of biological constraints, the ability to deliver DNA to different types of cells, the absence of vector requirements, the simultaneous transformation of multiple genes and the presence of high molecular weight DNA that can be delivered to the target cells. In addition, only particle bombardment technique has achieved mitochondrial transformation until now (Johnston et al. 1988). The transgenic rice lines having Xa21 gene which shows resistant against bacterial blight disease as well as Bt cry1Ab–cry1Ac fusion gene for lepidopteran insect resistance have been produced by gene gun method at IRRI. These resistant Bt lines have been tested in field in China (Tu et al. 2000a, b) as well as in India (Datta et al. 2002).

4.2.9 Electroporation -Mediated Genetic Transformation

In this method, the target cells and tissues are applied with a short electrical pulse of high field strength, which causes certain types of structural changes in the host cell membrane, thus increasing the permeability of the cell membrane. The most used

use of the electroporation technique is now the introduction of DNA in vitro into the target cells. This method was developed earlier to transform protoplasts, but this method could also transform intact plant cells. In this method, protoplasts or intact cells in an ionic solution containing vector DNA are suspended between the electrodes. In a specially designed electroporation chamber, which alters the permeability of the cell membrane allows the absorption of suspended vector DNA from its surrounding solution, 25 mV voltages and 0.5 mA current are generally applied for a period of about 15 min. The surface concentration of DNA and the tolerance of cells to membrane permeation affect the efficiency of the electroporation. However, higher transformation rates could be achieved with the electroporation method by adding polyethylene glycol after the addition of DNA, giving a heat shock to protoplasts at 45 °C for only 5 min before the addition of DNA and using linear DNA instead of a circular form. There is a lot of monocot and dicot plants have been successfully transformed with protoplasts. The first successful fertile transgenic plant of rice was developed by the use of embryogenic protoplasts through this method (Shimamoto et al. 1989). One of the limitations of this method of delivery is the use of protoplasts in most cases and the absence of regeneration of protoplasts in plantlets in most plant species. However, transformed intact plant cells and tissues could also be obtained using the same electroporation principles as those required for protoplasts, and the first transgenic plants were produced in barley in this regard (Salmenkallio-Marttila et al. 1995). The gene was also transferred to intact sugarcane meristem tissue by electroporation (Seema et al. 2001). The thick cell walls of intact tissues are generally the key barrier in the electroporation method. This method is cheap and easy as compared to gene gun method but has lower transformation efficiency with success only in a few plant species.

4.2.10 PEG/Liposome- Mediated Genetic Transformation

By using chemical compounds such as polyethylene glycol (PEG), direct DNA delivery to target protoplasts can also be stimulated. The desired DNA and protoplast are mixed in this method, and the addition of polyethylene glycol facilitates the absorption of DNA by the protoplast. Higher PEG concentrations, i.e. 15–25%, precipitate DNA and stimulate endocytosis without damaging protoplasts. The transformed protoplast is then selected to produce transformed plantlets and regenerated. This method is very simple because no specialized machinery is required, but this technique achieves a lower transformation frequency due to the inability of protoplast to regenerate into whole plants. The transgenic maize and barley plants have been produced by this method (Daveya et al. 2005). Liposomes are referred to in small spherical lipid bags that contain a large number of plasmids and are formed by phospholipid hydration. The desired DNA is introduced into the target protoplast by the protoplasts through the endocytosis of liposome-containing DNA. In general, PEG helps induce liposome fusion with protoplasts. The positive charge of the liposome is attracted to the DNA and cell membrane, which are both negatively charged entities (Gad et al. 1990). The process begins with the adhesion of liposomes on the

protoplast surface with the subsequent merging of liposomes with protoplast at the site of their union and finally the discharge of plasmids into the protoplast. The positive charge of the liposome is attracted to the DNA and cell membrane, both of which are negatively charged entities (Gad et al. 1990). The process begins with the adhesion of liposomes on the protoplast surface with the subsequent merging of liposomes with protoplast at the site of their union and the discharge of plasmids to the target cell. The method of lipofection-PEG was used to transform intact YACs into tob accounts (Wordragen et al. 1997). The advantages of this technique are the protection against the digestion of nucleic acids by nucleases, lower levels of cell toxicity, stability of nucleic acids due to liposome encapsulation and wide range of applications for all cell types. Nonetheless, this method is very tedious and has very low transformation efficiency, as there are very few fruitful reports on the applicability of this procedure in plant transformation.

4.2.11 Microinjection/Macroinjection

Microinjection involves the direct as well as accurate DNA delivery inside the cells, protoplast or nucleus through glass microcapillary injection pipette of 0.5–1.0 μm diameter (Crossway et al. 1986). In this technique, the target cells are immobilised under the microscope and agar with low melting point is positioned under the microscope, and two micromanipulators, one holding the micropipette and the other holding a microcapillary needle, are used to penetrate the small amounts of desired DNA solution inside the cell membrane or nuclear membrane. This method is generally exploited to transform meristem, immature embryos and pollen, excise ovules and suspended embryogenic cells. The process is very time consuming and tedious, and expensive micromanipulator device along with highly skilled and experienced personnel are required. In addition, the transformation efficiency of microinjection technique is ten times lower than that of biolistics. Despite certain disadvantages, the precise nature of the delivery of this technique proved to be extremely effective, and genetic transformation of bacco (Crossway et al. 1986), petunia (Griesbach 1987), rape seed (Neuhaus et al. 1987), soya bean (Chee et al. 1989) was achieved using this method. In addition to inserting plasmids, this technique can also be used to introduce an intact chromosome into the plant cell genome (Griesbach 1987). Hypodermic needles with a diameter greater than the cell diameter are used to transfer DNA to the target cells in the macroinjection technique. This method is generally applied by conventional syringe to cereal plants in which DNA is injected into the section of the plant developing floral tillers. The area above the plant tiller node is injected with 0.3 ml of DNA solution until many droplets of solution emerge from the top of the young inflorescence (Jogdand 2006). It is important that the time of injection of DNA is 14 days before meiosis. The formation of chimeric plants is the main disadvantage of this technique, which transforms only part of the plant. But transformed plants from single cells could be subsequently produced from this chimeric plant. This procedure has also been used to transform other plant species, for example, rye (de la Peña et al. 1987), cotton (Zhou et al. 1983), rice (Xie et al. 1990), watermelon (Chen et al. 1998) and soy bean (Hu and Wang 1999).

4.2.12 Silicon Carbide (SiC) Method

In order to deliver DNA to maize and tobacco plants, the silicon carbide (SiC) method was first used (Kaeppler et al. 1990). SiC whiskers are able to puncture cells due to their physical and chemical characteristics without damaging the target cells. In this method, small needle-type SiC whiskers are mixed with plasmid DNA, which has a gene of interest, together with the suspension of callus /cell clusters/immature embryos. These contents are then mixed with the help of shaker or vortex (Kaeppler et al. 1992). The SiC whiskers pierce the cells and create small cell membrane holes through which DNA-coated fibers enter the target cells (Kaeppler et al. 1990).

The size of the fiber, the time required for vortexing, the type and speed of vortexing, the shape of the vessels used and the cellular characteristics of the host plant, such as the thickness of the cell wall, are the various parameters governing the efficiency of this technique (Mizuno et al. 2004). The elongated fiber with a length of 10–80 mm and a diameter of 0.6 mm is most frequently used in this method. The negative charge of SiC fibers and DNA molecules at neutral pH (Appel et al. 1988) leads to a minor rejection of plasmid DNA and SiC fibre. The transformation efficiency of earlier shaking of fibers with DNA has been shown to not increase on earlier shaking of fibres with a DNA suspension (Yamagishi et al. 2007). Therefore, it could be concluded that the fibers are not involved in the transport of DNA within the cells; instead, their perforation and abrasion mechanisms facilitate the transfer of DNA (Wang et al. 1995). Carborundum, silicon nitrate and glass with similar properties of silicon carbide fiber can also introduce DNA into plant cells; however, their transformation efficiency is lower. The SCMT is considered a simple and easy way to carry out transformations on a larger scale, as no sophisticated machinery or other costly resources or qualified engineers are required. The SCMT technique allows the stable transformation of various plants, including maize, rice (Takahashi et al. 2000), wheat (Sawahel and Saker 1997), tobacco (Kaeppler et al. 1990), etc. In addition, silicone carbide fibers have been reported to increase the efficiency of the Agrobacterium-mediated method of transformation (Singh and Chawla 1999). The disadvantage of this technique is its low transformation efficiency and the cell damage also reduces its impact. Furthermore, SiC fibres can produce extreme respiratory hazard, so the laboratory staff should take precautions to avoid inhaling fibres (Svensson et al. 1997). Recently, with the help of SiC fibers, the rate of callus transformation in rice is increased by 30–50% (Nagatani et al. 1997). The mesoporous silica nanoparticles formed after the reaction of tetraethyl orthosilicate with a micellar rod template (Nandiyanto et al. 2009) were also used for the transfer of DNA and other compounds within the cellular genome of the plant and whole leaves (Torney et al. 2007).

4.2.13 Ultrasonication-Mediated Transformation

The incorporation of exogenous DNA into the interior of target cells also known as sonication by ultrasound (high frequency sound above 20 kHz) is one of the other

potential techniques of genetic transformation. It has been reported earlier that ultrasound can change the transient permeability of cell membranes (Tachibana et al. 1999), allowing large molecules such as DNA to enter cells (Wyber et al. 1997). The breakdown of cell membranes can be induced to medium-frequency sounds such as clinical shock waves and ultrasounds with frequency in MHz by acoustic cavitations bubbles generated from sounds with a lower frequency, i.e. in kHz (Miller et al. 2002). Ultrasonic waves with a frequency exceeding 20 kHz propagate in aqueous media as longitudinal pressure waves. Acoustic cavitations are the phenomenon in which rapid pressure change leads to the development of microscopic gas bubbles with their subsequent collapse (Frizzel 1988). The first possible mechanism of acoustic cavitation-induced absorption of DNA may be the generation of high pressure and temperature shock waves resulting from the violent collapse of cavitation bubbles, which leads to plasmalemma rupture and subsequent absorption of exogenous DNA, followed by the restoration of membrane integrity. The second hypothetical mechanism is the electromechanical cal model (Zimmermann et al. 1974), which states that there is a critical hydrostatic pressure at which the intrinsic membrane potential is sufficiently large to induce mechanical disruption of the plasma membrane. The collapse of microbubbles carrying DNA leads to the release of DNA trapped in microbubbles or layered into plant cells on the surface of microbubbles (Unger et al. 2001). The cavitation method is more effective for lower plants, which do not carry flowers such as mosses, lichens and algae, in which the ducts and fibres are absent, since cavitation is governed by gas bodies. The explants are suspended in a sonic medium (few mm) in a microcentrifugal tube, followed by the addition of plasmid DNA (perhaps carrier DNA). The above sample is also used after rapid mixing for sonication. The cavitation phenomenon is not only influenced by the exposure time, strength and main frequency, but also by the application type, such as continuous or pulsed, the pulse rate and the duty cycle (Santarem et al. 1998). The stable transformation in tobacco was reported for 30 min by sonicating leaf tissue of approximately 4–8 mm at 0.5 W/cm^2 (Zhang et al. 1991). The intensity used to sonicate leaf tissue was approximately similar to the intensity used to sonicate protoplasts, but the exposure time increased to 1500–2000 times. This technique is mainly used in tissues in conjunction with the biological method of transformation of plant cells or tissues (Weber et al. 2003), i.e. sonic agrobacterium-mediated transformation (SAAT). The target tissues are exposed to short ultrasound periods in the presence of Agrobacterium in this technique, thereby improving the transformation efficiency by hosting a great number of micro-wounds into the host plant cells or tissues (Subramanyam et al. 2011).

4.2.14 Gene Expression in Transgenic Plants

The transformed cells are selected using a marker gene (scorable and selectable marker), which may be linked to the gene of interest (as part of the cassette) or

unlinked, as in the case of co-transformation. The gene products of selectable markers (herbicide or antibiotic resistance, antimetabolite marker) and scorable markers (luciferase, GUS, GFP, acetyltransferase chloramphenicol, anthocyanin) should not induce variation or affect the performance of the plant. In addition, molecular analysis is carried out to confirm the transgenic status of regenerants in which the transformed status is indicated by the PCR amplification of the marker gene or transgen indicates the transformed status. Further the successful incorporation of desired foreign gene into the genome of target plant is confirmed by Southern hybridisation, which is also helpful in revealing the number of independent insertions of introduced genes (Potrykus 1991). In order to assess the expression of the introduced gene, other techniques such as RT-PCR and northern and western hybridization are used. The functionality of the transgenic product can also be evaluated in the bioassays available. In primary transgenics, however, somaclonal and transgenic effects are confused, so progeny analysis is recommended. The presence of a single copy of the transgene, which is separated as a Mendelian trait and expressed uniformly from one generation to the next, is the characteristic of perfect transformants. The production of ideal transformants is a difficult task, whose success is to some extent governed by the transformable plant material as well as nature and transgenic complexity. In addition, variability from one transgenic plant to another is often observed due to the random integration of genes into the genome phenomenon known as' position effect variation (Vaucheret et al. 1998).). Sometimes high levels of introduced gene expression have been observed, as the introduced gene is close to an enhancer element. The transgenes lodged in the subtelomeric region could have a positive effect on the position, since it is known that these regions are highly expressed (Topping et al. 1991). The sufficient production of transgenic plants and finding out some transgenics with the desired level of expression could overcome this problem. Another problem that is mainly caused by increased DNA methylation or homology-dependent gene silencing is partial or complete inactiveation of transgenes often referred to as gene silencing is another problem, which is mainly caused by increased DNA methylation or homology-dependent gene silencing or transgene suppression by its antisense counterpart or RNA interference. Efforts are made to achieve stable expression and inheritance of transgenes, thereby eliminating the random integration of transgenes. Scaffold attachment regions could achieve this, which could protect the transgene from the influence of its surroundings. Information on gene expression control elements may come from genome sequencing. The ability to target integration could also lead to transgenic expression control (Puchta 1998). Site-specific recombinases are expected to help in this effort (Ow 1996). In order to produce selectable markers free transgenic plants, the cotransformation strategy in which markers and genes of interest are placed on two separate T-DNAs in a single plasmid or on separate plasmids in one or more agrostrains could be used. The selectable marker is segregated from the gene of interest in the next generation. The other method includes the removal of marker genes by transposases in

which either the marker gene is placed on a mobile element lost after transposition, or the mobile transgene is transferred to a new chromosomal position.

