Chapter 1
Generation and Deployment of Transgenic Crop Plants: An Overview

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1.1 Introduction

As biotechnology increasingly affects almost all aspects of human life, it is essential that the science behind this technology is explained in simple terms to the public to eliminate the misconceptions that may inhibit its acceptability. The basic question that is often asked is what is a gene, a promoter and a terminator? Genes are the basic units of heredity, composed of DNA sequences, which are transmitted from parents to offspring and which, independently or in combination with other genes, control specific traits in an organism. These traits may be, for example, plant height, flower color, fruit and seed size together with regulatory processes, such as assimilate partitioning and drought resistance. Genes are the basis for both the similarity and differences that exist among organisms, and are transmitted from one generation to another. Promoters are DNA sequences that are recognized by RNA polymerase in plant cells and that initiate and regulate transcription, the initial and most important step of gene expression. Terminators are those sequences that command or signal the termination of transcription.

It is possible to identify and to isolate genes from plants, animals, and microorganisms, to modify their promoters, structural sequences and terminators, and to introduce and express chimeric genes in the same or other genus, species, or cultivar. Consequently, it is feasible to control or modify physiological processes. Gene manipulation, combined with the ability to induce cultured plant cells to express their totipotency leading to the regeneration of fertile plants, provides a

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unique opportunity to extend the genetic pool available to breeders for crop improvement.

The successful development of transgenic plants necessitates a reliable tissue culture regeneration system, gene construct(s), suitable vector(s) for transformation and efficient procedures to introduce desired genes into target plants. Once transformation has been performed, it is essential to recover and to multiply the transgenic plants. The latter must be characterized at the molecular and genetic levels for stable and efficient gene expression (Sharma et al. 2005). It may also be necessary to transfer the introduced genes to elite cultivars by conventional breeding.

Prime targets for genetic manipulation include modification of plants to enhance their tolerance to the herbicides used to control weeds, and to confer resistance to insects, bacteria, fungi, and viruses, since these agents account for major crop losses. Other targets include the genetic engineering of plants for biosynthesis of health-care products, increased nutritional value, extension of the shelf-life of crops that deteriorate rapidly following harvest, and tolerance to abiotic stress. Similarly, although not essential for human existence, modification of the esthetic appeal of plants has considerable commercial potential.

1.2 Target Cells and Organelles for Genetic Transformation

A reliable tissue culture-based shoot regeneration system is a pre-requisite for plant genetic transformation. The recognition that, under optimum hormonal and nutritional conditions, somatic cells are totipotent and can be stimulated to develop into whole plants in vitro via organogenesis (shoot formation) or somatic embryogenesis, forms the basis of regeneration in tissue culture (Sharma et al. 2005). Genetic transformation without plant regeneration is of limited or no value. Hence, the identification of explants (cells/tissues/organs) that are capable of regenerating into plants is fundamental to any transformation procedure. Isolated protoplasts (Davey et al. 2005), callus and suspension cultured cells (Rachmawati and Anzai 2006), thin cell layers (Soneji et al. 2007a), leaf disks (Li et al. 2007), root sections (Huang and Ma 1992), stem segments (Song et al. 2006), floral tissues (Zale et al. 2008), epicotyls (Soneji et al. 2007b), hypocotyls (Wang and Xu 2008), cotelydonary nodes (Yi and Yu 2006), and axillary buds (Manickavasagam et al. 2004) have been used for genetic transformation. Explants of mature organs have also been used as target material in transformation experiments to overcome juvenility (Cervera et al. 1998). Tissue culture systems for several plants have been summarized (Khachatourians et al. 2002; Curtis 2004; Loyola-Vargas and Vázuez-Flota 2005) together with aspects of gene introduction into target plants using such systems (Birch 1997; Newell 2000; Sharma et al. 2005; Davey et al. 2008).

Shoot regeneration from cultured cells may lead to chromosomal or genetic variation known as “somaclonal variation.” This variation may be useful or detrimental. Tissue culture also requires extensive facilities for maintenance and manipulation of axenic explants, which is labor intensive and expensive.

Consequently, approaches have been reported that reduce or eliminate in vitro procedures. For example, genes have been inserted into pollen and the latter used for fertilization to produce transgenic seed (Saunders and Matthews 1995; Häggman et al. 1997), while Clough and Bent (1998) described a "floral dip" procedure that is discussed later.

In most investigations, gene insertion has been directed primarily to the nuclei of recipient plant cells. Additionally, plastid transformation has been established in several laboratories (Heifetz 2000; Daniell et al. 2002; Maliga 2002, 2004). Extension of plastid transformation to more species constitutes a logical step in the development of genetic manipulation technology (Bock and Khan 2004) as plastid transformation has several advantages for the engineering of gene expression in plants. These advantages include 10–50 times greater transgene expression in plastid genomes, compared to nuclear-