4.2.15 Engineering Plants for Useful Agronomic Traits

The genetic transformation approach has provided an important platform for increasing the efficiency of the crop production system, firstly by producing transgenic plants with useful phenotypes, which could not be achieved by conventional plant breeding, and secondly by correcting any shortcomings of cultivars more effectively than conventional breeding, or by allowing the capture of commercial valves. Production of "transgenic crops of the first generation," i.e. commercially improved herbicide, insect cultivars, viruses or postharvest deterioration resistance foreign genes, as well as the accumulation of modified and highly useful storage products have resulted in meeting one of the expectations (Shah et al. 1995).). Plants are modified to increase resistance to biotic stresses such as insect, viral, fungal and bacterial diseases, which have caused severe losses in crop yields. Tobacco (Vaeck et al. 1987) and tomato (Fischhoff et al. 1987) first reported resistance to insects. Several strategies for insect control have been proposed, the most effective of which is Bt. The first feature introduced in crop plants was resistance to virus infection. The most important molecular strategy for increased virus resistance in plants using a transgenic approach includes cross-protein coat protection, which was first shown in transgenic tobacco mosaic virus (TMV) coat protein showing resistance to TMV (Powell Abel et al. 1986). Genes that encode antimicrobial protein have now been identified and cloned to help respond to plant defence. These antimicrobial proteins include hydrolytic enzymes (chitinase, glucanase and other proteins related to pathogenesis (PR), proteins that inactivate ribosomes (RIP), antifungal proteins, biosynthetic enzymes for the production of antimicrobial phytoalexins, etc. Abiotic stresses (drought, low temperature, salinity, and alkalinity) have caused severe losses in crop productivity (10–20%), which has become a major challenge, especially in developing countries. Genetic transformation approaches to abiotic stress resistance include improving or reducing stress protection. For the production of transgenic plants resistant to stress, genes encoding enzymes for the production of osmoprotectants, late embryogenesis abundant proteins (LEA), antifreeze proteins, chaperons and detoxification proteins, as well as proteins involved in the transcription of stress-responding genes were identified and used. Increasing photosynthetic efficiency is another important application of genetic transformation. The intact phosphoenolpyruvate kinase enzyme from maize has been transferred to C3 rice plants, and the phosphoenolpyruvate kinase enzyme activity in transgenic rice plants has increased two to three times compared to maize (Ku et al. 1999). Most recently the researches are undertaken to increase the development strategies for molecular stacking of many desired traits in a single transgene locus. Potato line containing seven transgenes developed by Monsanto Company (APHIS Application 98-069-23 N) is an interesting example in this respect. Amongst seven genes, one is Colorado potato beetle resistant cry gene (cry IIIA Bt); other three are selectable

markers, viz. npt II, gus and CBI. Another CBI gene serves to provide resistance against Verticillium results in changed metabolic carbohydrate pathway as well as improved resistance in bruising. Virus coat protein gene and replicase gene are the remaining two genes, which provide resistance against two viral diseases. However, the production of efficient lines through transformation technology, having required phenotype without any unwanted side effects, governs the extent of meeting the other commercial or practical expectations of plant transformation.

4.2.16 Implications of Plant Genetic Transformation

Specific cultivation conditions are required for each crop species to be transformed. However, in the last two decades, numerous methods for gene transfer to target cells have been developed for various plants, among which Agrobacterium and particle bombardment are now standard laboratory techniques that have been sufficiently used to transform essentially any plant species. Despite this progress, the use of this technology is limited by the recalcitrant nature of many economically important crops and tree species. However, efforts are being made to efficiently integrate foreign genes to produce stable transgenics using both agrobacterial and biolistic transformation and improved tissue regeneration. In order to produce genetically transformed plants with desired characteristics, new techniques are still being developed (Veena 2008). The lower frequency of transformation combined with the high frequency of undesirable genetic change and unpredictable transgenic expressions constitute two major limitations in the practical transformation of many plant species. These problems require costly transformation and screening programs on a large scale to produce useful transformants. Another problem that needs to be addressed is the presence of different selectable marker genes along with the gene of interest, which requires the future development of marker-free transgenic plants. More progress in genomics, cloning technology and vector design is therefore needed in the future to eliminate the need for a selectable bacterial marker gene. A clearer understanding of the various events that occur during gene transfer through Agrobacterium is also required. The different questions raised in the use of Agrobacterium-mediated transformation include whether transient expression is a satisfactory test for Agrobacterium-mediated transformation, or whether another convenient test for the rapid detection and optimization of this key event is necessary. Do cell types influence Agrobacterium-mediated transformation and, if so, what are the key characteristics of gene transfer determination in these favored cells? Can these features be imparted to cell types that are highly regenerable? Stable transportation is observed when the naked DNA is transferred into many actively dividing and regenerable cells using direct gene transfer experiments offers a unique advantage of gene stacking with the production of multivalent vaccines in a single transformation step. Also, there is no concern about gene silencing in plastid transformation. Zinc finger nuclease technology (ZFN) is another promising technique that can be used in basic and applied agricultural biotechnology. The gene functions in plants could also be determined by ZFN-assisted gene targeting and

chromatin re-modeling studies. Today, however, different resources are used to develop zinc finger nuclease technology in various plant species. The introduction of mini-chromosome technology provides a solution to gene stacking technology in which large DNA sequences containing multiple genes could be integrated into the targeted genome of plants leading to genetic engineering advances. Hence, there is a hope that the GM plants would provide a solution to meet the world's demands for food, feed, fibre and fuel by the production of improved crop species with minimal genomic modifications (Chapotin and Wolt 2007).

4.3 GM Crops: History and Scope

Recombinant DNA technology is the combination of DNA molecules from two different species to produce new genetic combinations in a host organism. In medicine, agriculture and industry, the recombinant organisms have value and promise. Recombinant DNA technology allows a specific protein to be isolated from a segment of DNA or gene. The nucleotide sequence can be determined with this fragment, the transcripts can mutate the sequence in very specific ways if necessary and the modified sequence can be reinserted into a living organism. This technology has significantly benefited both agriculture and medicine (Slater et al. 2008). It should be noted that the timeline of plant science and the improvement of our crops is one more step. Genetically modified organisms or GMOs are currently referred to as crops or foods modified by modern genetic technology. GMOs have simply altered their genetic composition, so they can code for a new property. To turn it on, the gene needs a mechanism. This is called the promoter segment on the switch. One of the most commonly used promoters is 35S. When a new GMO with a new feature has been developed, the resulting gene construct is called an event with regular events. Before approval for use, these events are subject to various regulatory and security reviews. One area that unfortunately is growing is the development of unapproved events (James 2006; James 2015). Genetically modified (GM) foods were first approved for human consumption in the United States in 1994, and approximately 90% of maize, cotton and soybeans planted in the United States were GM by 2014–2015. GM crops covered more than ten million km^2 (3.86 million square miles) of land in 29 countries around the world by the end of 2010. Most GM crops in the Americas have been grown. The technique was applied to soybeans in the agricultural arena in 1988, paving the way for one of the most successful crops tolerant to glyphosate soy (FAO 2015). Although this development was of significant importance for commercial agriculture, very few consumers were aware of it. The introduction of tomato "Flavr Savr" in 1994 was probably the first GMO crop many saw. In the 1980s there was anecdotal information that the enzyme polygalacturonidase was a key since it dissolved cell wall pectin. A group from Celgene suggested the development of an antisense gene to limit this enzyme. The researchers hoped this would delay maturation and allow it to stay firm longer. Calgene identified and cloned the tomato fruit pg gene in 1987 and submitted a petition to the FDA in 1992 and in 1994 approved the addition of a kanamycin resistance gene

for the creation of PG-antisense tomato. Work continued and tomatoes from Flavr-Savr were introduced at the end of 1994. Demand was high and remained high, but also high production costs and the product was not profitable. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become the focus of commercial agriculture. There is currently a significant amount of food grown with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMOs and nearly 90 percent of soybeans comprise a significant percentage. GMO crops have different characteristics such as two of the most common crops with their associated characteristics. Demand was high and remained high, but the cost of production was also high and the product was not profitable. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become the focus of commercial agriculture. There is currently a significant amount of food grown with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMOs and nearly 90% of soybeans comprise a significant percentage. GMO crops have different characteristics such as two of the most common crops with their associated characteristics. Roundup ready to contain soybeans contains proteins that interfere most with the EPSPS pathway. Round Up known as glyphosate is a general-purpose pesticide used not only in agriculture but also in homes to eliminate weeds. Although it is good to eliminate weeds, healthy crops such as flowers, crops and ornamentals are also eliminated. In the case of Roundup Ready Soy, the GMO feature enables the farmer to use Round Up to remove weeds without killing soy. In addition, a farmer can be more productive if tedious weeding is eliminated. The second example is that BT maize has been encoded with a gene that eliminates the maize borer, which allows more maize per acre. The production of 170 million hectares, including 312 events in 29 species with 3497 approvals in 59 countries, was based on data from the end of 2012.

The use of Agrobacterium as a vector to insert the new DNA into a plant was one of the earlier techniques used to insert genes into plants. Agrobacterium tumefaciens in plants causes a disease known as a disease of the crown gall. A tumor-like growth or gall in the infected plant characterizes Crowngall. The transfer of a segment of DNA from the bacterial tumor-inducing plasmid initiates tumors. The plasmid T-DNA is semi-randomly integrated into the host cell genome in which the tumor morphology genes are expressed on the T-DNA, causing gall formation (Francis and Spiker 2004). In biotechnology, in particular genetic engineering for plant improvement, the ability of Agrobacterium to transfer genes to plants and fungi is used. A modified plasmid of Ti or Ri can be used. The plasmid is “disarmed” by deleting the genes that induce tumors; the only essential parts of the T-DNA are its two small border repeats (25 base pairs), at least one of which is necessary for plant transformation. Marc Van Montagu and Jozef Schell at the University of Ghent (Belgium) discovered the mechanism for gene transfer between Agrobacterium and plants, which led to the development of methods for the transformation of Agrobacterium into an efficient system for gene engineering in plants

(Schell and Van Montagu 1977; Joos et al. 1983). This work laid the basis for the insertion of specific genes into a plant using Agrobacterium. One can also argue that the gene transfer has been going on for a very long time and we have learned to use it effectively for specific crop improvements. The genes to be introduced into the plant are cloned together with a selectable marker into a plant transformation vector containing the bacterial plasmid T-DNA region. In conjunction with the other desired genes, an antibiotic marker gene was often incorporated into the plasmid to allow the selection of successfully transformed plants. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die. Transformation with Agrobacterium can be accomplished by incubating either protoplasts or leaf discs with the Agrobacterium to cause the plasmid insertion. From the callus that results, whole plants regenerated using plant tissue culture. Agrobacterium does not infect all plant species, but other plant transformation techniques, one of which is the gene gun, have been used. A genetic weapon is a biolistic particle delivery system that was originally designed to transform plants by injecting genetic material into cells. The plasmid DNA is coated on heavy metal elementary particles. The genetic weapon can transform almost any cell type, including plants, and is not limited to the nucleus' genetic material: It can also transform organelles, including plastids. Gene insertions intended to transform prokaryotic genomes generally have an interesting gene or genes, at least one sequence of promoters and terminators and a reporter which is a gene used to ease detection or removal of those cells which didn't integrate the construct into their DNA. These genes may each have their own promoter and terminator, or they may be grouped together to produce multiple gene products from a single transcript, in which case binding sites for translation machinery should be placed between them in order to ensure maximum translation efficiency. In any case, regions called border sequences, which are similar in sequence to locations within the genome, flank the entire construct; this allows the construct to target a specific point in the existing genome (Slater et al. 2008). A gene gun often targets a callus of undifferentiated plant cells growing in a Petri dish on a gel medium. The gel and callus are largely after the gold particles have affected the dish the gel and callus are largely disrupted. However, some cells are not killed in the impact, and have incorporated enveloped a DNA coated gold particle, which eventually migrates to and integrates into a plant chromosome. The term "genetic modification" and "genetically modified organisms" is often misused. All types of agriculture (organic, conventional) modify plant genes so that they have desirable characteristics. Traditional breeding forms indirectly change the genetics of the plant by selecting plants with specific characteristics, while genetic engineering changes the characteristics by directly modifying the DNA. Crosses are made relatively uncontrolled in traditional breeding. The breeder selects the parents to cross in conventional plant breeding, the results are unpredictable because the parents 'DNA recombines randomly. Genetic engineering, by contrast, enables highly precise gene transfer, rapid and efficient gene tracking in new varieties. This ultimately leads to increased efficiency in the development of new and desirable crop varieties (Popping 2010).

4.3.1 Scope

The first GMO crop to be introduced to the market was the introduction of "Flavr Savr" tomato in 1994. Anecdotal information was available in the 1980s that the enzyme polygalacturonidase (PG) was key to softening tomato fruit because it dissolved pectin in the cell wall. By developing an antisense gene, Calgene proposed to limit this enzyme. The goal was to delay the maturation so that the tomatoes could stay firm longer. Calegene identified and cloned the PG gene for tomato fruits in 1987 and submitted a petition to the FDA in 1992. In 1994, the FDA approved the addition of a gene construct for kanamycin resistance to the creation of tomato PG-antisense (FAO 2015). Work continued and the Flavr-S continued in late 1994 was introduced. Although it may have been a technological success, it has been a commercial failure and has not done anything for the cause of biotechnology in general, the use of biotechnology and transgenic food has become a major issue in agriculture. There is currently a significant amount of food produced with approx. DNA recombinant technology. Eighty five percent of maize grown in the US is GMO and nearly 90% of soybeans. GMO crops have different characteristics. There are examples of two of the most common crops with their associated characteristics. Roundup Ready Soybeans contain a protein that interferes most with the EPSPS pathway. Round Up, known as glyphosate, is a pesticide used to eliminate weeds not only in agriculture, but also in homes. While it is good to remove weeds, healthy crops such as flowers, crops and ornamentals are also eliminated. In the case of Roundup Ready Soy, the GMO feature allows the farmer to use Round Up to remove weeds without killing soy. A farmer can also be more productive in eliminating tedious weeding (James 2015). The second example is that BT maize has been encoded with a gene that eliminates the maize borer, which allows more maize per acre. The production of 170 million hectares, including 312 events in 29 species with 3497 approvals in 59 countries (NAS 2016), was based on data from the end of 2012. Approximately 12 genetically modified crops were used in 2015 (FAO 2015; James 2015). Nine food crops, three non-food crops and two types of flowers were commercially available for production in 2015. Maize and soybean were the genetically modified crops most widely grown. Since its first commercial release in 1996, the production of genetically modified maize has increased substantially to 53.7 million hectares by 2015. Genetically modified soybean rapidly increased from its introduction in 1996 to more than 92 million hectares in 2015 (James 2015). The seven other food crops of which GE varieties were grown in 2015 were apple (*Malus domestica*), canola (Brassica napus), sugar beet (Beta vulgaris), papaya (Carica papaya), potato, squash (Cucurbita pepo), and eggplant (*Solanum melongena*) (James 2015). The contribution of GE varieties to the production of those crops was small, except for canola; GE varieties of canola constituted 24% of the 36 million hectares planted in 2015 (James 2015) rd of all land planted to maize worldwide that year (James 2006, 2015). Herbicide resistance, insect resistance and virus resistance are the most economically important crop changes to date. Herbicide resistance introduces a crop's ability to resist the use of certain weed control herbicides. For nine different herbicides, herbicide-resistant traits have been developed and

introduced into eight herbicide-resistant traits for soybeans, six for cotton, three for canola, three for maize, two for sugar beet and one for alfalfa. Some varieties of crops with stacked resistance to two herbicides (e.g. glyphosate and 2,4-D or glyphosate and dicamba). Glyphosate has been introduced for soybeans since 1996 while as glyphosate resistance has been introduced in alfalfa, cotton, canola, maize, and sugar beet by 2015 (FAO 2015).

Insect-resistant (IR) characteristics include insecticidal properties produced internally by a plant. An example of insect resistance is the transfer of gene coding from the soil bacterium Bacillus thermogenesis for a crystalline (Cry) protein. When the insect feeds the plant, the Cry is toxic to the target insect. Cry proteins can control many insect pests-moths, beetles and flies in particular (Höfte and Whiteley 1989). Cotton, eggplant, maize, poplar and soybean insect-resistant varieties were commercially produced in 2015 (NAS 2016). The resistance of the virus prevents the susceptibility of a plant to specific viral diseases. The resistance of the virus in crops targets the targeted virus 'coat-protein gene. The transgene prevents the virus from successfully replicating in the host plant. In 1998, commercially grown varieties of papaya resistant to viruses were first introduced in Hawaii. In the late 1990s NAS, 2016, virus-resistant squash was also marketed in the United States.

4.3.2 Testing

The ability to determine whether a crop has been genetically modified is important because consumers and regulators need this information. On selected commodities, there are two basic types of testing: Protein and DNA. The new gene is sandwiched between two segments in the development of the gene sequence for a crop, a promoter and a terminator. There are a number of promoter and terminator segments that are easily identified from a new source. 34S and 35S, which come from the Cauliflower Mosaic Virus (CaMV), and the Figwort Mosaic Virus (FMV), are two of the most common promoter segments. Nopaline Synthase is a relatively common terminator marker. There are two approaches to testing GM content. In the first approach, an ELISA or immunochromatography method can be used to test the expressed protein. For decades, ELISA tests have been used for a large number of compounds. While these are useful, the number of possible proteins to be tested is limited and the levels of proteins are very low. A second approach is to test fragments such as 34S, 35S and NOS using PCR or RT-PCR using several commercial test protocols with kits for testing the specific insert (Slater et al. 2008). Samples must obviously be extracted and prepared for analysis using one of several available techniques before any of these techniques. In qualitative PCR, the DNA polymerase specificity is used to amplify target sequences. Two pairs of primers are used in standard PCD with one being a sequence of senses and the other antisense. These sequences are multiplied by approximately a million times. These segments can be separated by electrophoresis of agarose gel after amplification, but other techniques such as HPLC have been used. The alternative approach to qualitative PCR is quantitative real-time PCR, in which fragment separation is performed automatically.

Should an organization not choose to perform testing, there are several contract labs that can perform this assay (Ahmed 2002). Although the various technologies involved in GMO testing are of interest, a new phenomenon has emerged in recent years, which is GMO verification services, the most visible of which is the non-GMO verification project.

4.4 Environmental Implications of GM Crops

The debate on the impact of GM crops on the environment has focused on questions such as: What are the potential environmental risks of GM crops? If we market genetically modified crops, how far will it have unwanted effects on non-target species? First, toxicity produced by chemicals used in GM crops is a major challenge for both the environment and hereditary plants (De Schrijver et al. 2015). Second, such crops may be toxic to non-target species, in particular to "friendly" species such as beetles, bees and butterflies (Yu et al. 2011). Generally speaking, the effect of subsistence, organic or intensive agriculture on the environment is evident, which demonstrates strongly that GM crops must have implications on the environment. The International Council for Science (ICSU), the GM Science Review Panel and the Nuffield Council on Bioethics (www.nuffieldbioethics.org), among many environmental protection platforms, approve that GM crops have a positive or negative impact on the environment, depending on how and where they are used. The role of genetic engineering is plausible in more sustainable crop production and conservation of natural resources, including biodiversity. Its role in accelerating the harmful effects of agriculture cannot be avoided, however. The issue of basic environmental impacts in relation to the release of transgenic commercial crops is particularly relevant (Domingo 2011). Direct impacts include gene transfer, non-target species trait effects and wildlife, invasiveness, weediness and genetic recombination of free DNA in the environment. By contrast, indirect effects include harmful and adverse effects of chemical control, i.e. reduced efficiency of pest control, disease and weed control, effects on water and soil, and global biodiversity decline (Tutelyan et al. 2010). The most debatable environmental implications are discussed below.

4.4.1 Direct Impact of Transgenes on Environment

4.4.1.1 Gene Flow

Gene flow is considered to be a major evolutionary force, leading to changes in gene frequencies, mutation, genetic drift and selection (Lu and Yang 2009). Gene flow can affect the environment by reducing population differentiation and increasing the diversity of people in a population (Mertens 2008). One of the effects of gene flow is also the structure of genetic diversity (Gepts and Papa 2003). The introduction of non-native GMOs into ecosystems poses potential long-term risks to the environment and its consequences are quite difficult to predict. Scientists from different streams around the world are concerned with the possibility of transferring

transgenic sequences to related wild species or weeds through horizontal gene transfer (HGT) or hybridization. There is no doubt that the environmental effects of gene flow are variable, but some of the effects of gene flow could be generalized on the basis of general findings in many cases, such as the development of superweeds, the development of new viral pathogens, the instability of transgenes in the environment, the creation of GD, the development of pests and pathogens with resistance to new compounds (Egan et al. 2011). At the same time, it is also necessary to address the secondary effects of gene flow, including effects on non-target species, disturbance of biodiversity, displacement and extinction of species, disturbance in the microenvironment of the soil and species of environmental concern (Layton et al. 2015). The possibility of new species evolution cannot be ignored and could also lead to an infinite number of biotic interactions (Beusmann and Stirn 2001). It is implicitly expected that gene flow from GM crops will be considered, as it has occurred between sexually compatible species for millennia (Keese 2008). However, this expectation is based on certain basic concepts, such as the distance between compatible plant species, the synchronization of flowering time, the ecology of the recipient species and sexual compatibility off course (Han et al. 2015). Some transgenic features make them more suitable for introgressing into wild counterparts, such as dominance, no association with harmful crop alleles, and location on shared genomes and/or homologous chromosomes. To predict the possibility of gene transfer through this mechanism, mathematical models of pollen movement are being developed (Dale et al. 2002. Examples of such investigations are reported in rapeseed, maize, cotton, wheat, barley, beans and rice (Han et al. 2015). The transfer of the pollen-mediated gene depends solely on the biology of the plant's pollination, the amount of pollen produced, the matching system between donor and recipient species, the excess rate, the relative density of the donor and recipient species, vector types, wind, air turbulence, water current, temperature, humidity and light intensity (Hancock 2003). A recent investigation by Dong et al. (2016) reported that the wind direction significantly affected a pollen-mediated gene flow. In addition, an increasing distance from the pollen source in WYMV-resistant transgenic wheat N12-1 reported a drastic decrease in pollen-mediated gene flow. In transgenic corn, canola and creeping bentgrass, pollen transfer rate decreased rapidly when the distance was increased just by 30 m, 20 m and 20 m respectively (Van de Water et al. 2007). In creeping bentgrass and rigid ryegrass, the highest frequency of gene flow was also reported as a result of pollen flow with a pollen donor only 2000 and 3000 m away (Van de Water et al. 2007). In self-pollinated crops, comparatively low frequency of gene flow was observed than in cross-pollinated crops (Warwick et al. 2009), as in the case of direct and indirect pollen-mediated gene flow from rice to red rice and vice versa <1%. Two other possible mechanisms for gene flow are seed mediation and vegetative propagulate mediation (Lu 2008). The transmission of seed-mediated genes is supported by human error in the seeding, harvesting or post-harvesting of adventitious plants (Schulze et al. 2014). In maize, wheat and canola, adventitious presence of herbicide resistance genes was observed in farm-harvested seed (Friesen et al. 2003). The transgenic transmission of vegetative propagules is caused by vegetative plant organs or by different animals (Schulze et al. 2014).

Scientists argue that whether or not such a transgenic flow really matters and what would be the consequence if it really matters? As discussable, such events occurred in nature between conventional crops and land races without constitution of any environmental problem. Introduction of new traits and novel genes into ecosystems as a result of genetic engineering raises additional concerns allowing flow of genes into diverse crops with variable outcrossing potentials (Ellstrand 2003). Here we discuss the detailed impacts of gene flow on the environment accompanied with relevant underpinning research.

4.4.2 Transgene X Wild Hybridization

Because of the ability of plants to hybridize with sexually compatible species and the release of hybrids into the environment and the spread of transgenic contamination, scientists recognize the possibility of transgenic flow. Ecosystem disturbance can be attributed to the persistence of a possible transgenic hybrid with a competitive advantage over the wild population. In theory, a rare hybridization event would be sufficient to develop such a hybrid under natural conditions (Cruz-Reyes et al. 2015) and the developed hybrid could be more fit than its parents. Fitness is a hybrid's relative ability to survive and reproduce thereafter in an environment (Haygood et al. 2003). The development of such a hybrid depends on certain factors, such as the synchronization of the flowering period, the hybrid's reproductive fitness and survival rate (Lu and Yang 2009). Fitness may be reduced in the first hybrid progeny F1, but is recovered in the next hybrid progeny as seen in sunflowers resistant to imidazolinone (IMI) (Presotto et al. 2012). In Brassica rapa/Brassica napus F1 hybrids and both parental species, ample fitness differences were observed. In regions where the crop species originated and had wild relatives, the risk of unintended gene transfer is greater (Lu and Snow 2005). Detection of the terminator of NOS (Nopaline Synthase) and the promoter of CaMV (Cauliflower Mosaic Virus) 35S in Mexican maize populations has strengthened the idea of gene transfer from GMO to land races and wild relatives (Pineyro-Nelson et al. 2009). Some factors such as hybrid vigor, selection and heterosis will play a role in determining the frequency of transgenes in wild populations after transgene flow to host plant genomes. Hybrid fitness depends solely on the ability to cross wild counterparts or related species, the life cycle of hybrids and their parents, fertility, changes in the survival rate of seed banks, seed persistence and seed dormancy (Lu and Snow 2005). Fitness costs in wild plants and crops must be different due to their diverse genetic background and the possible causes are pleiotropy, the physiological costs of new features or the effects of specific insertion sites in the genome and genetic changes in plant genomes as a result of mutagenesis (Schnell et al. 2015). The fitness of crop wild sunflower hybrids was higher in relative competitive wheat intercropping conditions compared to crop lines and was greatly affected by the interactions between the genotype environment (Mercer et al. 2014). The influence of the above random

and unintended effects on other associated characteristics is not negligible, but may remain unnoticed until the establishment of transgenes as wild populations; one such example is the transgenic sugar beet × swiss chard hybrids for their bolting pattern (Ellstrand 2003). The evidence of GM/wild interspecific hybridization was presented by a collection of triploid individuals in commercial canola fields in Chile (Prieto 2006). In Arabidopsis thaliana, Gressel (2000) also hypothesized a fitness penalty resulting from the resistance to the target site and increased ability to donate pollen to nearby non-GM mothers. Such gene flow cases are always accompanied by selection pressure against herbicides, insecticides, abiotic stress or pathogens. However, even if selection pressure introgression is not present, the persistence of transgenes in wild populations is still possible due to the recovery of selective fitness through successive backcrossing (Wang et al. 2001) and was noticed by Schulze et al., (2014) who reported the presence of glufosinate-resistant (particularly, events MS8 × RF3, MS and RF3) feral plants of oilseed rape in Switzerland even if there was no transgenic oilseed rape in the surrounding area at the time of sampling. On the other hand, genetic bridge is also responsible for gene flow, as crop hybrids and a sexually compatible wild plant can also provide transgenes directly to non-hybridizing species (Lu and Snow 2005). The Poaceae and Brassicaceae families have been reported to have a maximum number of natural hybrids among the target families of transgenic introgression (European Food Safety Authority 2016). Ellstrand (2002) reported that in different agro-ecological areas of the world at least 44 cultivated plants could cross with one or more wild relatives. Twenty-eight cultivated species, including 22 world food crops, have seen natural hybridization with one or more wild associations. He further confirmed hybridization with related wild plants for 83 species, due to the presence of sympatry, it was evident that 48 species had something more than just morphological intermediaries. Recently, the outcrossing potential of 11 GM crops with vascular flora in Chile was documented by Sanchez et al., (2016). 810 of 3505 introduced species and 824 of 4993 native species had interrelationships either based on genus or species correspondence.

In addition, the progeny of hybrids of GM/sexually compatible species may carry hemizygous allelic conditions that may not be expressed at the phenotypic level unless the condition is homozygous as a result of additional self-pollination or cross-pollination events (Sanchez et al. 2016). The GM Science Review Panel (2003) confirmed the absence of such hybrids, which in the UK could have become wildly invasive. In addition, the transgenic transfer of maize, cotton, canola and soybean has not been documented (Heuberger et al. 2010). In the case of B, though. Transgenic herbicide resistance napus transferred to its relative wild weed type B. Québec rapa and its persistence for the following 6 years, it was observed that no herbicide selective pressure in natural conditions occurred (Warwick et al. 2008). Based on the above reports it is obvious that hybrids may develop by introgression of GMO with its wild relatives and hence the possibility of transformation of resistant genes exists.

4.4.3 Transgene Stacking

With the development of transgenic plants with improved resistance to herbicides and insect pests, the use of GM crops worldwide is increasing. The area covered by single transgenic characteristics, such as glufosinate tolerance, is still high, but the relative percentage of GM crops with stacked characteristics (herbicide tolerance, insect resistance, restauration of fertility, male sterility, mannose metabolism, visual marker and antibiotic resistance) has increased. In 2012 alone, 43.7 million hectares were planted with biotech characteristics, with an average annual increase of 31% (www.isaaa.org). Many businesses such as Bayer Crop Science, Syngenta, Pioneer, Monsanto and Dow Agro Sciences are pursuing to achieve GM crops with stacked traits. The environmental and ecological consequences of transgenic stacking must also be taken into account. Transgenic contamination may include approved transgenic constructs and sequences and constructs not approved in a given country (De Schrijver et al. 2007). Kok et al. (2014) classified three possible risk scenarios from stacked plants. These include gene stability, gene expression changes and synergistic or antagonistic effects. Initially, the probability of stacked gene escape may be low, but multiple transgenes are likely to occur in wild plant populations in the long term (De Schrijver et al., 2007). Nuclear-coded and in rare cases, nuclear-encoded and plastid-encoded genes may even be combined (Halpin 2005). Accidental stacking, as well as intentional breeding between sexually compatible GM plants, may lead to accumulation of many genes in the same area. Consecutive generations of related and sexually compatible weed species would be able to receive transgenes with a wide range of action modes, such as pest resistance, various stresses, herbicide tolerance, etc., and would persist more forcefully in the environment (Mertens 2008). Recent developments in plastid genetic engineering have allowed multiple genes to be expressed in a single operation. On the contrary, gene escape from such events paves the way for introgressing perhaps the entire stack of transgenes that are often linked to a single metabolic pathway. As a result of this gene flow, environmental risks could only develop resistant and tolerant weeds in one generation (Bock 2007). In comparison to a single event or conventional counterparts, significant changes in endogenous gene expression and protein levels can be observed in GM plants with stacked characteristics. The expression of two stacked genes (Enolpyruvulshikimate-3-phosphate synthase and cry genes) led to changes in maize energy/carbohydrate and detoxification. In comparison to single event hybrids, both stacked genes had a 34% lower expression (Agapito-Tenfen et al. 2014). Some reports indicated that these reduced expressions could lead to the development of resistance in target insect pests (De Schrijver et al. 2015). The synergistic and antagonistic effects of stacked transgenes can present risks at two levels. Firstly, the interaction of proteins or stacked event components at the level of the GM plant may affect certain pathways, such as high oleic acid GM soybean, which may have a synergistic or antagonistic effect on other components of the oleic acid pathway. Secondly, the effect can be expected at the cellular level, where the expression of transgenes may affect the levels of cell components (Kok et al. 2014). At the cellular level, however, the risk cannot only be associated with stacked

characteristics, since transgenes of single events could also present the same risk. Open pollinated crops are at higher risk of developing polygenic transgenic characteristics as a result of the recombination of multiple transgenes compared to self-pollinated crops. What would be the possible impact on the environment and biodiversity of such gene flows? The most important thing is weed management and stacked volunteers of transgenes. Stacked transgenic volunteers have been resistant to various herbicides in Canada (oilseed rape) (Dietz-Pfeilstetter and Zwerger 2009). The question is, how can such a threat to the environment be managed? Orson (2002) suggested that such volunteers in the field of volunteering are inevitable to practice such volunteers in the field of agriculture. De Schrijver et al. (2015) proposed theoretical scenario tests to estimate the effect of stacked Bt proteins on non-target invertebrate species. He stressed that current knowledge of interactions with Bt toxins is limited and should be evaluated using more precise data. Schuppener et al. (2012) reported that lepidopteran and chrysomelidae were not significantly affected by stacked maize (Cry1A.105 andCry2Ab2) in European agricultural landscapes. Another, study involving Bt11 ut MIR604 maize, which expressed Cry1 Ab andmCry3A proteins, revealed unbelievable results that the cultivation of stacked GM maize did not differ more than single maize events (Raybould et al. 2012). In milk cows, beef heifers, swine, laying hens, broiler chickens and rodents, the combined toxicological impact of Cry1F and phosphinothricin acetyltransferase (PAT) proteins from TC1507 maize was considered. The report showed negligible or no allergic or toxic effects on humans or any of the organisms studied. There was no detection of gene flow and HGT (Baktavachalam et al. 2015).

4.4.4 Horizontal Gene Transfer

Stable transfer of genes to offspring other than parents (sexual/asexual) is considered to be horizontal gene transfer (HGT) (Keese 2008). The transfer takes place through the passage of genetic material from donors across cell boundaries, followed by heritable incorporation into the recipient organism's genome. The most popular strategy for genetic transformation is The result of HGT is agrobacterium tumefaciens (Conner et al. 2003). In addition to transduction, transformation and conjugation, many different mechanisms are naturally involved in the absorption and establishment of genetic material. With the advent of genetic engineering, the possibilities of HGT question the risks associated with the environment and biodiversity. The role of HGT in the evolution of microorganisms and macroorganisms under natural circumstances has already been recognized, and it is well understood that mechanistic HGT has no direct adverse effects, but changes in the fitness of the recipient organism have a drastic effect (Conner et al. 2003). Keese (2008) explained in detail the risks associated with HGT and possible factors that play a role in gene transformation. HGT from genetically modified plants raised further concerns about the possibility of transgenic transfer to another organism. Such gene flow could constitute a potential risk to humanity and the micro and macro environment (Conner et al. 2003). Possible HGT cases may include the transfer of transgenic

antibiotic resistance to pathogens and transgenic flow to viruses and/or humans (Ho et al. 2000). Such gene transfers could occur in soil, water and a human or animal gastrointestinal tract. These cases are still highly speculated, however, and detailed experimental evidence is expected. It is important to consider the interplay of alleles between bacterial communities with special consideration for HGT, which highlights the possibility of overcoming ecological barriers to the transfer of alleles among bacterial communities with special consideration of HGT, which highlights the fact that ecological barriers to allele transfer could be surpassed in different ways. Many bacterial species adopt such a strategy to maintain genetic similarity in the population, but this characteristic phenomenon poses a threat to the environment when considered at microclimate level in the context of genetically modified plants and bacterial interaction. Another major concern is the acquisition of multiple antibiotic resistances in a wide range of bacterial populations due to the widespread use of antibiotics in humans and animal medicine (Lawrence and Retchless 2009). Transgenic transfer from genetically modified plant roots and leaves to microorganisms was demonstrated by Tepfer et al. (2003), and such studies confirmed that Arabidopsis, oilseed rape, tobacco, alfalfa and carrot could transfer genes (nptII gene system as a marker) to Acinetobacter spp. Many experiments have shown that intact tobacco leaves with plastid transgenes can consistently produce bacterial transformants. Some factors were considered important in HGT, such as the size of the transgene, nuclear or plastic transgene, sequence mosaicism, selective pressure, transgene copy number, the genome size of the recipient species, the use of codons between the donor and the recipient, the type of promoter used in the insert, compatibility of RNA and protein synthetic machinery. (Tepfer et al. 2003; Daniell et al. 2001). Natural GM sweet potato (*Ipomoea batatas* (L.) Lam.) harboring many A. tumefaciens DNA (particularly two T-DNA regions i.e. IbT-DNA1 and IbT-DNA2) sequences strengthen the hypothesis that HGT can be a possible route of transgene movement from microflora to GM plants and vice versa (Kyndt et al. 2015). During evolution, when A. tumefaciens infected sweet potato these regions were transferred naturally. Recent investigations targeted at the transfer of CaMV-P35S promoter from a GM diet to blood in liver and brain of male Wistar albino rats suggested that this promoter have affinity of incorporation. The report suggested that larger segments had a higher incorporation frequency than shorter sequences and affinity increased with the increase of feeding duration (Oraby et al. 2015). Many researchers are in a debate that HGT frequency from plants to prokaryotes is as low as $2 \times 10–17$, while some scientists argue that 10 recombinants per 250 m^2 could be predicted considering a transgene transmission frequency of 10–17 (Mertens 2008).). Matthews et al. (2011) predicted HGT of *Rhodnius prolixus* less than $1.14 \times 10–16$ per 100,000 generations with 99% certainty level. Apart from traditional marker transgenes, novel transgenes having no natural counterparts i.e. those genes which are being engineered for production of pharmaceuticals, chemicals and vaccines, necessitate investigation in relation to HGT which may frequently include

unique combinations of toxin protein domains and regulatory elements, derived from diverse species which will probably differ considerably from those arising by natural evolution. HGT of dsRNA from GM crops to other related organisms should also be accounted for (Heinemann et al. 2013).

4.4.5 Structure of Genetic Diversity

Gene flow can affect the environment by reducing population differentiation and increasing the diversity of people in a population. The structure of GD or so-called "domestication bottleneck" is also a result of gene flow and can be determined by taking into account the history of life and demographic factors of domesticated crops (Lu and Yang 2009). Those crops that domesticated from a small initial crop population show a reduction in genetic variation known as the bottleneck of domestication. The main driving force for partial restoration of GD and GD is the natural flow of genes from wild to domesticated crops and new alleles and introduction (Marri et al. 2007). Such gene flow also plays an important role in development. By the advent of modern genetic engineering and plant breeding, characteristics including resistance to many pests and pathogens and quantitative quality and yield characteristics have been incorporated into crop plants grown on a commercial scale. The flow of these transgenes from GM crops to wild families reduces GD and sometimes completes the genetic extinction of wild populations (Gepts and Papa 2003). The frequency of genes is primarily affected by mutation, selection, genetic drift and migration (Papa and Gepts 2004). Migration of gametes to wild relatives through the movement of pollen between GM plants could be a strong factor in reducing GD between subpopulations. With such transgenic migration, gene frequencies in the entire genomes of the recipient species will be disturbed mainly by genetic recombination on target loci (Cruz-Reyes et al. 2015). In the GM cropping system, GD of rhizosphere bacteria can also be affected. There has not yet been such a detailed report, however. In major rhizospheric bacterial groups such as Proteobacteria, Actinobacteria, Chloroflexi and Firmicutes in the root zone of MON810 maize, no significant genetic variations were detected (Ondreickova et al. 2014). Overall, the possibility of controlling disturbance in GD is considered to be the decision of the farmer by compensating crop production with non-GM crop plants instead of agreeing with current scenarios of widespread GM monocrop crops. In any particular case, the extent and quality of gene flow unfolds the possible risks associated with it. It is now clear from the above discussion that gene flow is a strong evolutionary force and strongly demands that special containment strategies be developed to reduce it as much as possible (Ellstrand 2003). Possible strategies include (1) isolation zones or border areas (2) trap crops (3) molecular strategies such as limiting the opening of the flower, chloroplast engineering, male-sterility, genome incompatibility, seed sterility, apomixes, transgene excision and cleistogamy (Husken et al. 2010).

4.4.6 Fate of Naked DNA

In the natural environment, nDNA encoding a resistance or tolerance feature may persist (Barnes and Turner 2016). There are several possible sources of nDNA to be transferred, such as compost of GM plants and manures of animals with GM fodder (Gulden et al. 2005). There is another possibility of transgenic movement of meat and milk from animals fed with GM diets to natural habitats. Naked dsRNA from GM plants produced by dsRNA silencing may pose additional risks in addition to nDNA (Heinemann et al. 2013). Once nDNA has escaped, its persistence in the environment depends only on certain factors, i.e. transgenic size, DNA type (plastid/nuclear), kind of mineral or particle in soil to which DNA will bind, physiological state of recipient micro/macro-organism, stress on recipient microbe as well as availability of nutrients, pH of soil, amount of humic acid and soil temperature (Dale et al. 2002). The size of naked and degraded DNA (possibly transgenic and its regulatory sequence) and its facilitating sequence of flanking DNA are key factors for successful integration. In order to gain a perspective on the impact of nDNA on the environment, let us consider the amount of such DNA added to the environment. In contrast to immense amounts of DNA from non-GM plants added to the environment by pollen, leaves, fruits and compost and decaying plants, the relative amount of DNA from GM plants is relatively low (Dale et al. 2002). Once nDNA has escaped from a GM host and reached the environment, what damage to the environment could be possible and what is the risk? This DNA can create interruption in ecosystems? Well, the risk from such events is not negligible. First, such naked-extracellular DNA could be a source of the gene pool for microbial communities in the vicinity, especially bacteria and fungi with natural intake of DNA. Secondly, viral pathogens residing in microflora that could receive nDNA could be the most devastating danger. Third, there is another possibility of gene transfer from bacteria residing in GM crops to microbes in the intestines of animals feeding on GM crops (Dale et al. 2002). The intake of GM DNA in dairy cows fed transgenic Bt maize was 0.000094% of the total intake of DNA, which was nearly 54 μg/day. Although it was found that the daily intake of non-GM DNA in cows was 54–57 g/day (Phipps et al. 2002). Although the possibility of such a transfer is quite negligible due to nucleases in the intestine of the animal, nDNA would degrade (Flachowsky et al. 2005). Fragments of degraded DNA of 680 bp were detected in maize cob silage within 28 days, while only 194 bp were detected in whole plant silage for up to 35 days (Einspanier et al. 2004). In response to different acids, endonucleases and microbial activities, this fragmented DNA was immediately degraded in the animal digestive tract. A case study to detect CP4EPSPS in sheep fed with Round Ready canola detected fragments of 527 bp after 2 min (Alexander et al. 2004). The likelihood of risk in the digestive tract of an animal is quite negligible. However, it is possible that microbes residing in animal intestines can endocytise these small fragments and can be incorporated into host microbial genomes. Third, highly degraded segments of DNA may introduce amino acid substitutions or indels to bacterial genomes by transposition or homologous recombination, apart from the fact that these highly degraded segments are unlikely to transfer new protein encoding

capabilities (Van Hoek et al. 2011). Finally, if the decomposed GM material is exposed to aquatic ecosystems, it may be in aquatic animals 'gastrointestinal tract of aquatic animals and fish, fungal species could possibly up take nDNA (Mullany 2000). Persistence of nDNA from Bt corn (event MON863) containing Bt3Bb1 and nptII genes and DNA from plasmid Pns1 in water was reported to decrease by two orders of magnitude within >4 days (Zhu 2006). As far as the persistence in agricultural ecosystems concern, the possibility of nDNA perseverance is not zero. The persistence of nDNA in root zones of Roundup Ready GM corn and soybean is for a very short duration of 26.7 h if temperatures are high (> 15 °C) while, its persistence increases when temperatures are <15 °C and frequent rainfalls can distribute their DNA into various soil layers and across the agricultural fields (Gulden et al. 2005).

4.4.7 Weediness

Another growing concern that has severe and irreversible effects on biodiversity is the change in invasiveness or persistence of crops in agricultural and natural habitats. The establishment of a transgenic or transgenic hybrid as a weed is referred to as weediness in other fields or other habitats. Weediness is one of the possible effects of herbicide-resistant crops (HR) (Ammann et al. 2000). The ICSU, GM Science Review Panel agreed that domesticated crops are at low risk of weed establishment because domesticated characteristics are often less fit in the wild. Recent studies, however, support domesticated crops as it can escape cultivation (ferality) and turn into a potential weed. Features such as rapid growth rate, self-compatibility (crop features) could promote weediness (Ellstrand 2012). Increased herbicide resistance by hybridization with GM plants could lead to its persistence in agricultural habitat (Guan et al. 2015). Scientists have a contradiction about the establishment of transgenic recipients as weeds in the environment. For example, Williamson et al. (1990) reported that small genetic modification of domesticated crop hybrids by GMOs could cause major environmental changes. On the other hand, Luby and McNichol (1995) argued that it is unlikely to establish a crop as a weed by adding a single transgen. Based on the risk of increased fitness, some characteristics are strong candidates who can increase the chances of competitiveness, such as herbicide tolerance, stress resistance, pathogens and pests and characteristics responsible for increased growth (Yang et al. 2012). In view of the dispersal, plants with perennial, robust, prolific and competitive characteristics and the ability to withstand a variety of natural habitats could be regarded as plants with high impact (Mertens 2008). Furthermore, the rate of weediness through gene flow relies on the frequency of hybridization and net selective effects of target transgenes (Lu and Yang 2009). Certainly, weeds and crops exist in some plant species (Ammann et al. 2000). What could be the risk of such species? A change in habitat could obviously put potential pressure on the development of a weed from a cultivar or from a closely related feral plant. Plants can develop several mechanisms of herbicide resistance, such as herbicide detoxification, changes in the intracellular compartmentation of herbicides,

insensitivity to target sites, reduced entry of herbicides and translocation of herbicides and overproduction of target sites (Guan et al. 2015). According to the GM Science Review Panel, "there have been detailed field experiments in a variety of environments on several GM crops in a range of environments have demonstrated that the transgenic traits do not significantly increase the fitness of the plants in semi-natural habitats". Resistance to disease or pests are characteristics that could give weeds a fitness advantage and could have negative environmental penalties, but the possibility is little as present evidence shows. Current evidence is insufficient to determine this probability and more experimental investigations and field surveys are needed. The hybrid progeny had limited fitness advantages in the case of reduced ambient selection pressure of selective insects in Bt/CpTI GM rice in the intensive cultivated agricultural area (Yang et al. 2012). The herbicide resistance transgene from GM soybean to its wild counterpart (i.e. glycine soy) can still persist with zero herbicide selection pressure, escaped herbicide resistance transgene from GM soybean to its wild counterpart (i.e. Glycine soja) can still persist in nature (Guan et al. 2015). A notable case of amaranth (Amaranthus palmeri; cotton weed), first reported in Georgia in 2004, spread to 76 countries in the next 7 years (Gilbert 2013). This report also revealed that after release of many RT crops since 1996, 24 glyphosate tolerant weeds have been identified. Interestingly, from 1996 to 2011, PG Economics reported an 8.9% improvement in the environmental impact quotient. WeedScience (Ondreickova et al. 2014) published a chronological increase in resistant weeds on a global scale from 1955 to 2014. The report describes that around 145 plant species have become resistant to eight herbicide groups including acetolactate synthase (ALS) inhibitors, triazines, Acetyl-CoA Carboxylase Inhibitors, synthetic auxins, bipyridiliums, glycines, ureas, amides, and dinitroanilines. Current GM crops undergo the most extensive risk assessment studies so that the likelihood of invasiveness of these crops tolerant to herbicides in natural or agricultural habitats could be speculated (Dale et al. 2002). Although the risk of pervasiveness or invasiveness is considered relatively low, there are possible biological changes that could lead to weediness, such as tolerance to extreme temperature regimes, water and soil salinity, changes in the characteristics of seed propagation and dormancy, and the introduction of pest or pathogens resistance (Mertens 2008). In response to competition, an increase in the fitness of a cropwild hybrid was reported in wild sunflower hybrids and most importantly to the application of the herbicide (Mercer et al. 2014). However, the competitive fitness of susceptible and resistant common cocklebur against acetolactate synthase was not significantly different suggesting that case-by-case risk assessment studies are needed before approval of any GM crop for commercial cultivation (Crooks et al. 2005).

4.4.8 Chemical Toxicity

Plants naturally use toxins to fight threats such as pests and pathogens. Such chemicals cause biotic and abiotic environmental factors toxicity. Toxins such as glycoalkaloids, ricin and endotoxins from delta are of greater risk and are thoroughly

investigated. In most GM plants, Bt delta endotoxins were targeted and the effects of their proteins on the environment and friendly organisms were extensively studied (Yu et al. 2011). Bacteria are the most common sources of transgenes, while fungi, plants, animals and humans are also used as sources of different transgenes. Transgenes are used for plant codon from these hosts are used either for plant codon usage or for direct molecular evolution (so called molecular breeding) (Keese 2008). Direct gene transfer expresses the desired proteins in the recipient organism, while numerous parental genes are fragmented and reassembled through molecular breeding in order to express new proteins that are not present in nature. In Escherichia coli, for example, a new carotenoid was expressed by shuffling DNA coding for a pair of enzymes involved in the pathway to carotenoid biosynthesis (Schmidt-Dannert et al. 2000). There are therefore risks associated with natural and novel toxins in the body of the plant. Natural toxins could be assessed on the basis of certain developed models. However, new toxins can affect life both target and non-target. We are concerned with the risks from both natural and novel toxins. In some negative interactions, engineered toxins responsible for growth or stress resistance could have unintended effects on the ecosystem. The environmental impact of herbicide tolerance toxins and resistance to insects/pests is analyzed below.

4.4.9 Herbicide Toxicity

The risks of herbicide toxicity can be regarded as a qualitative estimate, including the possibility and severity of immediate or delayed adverse effects on the environment, human health and the economy of the farmer. However, there are some factors associated with the probability and severity of each toxic effect, such as crop and characteristics, local weed flora, farm management practices and climatic conditions (Madsen et al. 2002). The cultivation of herbicide-tolerant GM crops is associated with potential threats to farmland and wild habitats. Eighty percent of transgenic crops grown in laboratories or in commerce have transgenes expressing glyphosate, glufosinate and glyphosate tolerance and/or stacked with insect resistance. There is also the possibility of toxicity to other forms of life in addition to toxicity to plants themselves. Johal and Huber (2009) explained in detail the direct weakening and increased pathogen virulence of plant defense induced by glyphosate. Glyphosate inhibits the defense and structural barriers of the plant and immobilizes micronutrients such as manganese (Mn), which play a key role in disease resistance. The metabolism of plant nitrogen is modified in response to applied glyphosate in a manner similar to changes caused by high temperatures. By modifying the nitrogen and carbohydrate metabolism, the transient resistance of soybean and wheat rust was reduced. Some reports confirmed lethal effects of roundup on amphibians, larval amphibians, fish, tadpoles, snails, insect predators, small arthropods, fungi and bacteria (Relyea 2005). There was almost a complete mortality (96–100%) rate of post-metamorphic amphibians and North American tadpoles in response to direct application of roundup (Relyea 2005). Even, concentrations below environmental protection agency (EPA) levels harmed Pacific Northwestern Amphibian larval

community when exposed to 0–5.0 mg dilutions (King and Wagner 2010). Herbicide stratification was directly linked to temperature stratification and implicated the habitat choice in ectotherms (Jones et al. 2010). Application of roundup on rice has proven the increase of mortality in water weevil (Lisorhoptrus oryzophilus) in terms of 20% reduced larval incidence on herbicide treated rice (Tindall et al. 2004). Liver congestions, necrosis (2.5–5.5 times higher) and sever nephropathies (1.3–2.3 times higher) was found in male Sprague-Dawley rats fed with roundup applications in drinking water and GM maize diet (DKC 2678 R-tolerant NK603) for 2 years. The noticeable point is that even lower concentration than field application rates was also tested and found to be of concern. In the case of female rats, mortality increased two to three times and pre-mature death was observed whilst, mammary tumors appeared more frequently (Seralini et al. 2014). Antimicrobial activity of glyphosate and glufosinate is another rising concern (Samsel and Seneff 2013) as Kruger et al. (2013) clearly stated that glyphosate disrupts intestinal bacteria in cattle and poultry. Some scientists suggested altered defense response of plants against microflora (Benbrook 2016). Increase in bacterial biomass, enhanced activities of urease, alkaline phosphatase, and invertase have been observed in the rhizosphere of Basta-tolerant oilseed rape grown with the application of Basta (glufosinate) and Butisan S (metazachlor) depicting that GM plants and applied herbicides modify activities of the associated microflora (Sessitsch et al. 2005). Decreased activity of *Bradyrhizobium japonicum* (a nitrogen-fixing bacteria), *Azotobacter chroococcum, A. vinelandii* and entomopathogenic bacteria have been reported (Morjan et al. 2002). Such decreased activities of microorganisms especially of nitrogen-fixing bacteria indirectly reduced soybean yield by 8–10% because of inhibition of nodule formation, reduced nodule biomass and reduced nitrogen fixation (King et al. 2001). Alteration of Cytochrome P450 raised another affiliated risk of glyphosate use. Suppression resulted in a synergistic effect with intestinal bacteria and disrupted aromatic amino acid biosynthesis and could be a pathway to many modern diseases (Samsel and Seneff 2013). Apart from such effects on other life forms, the health of GM plants itself is another issue. Frequent application of glyphosate could possibly increase the susceptibility of crop plants by increasing the incidence of microflora in the rhizosphere. For example, Fusarium solani was reported to have higher incidence after glyphosate application (Njiti et al. 2003). Increased disease severity is a common hypothesis among plant pathologists in terms of weakening plant defense mechanisms and increasing the population of casual organisms. This can be indirectly linked to the immobilization of disease-related micronutrients, impeded plant growth, altered physiology and changes in soil microflora behavior (Johal and Huber 2009). In response to GM crop cultivation and cultural practices, Kremer et al. (2005) documented that microbial components of GM soybean and maize rhizospheres have been altered. In a comprehensive review, Duke et al. (2012) concluded that the balance of minerals in herbicide tolerant plants is not significantly affected and disease incidence is negligible after using glyphosate and the fact that current amount of evidence in insufficient in this context. The reduced levels of aromatic amino acids, i.e. phenylalanine and tyrosine in RT crops, resulted in a reduced effectiveness of the plant defense mechanism against abiotic stress and

pathogens (Benbrook 2012). The continuous use of herbicides causes a differential expression of transgenes in specific tissues, such as cotton, in which reproductive tissues have higher glyphosate concentrations (Pline et al. 2002). If a plant part with a higher accumulation of glyphosate is used for food or feed, the health risk to humans and animals will increase depending on the part of the genetically modified plant to be consumed and the level of expression of the transgene in that part of the plant. Bohn et al. (2014) investigated compositional differences in GM soybeans and reported high residues of glyphosate and aminomethylphosphonic acid in glyphosate tolerance GM soybeans. Young et al. (2015) presented a detailed report on the role of glyphosate in human endocrine disruption and cytotoxicity to human cells. Such increased concentrations will also affect pollination problems in the plant itself, reduced pollen viability, retention of bolls and abortion of bolls (Pline et al. 2002). In addition to direct toxic effects, the indirect effects of herbicide tolerance include disturbed biodiversity of weeds, arthropods inhabiting weeds, parasitoids, predators and decomposers, which may lead to disturbances in symbiotic relationships, a decrease in the population of beneficial insects and rapid changes in the food chain of agricultural land (Schutte and Schmitz 2001). In conclusion, the cultivation of GM crops with resistance to herbicides, influences host plants and non-target soil life, weeds and farmland biodiversity depending upon the degree of adoption. Despite extensive laboratory, greenhouse and farmland studies, there are still significant gaps in knowledge about the potential induced toxicity of herbicides. For more information on the toxicity of mammalian herbicides based on glyphosate, see Mesnage et al. (2015). Glyphosate will prevail in the coming years as the herbicide of choice worldwide and the quantification of its effects on human health and ecological consequences will thrive (Benbrook 2016).

4.4.10 Insecticide Toxicity

In the development of a resistant GM plant, the most challenging consideration is to identify a resistance gene and direct its product to appropriate plant tissues so that it targets only the pest without any side effects on friendly organisms. Apart from proteinase inhibitors, α-amylase inhibitors, avidin, chitinases and lectinases, Bt delta endotoxins are the most important examples of engineered insect resistance (Dale et al. 2002). Previously, toxin-based bacterial formulations were used to directly spray targeted insects. Preferences have been shifted to the expression of toxins in transgenic plants, which seemed to be relatively efficient and safe at eliminating insect pests (Schutte and Schmitz 2001). GM plants produce toxins throughout their lives, but sprayed formulations are used for a certain period of time. Although the Bt toxins expressed differ from natural toxins, less specific but sprayed natural toxins are rapidly disintegrated in natural conditions. The marketing of GM plants expressing Bt toxins has been rapidly adopted by the farming community and the area of GM plants is increasing every year, so that a broad community of researchers is questioning the ultimate potential target and non-target impacts of transgenic toxins. Many laboratory studies have been carried out to answer the

question: "Does Bt toxins kill monarch butterflies? Well, the answer to the question is inconsistent. The first report on the mortality of monarch butterfly caterpillars in response to pollen from commercial Bt maize has shown that Bt toxins pose a potential risk to non-target life forms (Losey et al. 1999). This has been followed by numerous studies that have also reached agreement on toxicity concerns raised by Losey and colleagues (Obrycki et al. 2001). However, later investigations concluded that toxicity to the host plant and non-target species depends on a variety of factors such as pollen, weather conditions, local fauna and flora, alternative host species for non-target insects, event of transformation, promoter, level of expression of toxin, the tissue of GM plant where transgene is being expressed, likelihood of exposure and routes of exposure (Fontes et al. 2002). The hazards of Bt and other toxins on lacewings, earthworms, herbivores, honeybees, human fetuses are reported in numerous farms and laboratories (Aris and Leblanc 2011). In response to Bt-maize pollen, no significant risks were associated with larval survival and the prepupal weight of honey bees. Delayed growth and reduced weight gain were observed in herbivores feeding on sublethal doses of Bt (Agrawal 2000). Higher mortality, reduced egg production and a lower proportion of females reaching maturity were observed in Daphnia magna; a crustacean arthropod, when fed with Cry1Ab maize (Dekalb 818 YG) (Szenasi et al. 2014). Bt doses could then possibly affect tri-trophic interactions (i.e. plant-herbivores-their natural enemies) in synergistic, additive, or antagonistic ways. Effects of Bt toxins on other trophic-levels including vertebrate predators preying on lepidopteran pests are yet to be considered (Clark et al. 2005). The presence of Bt toxins in aphid (Myzus persicae) samples detected by a double enzyme-linked immunosorbent test confirmed the potential effects of these toxins on food chains and trophic levels of natural herbivore enemies (Burgio et al. 2007). In contrast, many researchers reported that non-target species were not toxic due to shorter persistence or degradation of Bt toxins in the soil (Oraby et al. 2015). However, the combined effect of Cry1Ab and Cry1Acas is not inert, as well as in response to 1–200,000 ppm was confirmed. Cry1Ab concentration of 100 ppm resulted in the death of human embryonic kidney cells (Mesnage et al. 2012). Domingo (2000, 2007, 2011, 2016; Domingo and Giné Bordonaba 2011) reviewed the adverse effects of GM crops on health and summarized the published studies and reported that GM crops have the same health effects as their counterparts with few exceptions which clearly indicates that it is difficult to consider GM food and feed safe due to the presence of controversial experimental results. Given the chemical toxicity, we can conclude that most of the chronic and sub-chronic studies that have been carried out so far to test the toxicity of genetically modified (GM) organisms used as food and feed do not show any potential health effects (Domingo 2016), but have many limitations, such as the exposure period, which is too short to assess the long-term effects and endpoints. Classical toxicological studies do not take into account the whole area of interactions that may occur in real life exposure between genetically modified organisms and other chemicals exposed to humans every day, even at doses below or around regulatory limits, which could lead to synergistic and potential effects (Hernandez et al. 2013). In addition, these types of single compound studies do not focus on various types of long-term toxicity, for

which neurotoxicity, cardiotoxicity, nephrotoxicity, genotoxicity, hepatotoxicity and endocrine disruption are currently of particular concern. The fact is that the international regulatory authorities have also begun to recognize the need for this cumulative risk assessment and new methodologies are being developed, but only for commercial artificial mixtures (EFSA journal, 2013; Regulation 1272/2008/EC 2015). For non-commercial artificial mixtures that represent the real scenario of real life exposure, no regulatory provisions have been taken. As for pesticides and other chemicals to which consumers are exposed during their lifetime, it is also necessary for genetically modified organisms to pass from a single compound risk assessment to cumulative risk assessments, which threaten the long-term exposure to low doses of chemical mixtures, which simultaneously monitor different endpoints associated with the investigation of systemic mechanistic pathways such as oxidative.

4.4.11 Indirect Impact of Transgenes on Environment

The environmental impact of transgenic crops is evident in response to changes and changes in current agronomic practices or agricultural practices in general. Indirect effects of GM crops include soil, water, and biodiversity of wildlife and reduced weed, insect and pest control efficiency. The level of risk depends primarily on the nature of changes in agricultural practices (ICSU, GM Science Review Panel). Nevertheless, it must be decided whether the overall impact of such a modified use of pesticides has positive or negative prospects, but there are reports that establish the concept that changing agricultural practices have disturbed the habitat of the fauna and flora of farmland.

4.4.12 Effect on Soil and Water

Scientists and farming communities continue to debate the effects of the introduction of GM crops on groundwater and water reservoirs. This debate is directly linked to the extent and extent of the use of herbicides in GM crops. GM crops are known to be herbicide tolerant and invite wide-spectrum use of herbicides (Benbrook 2012). This increase in the use of herbicides was indirect, i.e. the replacement of more toxic herbicides that persist with glyphosate in the environment (Duke et al. 2012). There is a general decrease in the use of toxic herbicides and an increase in herbicides based on glyphosate (Benbrook 2016). Glyphosate is probably the world's most common herbicide. Glyphosate can reach the soil by direct interception of spray in early season or post-harvest applications, by removal or leaching of herbicide from vegetation and by exudation from roots or death and decomposition of plant material (Duke et al. 2012; Kremer et al. 2005). The addition of glyphosate to agricultural water and ultimately to aquatic ecosystems and their impact on aquatic life is evident. However, due to a shorter half-life compared to many other herbicides and strong adsorption to the soil matrix, the risk of glyphosate toxicity to non-target soil biota is often considered to be marginal. Zabaloy et al. (2016) showed

no negative effects on soil microbial communities in fields that were exposed to glyphosate. This study suggests that glyphosate use at recommended rates poses a low risk to microbiota (Duke et al. 2012). The antimicrobial activity of glyphosate is a matter of debate too, because large scale applications of glyphosate would certainly disturb microbial communities at farm scale (Samsel and Seneff 2013).

At the same time, the transfer of Bt toxins from GM crops to soil and water has many possible routes, including pollen deposition during anthesis, root exudates and residues from GM plants (Yu et al. 2011). There is evidence that Bt toxins bind to clay and humic substances to biodegrade proteins (Clark et al. 2005). Once the protein is bound to the particles of clay, its susceptibility to degradation decreases, as Stotzky (2004) observed, with a special reference to Cry1Ab, Cry1Ac and Cry3A in the root exudates of GM maize, potato, rice, canola and cotton. But the unintended effects of these proteins on organisms residing in the soil have not been consistent and have not been taken up by non-GM roots. Statistically, non significant pH levels under Cry1Fa2 GM maize were observed as compared to soils under non-GM maize (Liu et al. 2010a, b). Most studies have suggested that Bt proteins from transgenic plants break down relatively rapidly in the early stage after entering the soil and that only a small amount of them can remain for a long time period, so that Bt proteins do not bio-accumulate in soil (Yu et al. 2011).). However, the persistence of Bt toxins in the soil depends largely on the type of toxin and type of soil, not on the number of expressed transgenes (Rauschen et al. 2008). In South Africa (Bennett et al. 2003), as a result of less chemical pesticides being sprayed on cotton, demonstrable health benefits for farm workers have been documented.

4.4.13 Effect on Biodiversity

Widespread commercial cultivation of GM crops, in particular herbicide-tolerant crops, poses serious threats to the complexity of the ecosystem and biodiversity reduction. Contrary to yield loss and contamination, weeds are ecofriendly in a sense too; consider the reduction of soil erosion by weeds and provision of habitat to a range of beneficial organisms (Mertens 2008). Studies have also shown that, contrary to conventional systems, the diversity, density and biomass of the seed bank in farmland are obviously lower in GM systems (Bohan et al. 2005). UK Farm Scale Evaluations (FSE) reported a reduction in weed seed banks of 20–36% (Andow 2003). However, the report found that weeds of dicot were more susceptible than monocots). Rapid changes in habitat destruction will have a significant impact on changes in food webs and food supplies. In addition to the impact on beneficial organisms, the balance of the predator-prey systems becomes even more critical. Of course, this will not result in disturbed tri-trophic interactions and symbiotic associations leading to complicated disruption in the food web. It is clear that such disturbances in the management of weeds, insects and pests will result in increased use of pesticides (Schutte and Schmitz 2001). In most cases, this change

in accessibility of resources has knock-on effects on higher trophic levels. The free-style foraging behavior can also be adapted by the frequent application of herbicides as in the case of glyphosate application where spiders moved to superfluous cricket killing behavior (Marchetti 2014). Other consequences are moving from herbivore to detritivore in the food web. The application of glyphosate resulted in an increase in fungal biomass in relation to bacterial biomass, which paves the hypothesis of a change in the food web on the basis of slower nutrient turnover and harnessed enrichments; based on resources of the carbon and nitrogen ratio (C: N ratio) (Powell et al. 2009). Types of herbicides and insecticides used, degree of adoption, frequency of use, timing of application of herbicides or insecticides, target crops, rotational and agronomic practices, local fauna and flora, alternative hosts for friendly insects, microclimate conditions, history of management and surrounding habitats (Merte) The emigration of agrobiont wolf spider (Pardosa milvina) was reduced when Baccaneer ® Plus (glyphosate) was used to indicate that there is a disturbance in the predator-prey relationship in food webs across the eastern United States (Wrinn et al. 2012). Bt crops were also questioned for their potential threats to biodiversity in parallel with herbicide-tolerant GM crops. Pesticides are often transported beyond crop fields and can have a significant impact on land and aquatic ecosystems or plant populations near crop fields. Most prominent targets are mammals and birds, and many studies have shown little or no evidence of Bt toxicity (Flachowsky et al. 2005; Aris and Leblanc 2011). In a broader sense, it can be concluded that the cultivation of HR GM crops has a negative impact on biodiversity (Bohan et al. 2005). The discussed dangers to biodiversity could be possibly observed on a long-term basis and of course, risks could not be left out of the equation. However, one short-term food web assessment (a 2-year investigation) in response to the cultivation of GM maize revealed the presence of stable and complex food webs and their persistence was not compromised. The study included GM maize having resistance against Coleoptera, Lepidoptera and glyphosate and mainly focused on arthropod food webs with an experimental population of 243,896 individuals (Szenasi et al. 2014).

4.4.14 Reduced Efficiency of Pest, Disease and Weed Control

The effects of changes in agronomic practices in response to the introduction of GM crops are few of the frequent crop swooping, increased use of broad-spectrum herbicides and increased impetus for minimal cultivation/zero-tillage agricultural systems (Dale et al. 2002). Of course, there are many advantages associated with changed agricultural practices such as soil erosion, less disturbance to earthworms and minimal disturbance to the microclimate of the soil, especially in the case of zero laying. In contrast, many indirect risks are also associated, such as the development of RT weeds, weed population shifts, cross-resistance development and multiple resistance and resistance to Bt toxins.

4.4.15 Evolution of Herbicide Resistance

The appearance of RT weeds is inevitable because weed species have a remarkable ability to develop herbicide tolerance in the weed gene pool (Agapito-Tenfen et al. 2014). Weeds can develop herbicide resistance in approximately 3 years, as polygenic herbicide resistance was reported in the progeny of F1, F2 and backcross in the case of low doses of diclofop methyl (Busi et al. 2013). Increased prominence of Asian dayflower (Commelina cumminus L), wild buckwheat (Polygonum convolvulus L) and common lambsquarters (Chenopodium albus L) were observed where there was significant selective pressure due to the concomitant use of herbicide and frequent cultivation of herbicide-resistant crops (Owen and Zelaya 2005). In the development of tolerance, different mechanisms could help the plant, such as target site over production, modification of intracellular herbicide compartmentation, minimal absorption and translocation of herbicides, herbicide detoxification and insensitivity to the target site (Brower et al. 2012). Although the probability of target-site resistance to a single herbicide is relatively low, but not negligible, i.e. one person in 10–5 to 10–10, while the frequency is nearly half when multiple-target site resistance is considered (Mortensen et al. 2012). As of February 2016, a total of 467 unique cases of RT weeds from 249 species (144 dicots and 105 monocots) have been recorded globally. These 249 species are resistant to 22 of the 25 known action sites for herbicides and 160 other herbicides (http://www.weedscience.org). The resistance of glyphosate and glufosinate from the weed gene pool is highly unlikely, mainly due to its chemical structure, no residual activity, limited glyphosate intake from soil plant roots, mode of action and persistence of near-zero soil (Baylis 2000). Few reports of the development of glyphosate resistance are annual rye grass in Australia and horseweed in the United States (Dale et al. 2002). This development of resistance may be over-expression of the target enzyme, reduced translocation of herbicides and different sensitivity of the target enzyme to glyphosate (Wakelin et al. 2004). Many independent evolutionary events could simultaneously interact with the emergence of herbicide resistance on a large geographical scale (Bonny 2016). Regular use of glyphosate in a significant proportion of GM crop fields makes the assumption of the development of glyphosate resistance a reasonable assumption. Weeds are not a poorer competitor than susceptible weeds, as no fitness difference between susceptible and resistant Lolium rigidum biotypes was detectable (Busi et al. 2013). Conclusively, although the development of biotypes of resistant weeds, the development of cross and multiple resistance and the shift in weed populations is inevitable, delay strategies for development could be strategies could though comprehend the herbicide resistance development (Schutte and Schmitz 2001).

4.4.16 Evolution of Insecticide and Pesticide Resistance

It has been shown that the control of pests by conventional and chemical techniques is challenging, as insecticide and pesticide resistance have developed in many cases

(Dale et al. 2002). In particular, due to the constitutive expression of Bt toxins in all plant tissue, the possibility of evolution of Bt-resistant insect pests cannot be negated imparts higher selection pressure on target species (Yu et al. 2011). The use of Bt bio-pesticides by organic farmers in Central America, Florida, Japan, the Philippines, Hawaii and China (Tabashnik et al. 2005, 2013). Gassmann et al. (2014) reported that Bt maize with a higher dose of toxin offers a higher selection pressure on the western corn rootworm, resulting in the development of cross-resistance between maize Cry3Bb1 and maize mCry3A. The selection of resistance to Bt toxins European maize borer, pink bollworm, cotton bollworms was also reported in many laboratory studies. The intensity of selection is an important driving force in determining the evolution of resistance, the size and arrangement of shelters, the mating behavior of insect pests, seasonal changes in habitat and population regulation by insecticides in GM crops and shelters (Caprio 2001). A decade-long report by Tabashnik et al. (2005) explained the presence of recessive alleles of the gene of cadherin (BtR) in the resistant strains of pink bollworm (Pectinophora gossypiella) associated with Cry1Ac resistance. Griffitts et al. (2001) reported a different resistance mechanism in nematodes and lack of encoded protein by bre-5 (a putative ß-1, 3-galactosyltransferase) in the Caenorhabditis elegans intestine resulted in no binding leading to resistance to the Bt toxin Cry5B. Tabashnik et al. (2013) surveyed 77 reports of developments in pest resistance to Bt toxins from five continents and confirmed the resistance of Bt toxins to fiel in 5 of 13 species. EPA has proposed two strategies to delay the evolution of resistance, i.e. high dose of toxins and high dose refuge (www.epa.gov). Dale et al. (2002) has also proposed a gene pyramiding strategy that delays the development of resistance in a much more effective way. Contrary to the development of resistance to Bt toxins and insecticides, the development of pathogen resistance is quite high because viruses, bacteria and fungi are known to adapt to selective forces very quickly. In principle, it is easy to overcome single gene-based resistance mechanisms. In addition, there have been frequent mutations in avirulence (Avr) genes of bacteria and fungi, so that resistance can be overcome through the integration of the corresponding resistance (R) gene. Other delaying strategies based on developments in the field include low initial frequency of resistance alleles, recessive inheritance, abundant refuge populations and the use of two-toxin Bt crops instead of single-toxin Bt crops (Tabashnik et al. 2013).

4.5 Safety Assessment of Genetically Engineered Crops

The modified crop is substantially equivalent to the unmodified parent, with the exception of one or a limited number of identifiable characteristics (such as the presence of proteins confering insect resistance or herbicide tolerance) resulting from the genetic change. In these circumstances, the safety of the new feature is sufficient to conclude that the modified crop or the food/feed products derived from it are safe. In most cases, the new feature was the presence of a specific protein and the safety considerations were addressed by determining the safety of this protein.

4.5.1 Protein Safety Evaluation

Protein safety assessment will be based on its structure, function, bioavailability, specificity and potential allergenicity. Proteins in the diet are generally not considered to pose a significant risk to human health, since proteases destroy almost all proteins ingested in the digestive tract. However, some adverse effects associated with proteins must be taken into account and specific safety assessment strategies must be taken into account. For example, proteins are some of the most powerful toxins known to humans (Rappuoli and Montecucco 1997). The other main adverse effects associated with proteins are, in addition to acute toxicity the other main adverse effects associated with proteins are anti-nutrient effects (e.g. soybean trypsin inhibitors), effects on the immune system (e.g. lectins) and allergenicity (Taylor and Lehrer 1996).

4.5.2 Protein Allergenicity

With the development of GM crop plants, there has been an increasing interest in available approaches to confirm the lack of allergenicity of new gene products or otherwise (Kimber et al. 2000). In a comprehensive analysis carried out by the International Life Sciences Institute (ILSI), the Institute for Allergy and Immunology and the International Food Biotechnology Council, the hierarchical decision tree (Metcalfe et al. 1996) was proposed for the evaluation of the allergenic potential of food derived from GM crops and was further recommended by the joint FAO/WHO expert consultation. If the food in question is in this scheme, containing gene from a source needs to be considered allergenic then the immunological identity of the novel protein with allergens deriving from the source material is determined. The purpose here is to protect those who have already been sensitized from accidental exposure to allergens induced. An example of the successful usefulness of this approach is the study of modified soybean expressing the storage protein Brazil nut 2S. Sera was found to contain IgE antibody reactive with 2S protein from eight of nine subjects with confirmed Brazil nut sensitization (Nordlee et al. 1996). However, if the protein of interest is a product of a gene derived from a source that is not normally associated with allergies, or if human consumption is not widespread, an alternative strategy is recommended. This is based on sequence homology considerations with known allergens and of protein stability (Gendel 1998). The homology of the linear sequence of eight or more contiguous amino acids between the test protein and one or more known human allergens (based on the minimum length of the peptide for immune recognition) is indicative of a sufficient immunological identity. If this linear sequence homology is identified and/or if other structural similarities exist between the test protein and known human allergens, the immunological identity as described above should be investigated. The stability (digestibility) of the protein in a simulated gastric fluid (SGF) containing the relevant protelotic enzymes is examined in the other approach (Astwood et al. 1996). The assumption is that rapidly digested proteins will not cause an immune response, and the

available data show that many food allergens are relatively resistant to SGF digestion (Astwood et al. 1996; Metcalfe et al. 1996). Since the correlations between the homology of sequences and stability are part of an overall safety assessment, the nether approach provides direct evidence of allergic potential. A number of laboratories are therefore developing suitable animal models (Atkinson et al. 1996). A recent review has resulted in a proposed amended strategy to predict allergens (FAO/WHO 2001).

4.5.3 Requirement for Animal Studies

If the food characterization indicates that the available data are insufficient for a thorough safety assessment, testing of animals may be considered necessary. This would be especially the case if the food is expected to make a significant dietary contribution, if the gene product is stable and if there is no history of consumption, or if the change affects several metabolic pathways. The studies should be designed to address specific safety aspects relating to the difference between transgenic and parental crops or their derived foods. The aim is to ensure that after prolonged consumption of GM crops and their derived foods, there is no concern for adverse health effects for humans or animals. Where toxicology studies are considered necessary to assess the safety of long-term food consumption in the diet, it is generally considered that a subchronic study of 90 days is the minimum requirement to demonstrate the safety of repeated food consumption in the diet. This may need to be preceded by a short-term pilot study to ensure that the diet is suitable for the test species and that the incorporation level of the test item is appropriate. The highest dose level used in any animal study should be the maximum possible without causing a nutritional imbalance, while the lowest level should be comparable to the expected intake of humans. The need for additional toxicological tests should be taken into account case by case, taking into account the results of the 90-day study and other studies. For example, proliferative tissue changes in the 90-day study may indicate the need for a long-term study of toxicity (FAO/World Health Organization 2000). In addition to animal studies specifically designed for safety assessment, nutritional or health tests can be carried out to determine whether the food or feed product of the GM crop poses any nutritional problems compared to the unmodified parent crop (Hammond et al. 1996). These studies involve the administration of the food or feed product in quantities representative of anticipated use to an appropriate test species. The best species are normally those that consume food or feed or can be selected due to particularly high growth rates, which would lead to an increased sensitivity to any nutritional problems. Studies would typically last 28 or 90 days and the end points are generally indices of growth and nutrition, such as food consumption, general condition, weight gain, yield and composition of milk (cattle), performance of laying (hens), or efficiency of food conversion and body composition (fish). Observations in some studies would also include simple pathological endpoints such as carcass quality and organ weights and their postmortem macroscopic appearance. Although these studies should not be confused with toxicology

studies, as they are not necessarily optimal for safety assessment, they provide useful data.

4.6 Viral Resistant Crops

Viral diseases are a major threat to crop production throughout the world. In developing countries, the problem is exacerbated, particularly in tropical areas where crop-free seasons are rare and plants are constantly under pressure from viruses transmitted by vectors from both cultivated and wild plants (Fargette et al. 2006). Africa suffers from major pandemics and epidemics of recurrent plant viruses in important crops. Known viral diseases caused by DNA viruses include cassava mosaic disease (CMD) caused by begomoviruses (members of the Geminiviridae family) (Zhou et al. 1997), banana bunchy top disease caused by babuvirus (member of the Nanoviridae family) (Blomme et al. 2013) and maize streak disease caused by maize streak virus (MSV), a mastrevirus (a member of the family Geminiviridae) (Thottappilly et al. 1993). Cassava brown streak disease is caused by two closely related RNA viruses (members of the genus Ipomovirus, family Potyviridae) (Alicai et al. 2007). In eastern Africa, maize suffers from lethal necrosis caused by co-infection by two RNA viruses, maize chlorotic mottle virus (a member of the genus Machlomovirus, family Tombusviridae) and the potyvirus sugarcane mosaic virus (a member of the family Potyviridae) (Mahuku et al. 2015). Sweetpotato virus disease (SPVD), the most devastating disease of sweetpotatoes, is also caused by co-infection by two RNA viruses, the crinivirus sweet potato chlorotic stunt virus (SPCSV, a member of the family Closteroviridae) and the potyvirus sweet potato feathery mottle virus (Karyeija et al. 2000).

Natural sources of resistance to many tropical viral diseases are known and used in breeding. However, resistance sources are often lacking, or the genetic complexity and difficulties of introgressing resistance genes to cultivars by crossing hamper crop enhancement efforts. The development and transfer of crop resistance by biotechnological means is therefore an attractive alternative. Powell Abel et al. (1986) studies have shown that the transformation of tobacco plants (*Nicotiana tabacum* L.) to express the tobacco mosaic virus coat protein has made the plants resistant to the virus. The transformation of plants into non-structural viral proteins (Golemboski et al. 1990) and truncated defective viral genes also protected against homologous viruses (Anderson et al. 1992). These findings have created a great deal of excitement and hope for a quick solution to problems with viral diseases in crop plants. With the discovery of post-transcription gene silencing (i.e., RNA silencing or RNAi), the resistance mechanism in genetically engineered plants became understandable as binary vectors expressing virus-specific inverted-repeat (hairpin) RNA to target the virus to degradation by RNAi (Waterhouse et al. 1998).

It was later found that some viral proteins suppress or interfere with antiviral RNAi (Anandalakshmi et al. 1998) and resistance derived from the virus could fail if the plant was infected with a virus that differed >15–20% at the sequence level from the donor of the transgene (Savenkov and Valkonen 2001). The use of chimeric

transgenes from pieces of genomes from viruses expected to infect the crop can overcome this problem (Chung et al. 2013). In developing countries, considerable efforts have been made to develop resistance to viruses affecting crops relevant to agriculture and food production. In addition to the above, the main target crops have been potato (*Solanum tuberosum* L.) (Orbegozo et al. 2016), tomato (*Solanum lycopersicum* L.) (Fuentes et al. 2006), peanuts (Arachis hypogaea L.) (Magbanua et al. 2000), sugarcane (Saccharum officinarum L.) (Ingelbrecht et al. 1999), peppers (Capsicum annuum L.) (Lee et al. 2009), rice (Oryza spp.) (Shimizu et al. 2009), papaya (Carica papaya L.) (Ferreira et al. 2002), passionfruit (Passiflora edulis Sims) (Trevisan et al. 2006) and soybean (Glycine max (L.) Merr.) (Reddy et al. 2001).

4.6.1 Transgenic Resistance to Potato Viruses

After rice and wheat, potato is the world's third-most cultivated food crop. The climate in highland areas is well suited to potatoes and Africa has grown rapidly. Root crops such as potatoes and sweet potatoes are also expected to be less affected by climate change than many other subsistence crops (Adhikari et al. 2015). The most common and devastating potato viruses in the world are potato viruses Y (PVY, a member of the genus Potyvirus; family Potyviridae), potato leafroll virus (PLRV, a member of the genus Polerovirus; family Luteoviridae) and potato virus X (PVX, a member of the genus Potexvirus; family Alpha flexiviridae). The PVY and PLRV transmitted by aphid may cause significant yield losses on their own. The PVX transmitted by contact becomes significant with PVY co-infection, which induces synergistic viral interactions leading to high PVX accumulation. In potato cv, virus-derived resistance was developed to PVY and PVX in order to solve this problem. Russet Burbank, which represents only the second example of genetically engineered resistance to the virus in crop plants (Kaniewski et al. 1990). In the late 1990s, "Russet Burbank" was also designed to resist PLRV, PVY and Colorado potato beetle (Leptinotarsa decemlineata Say) and approved for marketing under the name NewLeafTM (Lawson et al. 2001). Potatoes are propagated clonally and are therefore prone to viral infections over generations. In industrial countries, potato viruses are controlled by the planting of certified seed potatoes without viruses produced under special cultivation schemes. In combination with control of the aphid vectors using pesticides, this has reduced the prevalence of PLRV in the last 30 years. However, potato viruses are common in low-income countries and losses are severe, as healthy seed potatoes and pesticides are not frequently available or affordable (Valkonen et al. 2015). The introduction of virus resistance genes into new cultivars of potatoes is demanding and time consuming because of the highly heterozygous outcrossing and polyploid nature of potatoes. This combination of factors makes transgenic approaches to virus resistance especially appropriate for potato, as well as other major clonal crops such as bananas, cassava and yam (Dioscorea spp.).

Resistance to PLRV in "NewLeaf" reached commercial production in the USA, but the engineered variety only remained on the market a few years before it was

withdrawn due to the decision of major potato processing industries to refrain from the use of transgenic potatoes (Thornton 2003). The demand for "NewLeaf" was not high in the US market, as clean seeds can be purchased every year and pesticides are affordable, providing alternative means of controlling viruses. However, in low-income countries, virus-resistant potato varieties would be of great importance to prevent yield losses, as farmers rarely renew their seed potatoes. Resistance to both primary and secondary infections with PLRV has been achieved using efficient inverted repeat hairpin constructs (Orbegozo et al. 2016). High levels of resistance in transgenic plants expressing such hairpin constructs have also been obtained against PVY, PVX and the aphid-transmitted potato virus A (Missiou et al. 2004).

4.6.2 Viruses in Common Bean

Common beans (Phaseolus vulgaris L.) and vegetable crops such as tomatoes, peppers and cucurbits (Cucurbita spp.) are worldwide damaged by begomoviruses transmitted by whitefly (Leke et al. 2015), especially in Latin America (Morales and Jones 2004). The bean golden mosaic virus (BGMV) and the related bean yellow golden mosaic virus are one of Latin America's greatest constraints in bean production. The Brazilian Agricultural Research Corporation (EMBRAPA) was able to produce a transgenic line of common beans with high and stable levels of resistance to BGMV after almost two decades of work (Aragão and Faria 2009). In 2011, the EMBRAPA 5.1 transgenic line was approved for cultivation in 2011, and field trials for registration of several new cultivars developed from EMBRAPA 5.1 by breeding were initiated in 2012 (Faria et al. 2016). The resulting resistance is expected to allow the recovery of bean production in areas affected by BGMV, increase yields and quality, and reduce the need for vector control pesticides in Brazil. Due to differences between viruses and virus strains, these transgenic lines may not necessarily confer resistance to bean-infecting begomoviruses in other parts of the world. However, the approach to bean varieties that are resistant to the main begomoviruses found in other developing countries can be applied using an inverted repeat construct aimed at the viral replicase gene and a highly efficient transformation system.

4.6.3 Transgenic Virus Resistance and Impact on Low-Income Countries

Although no genetically engineered plants for virus resistance have been approved for cultivation in low-income countries, many efforts are ongoing, especially in Africa, where recurrent epidemics of viruses are a major constraint to crop production. These efforts are primarily focused on major staple and food safety crops such as manioc, sweetpotato, banana, rice and maize.

4.6.4 Cassava Mosaic and Cassava Brown Streak Diseases

As a subsistence crop in Africa, manihot esculenta Crantz is very important. The epidemic of manioc mosaics began and spread rapidly in East Africa in the mid-1990s, devastating manioc crops in many regions. The disease increased when African cassava mosaic virus and virulent recombinants co-infected cassava plants with other begomoviruses transmitted by whitefly, resulting in synergy, very severe symptoms, growth retardation and new virulent recombinants of the viruses (Zhou et al. 1997). More mosaic-resistant manioc germplasm was introduced to breeding programs in West Africa, and the new varieties eventually slowed the epidemic remains, and new means offered by biotechnology are being used in resistance breeding (Bart and Taylor 2017). As a subsistence crop in Africa, manihot esculenta Crantz is very important. The epidemic of manioc mosaics began and spread rapidly in East Africa in the mid-1990s, devastating manioc crops in many regions. The disease increased when African cassava mosaic virus and virulent recombinants co-infected cassava plants with other begomoviruses transmitted by whitefly, resulting in synergy, very severe symptoms, growth retardation and new virulent recombinants of the viruses (Zhou et al. 1997). More mosaic-resistant manioc germplasm was introduced to breeding programs in West Africa, and the new varieties eventually slowed the epidemic started in Tanzania, Uganda and Kenya in the mid-1990s (Alicai et al. 2007). Local resistance breeding programs have increased tolerance to symptom formation in new cassava varieties, but resistance to the brown streak viruses has not yet been achieved. However, transformation of the Ugandan farmer preferred cassava cultivar TME with a virus-derived inverted repeat construct appears effective against both brown streak viruses (Fondong 2017), but inadvertently resulted in the loss of resistance of CMD by the CMD2 gene, apparently as an unexpected consequence of the somatic embryogenesis process involved in regenerating transgenic plants (Beyene et al. 2016).

4.6.5 Sweetpotato Virus Disease

Sweetpotato (Ipomoea batatas Lam.) originates in South and Central America, but plays a particularly important role in Africa as a subsistence crop (Valkonen et al. 2015). During the above-mentioned CMD epidemic in the 1990s, the importance of sweetpotato increased. Sweetpotato is a crop that is generally healthy, with only a few diseases. More than 30 viruses infect sweetpotatoes, but most cause mild or no symptoms and only minor losses in yield. The main disease is SPVD, which has severe symptoms of leaf malformation and stunted growth of plants. Diseased plants may not produce for consumption any tuberous roots.

In sweet potato plants co-infected with SPCSV transmitted by whitefly and virtually any other sweet potato virus, SPVD develops (Cuellar et al. 2015). Targeting SPCSV with pathogen-derived resistance using different genomic regions of SPCSV as transgenes significantly reduces the accumulation of SPCSV in transgenic sweetpotato plants, but other sweetpotato viruses break down resistance and

cause severe symptoms (Kreuze et al. 2008). Studies show that SPCSV's double-stranded-RNA-specific RNase III enzyme suppresses antiviral RNAi by cutting small interfering RNAs used to target viral degradation of RNA (Cuellar et al. 2009).

4.6.6 Banana Bunchy Top Disease

Banana bunchy top virus (BBTV) is the world's most destructive viral pathogen of bananas and plantains. It comes from Asia, has probably been introduced to Africa from the South Pacific (Kumar et al. 2011 and Jooste et al. 2016) and has now reached most areas of sub-Saharan Africa (Cuellar et al. 2015). BBTV is currently a major concern for food security, as infected bunches are difficult for smallholder farmers to recognize and will not produce any usable fruit. By targeting the viral replicase gene with intron-hairpin RNA transcripts, Shekhawat et al. (2012) were able to generate high levels of resistance to BBTV by targeting the viral replicase gene with intron-hairpin RNA transcripts. This approach was also effective against other Nanoviridae family members of the virus. The challenge in introducing transgenic resistance to various banana cultivars is the sterility of vegetatively propagated cultivars and the need to separately transform each cultivar, which is not a trivial task. Since banana cultivars are essentially sterile, it is unlikely that transgenic flow to other cultivars or wild Musa species will occur. Since it is difficult to control the number of copies and integration sites of transgenes, the use of modern genome editing technologies could allow the targeting of specific host genes (Dale et al. 2017), for example, those that play a role in the susceptibility of viruses.

4.6.7 Rice and Maize Viruses

The rice yellow mottle virus (RYMV, a member of the Sobemovirus genus) causes a major disease in rice, which is used as an example of the key role of agricultural intensification in the emergence of plant viruses (Pinel-Galzi et al. 2015), and the virus can overcome recessive resistance genes in rice germplasm (Pinel-Galzi et al. 2007). However, as early as 1998, highly efficient resistance was achieved in transgenic lines of African rice varieties generated by the expression of RYMV's open reading frame 2. The resistance remained stable for at least three generations and conferred resistance to a wide range of RYMV isolates (Pinto et al. 1999), since rice is seed-propagated, transgenic resistance trait can be introgressed into local varieties via crossing. However, to our knowledge, these lines never progressed beyond the proof-of-concept stage. MSV causes maize streak disease, which is a major constraint in Africa's maize production (Shepherd et al. 2007). For the protection of crops from MSV, dominant and recessive natural resistance genes are available. In addition, the expression of a defective form of a viral gene involved in viral replication (Shepherd et al. 2010) is available in transgenic maize plants with engineered resistance to MSV and was the first transgenic crop plant developed in Africa. There

is a better system of transgenic expression that is only activated by MSV infection (Shepherd et al. 2014). Maize (corn) mortal necrosis caused by co-infection with maize chlorotic mottle virus and another potyvirus, maize mosaic virus maize dwarf mosaic virus was described in USA, but is currently ravaging East-Africa and threatening to spread further across the continent. The disease can be controlled by transgenic resistance against maize dwarf mosaic virus (Murry et al. 1993), which could provide a solution.

4.6.8 Naturally Transgenic Virus Resistance

Natural transgenes recently corresponding to Agrobacterium spp's transfer of DNA (T-DNA). The sweet potato genome was found to be integrated, raising the question of its possible role in host defense or crop domestication (Kyndt et al. 2015). Agrobacterium T-DNAs are also found in other plant species (Matveeva et al. 2012), similar to transgenic plants created by the transformation of Agrobacterium. However, viral sequences integrated into plant genomes are more commonly found, which is comparable to plants transformed by particle bombardment and leads to a random integration of DNA into the plant genome.

The integration of viral sequences in a plant genome was first carried out in bananas carrying fragments of banana streak virus, a Para retrovirus with a double-stranded DNA genome encapsulated in bacilliform particles (Harper et al. 1999), and later in other Para retroviruses. Petunia vein clearing viruses, for example, and tobacco vein clearing viruses-such as sequences are integrated into petunia and many solanaceous crops. In woody plants such as grapevine and fig, viral integrations have also been observed (Laney et al. 2012 and Bertsch et al. 2009). Some of the integrated sequences can be reactivated and cause disease if stress or other exceptional conditions affect plants.

However, para retroviruses are mostly dormant and the host plants are rather resistant to them; indeed, retroviruses integrated into the plant genome may confer resistance to infecting homologous viruses (Chabannes and Iskra-Caruana 2013) The most likely resistance mechanism is the silencing of RNA induced against endogenous sequences as a method to control viral expression. Sequences of other viruses of DNA and RNA are also found in plant genomes (Chiba et al. 2011). The integrated sequence of cucumber mosaic virus in soybean is structurally similar to the constructs of hairpin RNA designed to induce target-specific RNA silencing and resistance to viruses (da Fonseca et al. 2016).

4.7 Next Generation Quantitative Genetics in Plants

For almost a century, scientists have used quantitative trait loci (QTL) analysis to dissect the genetic architecture of quantitative traits in plants (Fisher 1918). These analyze associate genetic markers with the phenotypic variation of a quantitative trait in a segregating population. History has consistently improved the

techniques used to obtain markers and physiological phenotypes (Montes et al. 2007). The fall in prices of high-performance technologies has allowed plant researchers to quantify the general abundance of transcripts, proteins or metabolites in segregated populations (Drost et al. 2010). These studies show that there are multiple benefits in using "omic" technologies for QTL analyses, even when the goal is to characterize physiological phenotypic diversity. Firstly, molecular phenotypes are the first step towards the production of physiological phenotypes and their regulation is based on a great deal of phenotypic diversity (Stern and Orgogozo 2008). Secondly, the availability of information across the genome significantly increases the ability to identify candidate genes for QTLs (Jimenez-Gomez et al. 2010). Third, molecular characteristics measured at the system scale allow the estimation of the effect of QTLs on genetic pathways of interest, or the identification of other genetic networks altered by the loci responsible for the variation (Kliebenstein et al. 2006). Finally, molecular characteristics provide researchers with a better understanding of how mutation drives physiological variation and what are the evolutionary forces acting at primary levels. So it is clear that HTS will be the choice tool for QTL analyses very soon. An important limiting factor remains to be removed: Analysis of data. It requires long and computer-intensive pipelines to be customized for each specific experimental setup. An increasing number of new algorithms are constantly being released to the community, and the debate on which pipelines deliver the most accurate results continues. Comparing, combining and customizing these pipelines requires simple Unix or Linux commands and benefits greatly from knowledge in powerful statistical software such as R and in languages such as Perl or Python. For non-bioinformaticians, integrated solutions with convenient interfaces are becoming popular both from collaborative open projects and companies (Goecks et al. 2010). Www.seqanswers.com is a popular website that keeps an updated list of available software tools, where users and developers also discuss new technological advances and pipelines. The majority of tools are developed for Linux or Unix-based systems in terms of the computer equipment required for HTS data analysis. Although parts of the analysis can be carried out on any modern computer, machines with dozens of gigabytes of RAM are recommended in cases where reference sequences are available for the species concerned or with hundreds if no reference is available. An alternative option that is likely to be popular is to rent storage and computer power in specialist centers or "the cloud" (Stein 2010). Due to the rapid improvement in HTS, this review only aims to capture a snapshot of the opportunities it offers for the discovery, genotyping and molecular phenotyping of molecular markers in the segregation of plant populations. The purpose of this review is to help researchers who have not incorporated this technology into their work to think about HTS requirements and options. This review does not refer to all available experimental designs or analytical tools, and the solutions proposed here are merely suggestions that will soon be replaced by new and better ones.

4.7.1 Library Preparation

Sample preparation protocols are continually improved to use less biological material, to be completed faster and to reduce their output bias. For example, most current protocols allow multiplexing samples by adding a short sequence tag to all readings in a library, which is a convenient feature given the increasing number of readings per HTS run. The same companies that developed the HTS sequencers market library preparation protocols that are optimized for the most common experimental designs. Other companies also have kits that provide comparable results and can be more cost-effective. Finally, many researchers develop customized protocols for specific information such as the transcribed strand in RNA-seq experiments, the rate of RNA degradation, or the positions occupied by RNA polymerases, just to name a few (Parkhomchuk et al. 2009).

4.7.2 Quality Control and Pre-Processing

Detecting biases in the base composition, base quality and complexity of the sample assesses the quality of HTS readings. The quality of the sequences affects the reliability of the analysis 'biological interpretations (Dohm et al. 2008). The sample preparation protocols introduce parts of these biases, in particular during the synthesis of cDNA in RNA-seq experiments (Hansen et al. 2010) and PCR amplification (Aird et al. 2011). Further biases are specific to each HTS technology (Smith et al. 2008) or to each sequencer run (Auer and Doerge 2010). It is usually necessary to pre-process the readings by trimming low quality nucleotides and adapter sequences. At this stage, foreign sequences such as vectors or DNA from organisms contaminating the samples can also be removed.

4.7.3 Molecular Marker Discovery

Depending on the type of library, further pre-processing may be required, such as poly A or poly T tails and tails for terminal transferase in RNA-seq libraries. When multiplexing several libraries, reads should be separated by their barcode. With basic scripts written in Perl (Bioperl), R (Bioconductor) or Python, both quality control and pre-processing can be done easily. There are some convenient tools for non-programmers that can perform all or some of these tasks (Goecks et al. 2010; Schmieder and Edwards 2011). A cost-effective solution for obtaining molecular markers is the sequence of DNA or RNA from parental genotypes and polymorphisms from the resulting readings. These polymorphisms can later be used to design PCR markers or a genotyping test of high-performance for the entire population. This approach works remarkably well in diploid and polyploid species with a sequence of as low as 5, i.e. five times the genome size (Geraldes et al. 2011). A recent article reviews available methods and tools for the identification and genotyping of single nucleotide polymorphism (SNP) (Nielsen et al. 2011). In order to

align the readings with the reference, mapping software based on "seed methods" is preferred despite its slower nature because of its polymorphic strength. Before SNP, users can consider removing readings from the map to multiple locations in the reference and duplicate readings that may have been generated from PCR artifacts. A recent pipeline also recalibrates the quality of nucleotides in readings to correct high error rates in HTS, and realigns readings in complex genomic positions where rapid processing algorithms may have failed (Depristo et al. 2011). Commonly used indicators of the veracity of polymorphisms are based on the quantity and quality of readings showing polymorphism, frequency of observed alleles, alignment quality and/or proximity to other polymorphisms. There are some basic and popular options for calling polymorphisms from aligned reads (Depristo et al. 2011), tools for analyzing reads from specific sequencing platforms (Souaiaia et al. 2011), that have the ability to detect structural variation (Chen et al. 2008), or that have into account the quality of the reference in addition to the quality of the reads. High-performance sequencing sequences can be used to build the necessary reference to identify molecular markers if they are not already available. Although it is possible to assemble de novo a complete genome sequence with HTS, very deep sequencing and extensive bioinformatic analysis are required, especially given the relatively large size of most plant genomes. Sequencing mRNA is a more efficient option, which greatly reduces the complexity of the sample compared to genome sequencing and has the advantage of providing functional information such as polymorphism coding or levels of expression (Wei et al. 2011). A comprehensive compilation of the transcriptome assembly methods and tools has been recently published (Martin and Wang 2011). De novo assembly algorithms benefit greatly from long and paired readings, but are extremely sensitive to errors and polymorphisms and will not perform well during the assembly of mixed genotypes or highly heterozygous people. As the number of reads increases, the amount of new genomic positions detected in RNA-seq experiments decreases exponentially. The majority of medium and highly expressed transcripts in a sample are detected at low coverage, and increased coverage will mainly add non-coding RNAs and low expressed transcripts at very high costs (Tarazona et al. 2011). If the aim is to assemble complete transcriptomes, the sequencing depth is preferred to obtain samples from various tissues, time points and conditions. Even under the best possible conditions, RNA-seq reads will return only a subset of existing transcripts, many of which will be fragmented. This is expected due to the low expression of specific transcripts, the non-uniform reading coverage and the presence of various isoforms per gene. Researchers can use normalization protocols that deplete the most abundant transcripts from the samples to help assemble low-expressed transcripts (Christodoulou et al. 2011).). In any case, contigs resulting from *de novo* assembly can be effectively used as a reference for molecular marker detection and characterization of transcripts in un-sequenced genomes (Kaur et al. 2011). When comparing highly similar genotypes, RNA-seq may not be the best option, as it primarily targets less diverse coding regions than non-coding regions. In these cases, researchers can build reduced representation libraries by shearing DNA using endonucleases of restrictions and selecting the fragments to be sequenced. Readings from these libraries may be clustered by similarity and mined for polymorphisms near restriction

sites; or used to detect the presence of specific tags, indicating polymorphism at the restriction site itself (Etter et al. 2011). If a reference sequence is available, obtaining polymorphisms from reduced representation libraries is more efficient (Wu et al. 2010). However, researchers have already developed tools for genotype samples from these tags using a low number of readings from organisms without a reference (Ratan et al. 2010), or to reconstruct part of the targeted genome using paired-end sequencing (Willing et al. 2011). There are additional protocols for obtaining markers from reduced representation libraries in which different combinations of restriction enzymes are used for each of the genotypes involved (Hyten et al. 2010), or in which the DNA is not screened, but the readings are filtered for single copy sequences. The amount of reads necessary to perform this type of analysis depends on the size of the genome, the restriction enzymes used, and the availability of a reference (You et al. 2011).

4.7.4 Genotyping Populations

With the fall in prices of HTS technologies and the possibility of multiplexing samples, genotyping has become realistic for the entire population (Schneeberger and Weigel 2011). In the case of a sequenced system such as rice, the generation of readings from individuals in a population of 0.02–0.055 coverage allowed the genotyping of high density by comparison with parental genotypes (Huang et al. 2009), or by inferring the parental genotypes from the polymorphisms found in the population (Xie et al. 2010). Since erroneous calls for polymorphism are expected at low coverage, it is necessary to define more or less complex algorithms to correctly genotype each polymorphism in each individual (Huang et al. 2009;; Xie et al. 2010). In addition, a reference sequence can serve researchers to design enrichment essays that will target their preferred genomic locations, although at high cost (Kenny et al. 2011). For species where a genome sequence is not available, a very practical approach is to sequence reduced representation libraries as mentioned above (Hohenlohe et al. 2010).

4.7.5 Molecular Phenotyping

The list of molecular phenotypes that can be quantified with HTS is extensive and is rapidly increasing (Hawkins et al. 2010). Examples of these phenotypes are protein–RNA interactions, translation rates (Ingolia 2010), transcription rates (Churchman and Weissman 2011), protein–DNA interactions (Barski et al. 2007), RNA degradation rates (Addo-Quaye et al. 2008), RNA secondary structure (Underwood et al. 2010), transcription start positions (Plessy et al. 2010), chromatin accessibility (Boyle et al. 2008), methylation states (Cokus et al. 2008), natural antisense transcription (Parkhomchuk et al. 2009) or small RNA profiles (Lu et al. 2005). QTL analysis using these phenotypes as traits is an exciting field that remains un-explored. Therefore, the computational frameworks to quantitatively compare these phenotypes between individuals will need to be established.

Although many cases of phenotypic variation caused by coding polymorphisms have been documented, it has been shown that the variation in gene expression is the basis of much phenotypic diversity (Reviewed in Stern and Orgogozo 2008). One way to detect differences in expression between people using HTS is to sequence 26–27 tags of nucleotides from expressed transcripts (Hong et al. 2011). A recent study shows that this method reaches 6–8 million reads per sample saturation in mice (Hong et al. 2011). Its advantages over the sequencing of full transcripts are lower costs, higher sensitivity, reduced bias during amplification due to the fixed length of fragments and simplified statistical models to calculate differential expression. Methods based on tags, on the other hand, do not detect most coding polymorphisms and isoforms and require a sufficiently close reference sequence to extract biologically relevant results. Due to its simple preparation protocol, digital nature, large dynamic range and high sensitivity compared to previous technologies, RNA-seq is rapidly becoming a standard in expression profiling (Liu et al. 2010a, b). It can also be used to genotype people, identify new transcripts, characterize alternative splicing and quantify the specific expression of alleles (Reviewed in Costa et al. 2010). The novelty of the technique means that there is no consensus on the preparation of the sample preparation protocols presents fewer biases (Raz et al. 2011). Due to their increased precision, however, strand-specific methods could become a standard due to their ability to distinguish between sensory and antisense transcripts (Levin et al. 2010). As with any other type of genome-wide analysis (Auer and Doerge 2010), biological samples must be randomized and replicated in terms of experimental designs. There is little consensus on the sequence depth required for RNA-seq profiling. Recent estimates range from 30 million readings to compare the expression profiles of two samples, to 100 million readings to detect most transcribed genes and quantify isoforms, to 500 million readings to obtain accuracy including low expressed transcripts (Zhang et al. 2010). In any case, it is advisable to balance the number of reads between samples in the same experiment in order to perform accurate expression comparisons (Tarazona et al. 2011). The profiling of expression from HTS data sets is necessarily based on a reference sequence of the reads mapped to each transcript. If a reference genome or transcriptome is not available, at least one of the genotypes described above can be reconstructed using a de novo read assembly. The simpler and less computational intensive protocol for profiling expression is to map the RNA-seq reads to known transcripts (or de novo assembled) and a set of possible exon-exon junctions (when available) to detect alternative splicing. However, this protocol will not allow the detection of new exons, transcripts and isoforms in organisms with sequenced genomes this protocol will not allow detection of novel exons, transcripts, and isoforms. The preferred pipeline involves aligning the reads to the genomic reference using an alignment tool that splices the reads to detect intron–exon junctions (Wang et al. 2010; Lou et al. 2011). The need for robust quantification of readings generated from two or more alleles is a challenge for expression analyzes in samples from two unrelated persons. This implies that readings with the closer genotype to the reference are better aligned than readings from a more distant genotype, in which more polymorphisms can interfere with their mapping capability (Fontanillas et al. 2010). Aligners

based on seed methods will perform better in these cases than those based on the algorithm Burrows-Wheeler Transform (see Garber et al. 2011 for a review). Although most studies ignore this problem, the polymorphisms that cause these biases are identified and removed. (Degner et al. 2009), aligning the reads to all references from the genotypes involved (Bullard et al. 2010) or including the polymorphisms found in the references (Gan et al. 2011). When two references are used, a potential problem may arise from motifs that are more abundant in one reference with respect to the other if only uniquely mapped reads are counted. The use of longer reads and/or paired end reads greatly decreases the number of ambiguously mapped reads. In addition, there are robust methods to assign these multimapped reads to a single location (Wang et al. 2010; Ji et al. 2011). There are a number of tools to count the number of reads aligned to each transcriptional unit to calculate expression, most of which require knowledge of Perl, Phyton, Linux/Unix, or R (Anders and Huber 2010 and Morgan and Pagès 2010). Some alignment tools can directly calculate the number of reads per transcript and/or a measure of expression based in the reads (or fragments) per gene size in kilobases per million reads mapped, called RPKM (or FPKM; Mortazavi et al. 2008; Trapnell et al. 2010). However, these expression units show biases depending on the length, number, abundance of the transcripts present in the samples, or because of technical replication (Mcintyre et al. 2011). For this reason researchers have developed dedicated R/Bioconductor packages to calculate differential expression between samples based on raw read counts per transcript (Anders and Huber 2010). In addition, there are software packages that take into consideration the biases inherent to RNA-seq when calculating expression or performing downstream analyses such as gene ontology over-representation studies (Young et al. 2010; Zheng et al. 2011).

High-performance sequencing data sets allow expression quantification for each isoform separately, resulting in significantly more accurate estimates than gene expression calculation (Wang et al. 2010). Users must first identify splicing events from reads that align to exon-exon junctions for this purpose. Quantifying isoform expression is complicated because most reads can not be assigned to a single isoform in an alternative spliced transcript. The most promising methods of addressing this complex problem use the information provided by paired end and/or unambiguously mapped reads (Trapnell et al. 2010; Nicolae et al. 2011). One advantage of the complex process of identifying alternative splicing is that it can also be used as a feature for QTL analysis (Lalonde et al. 2011).

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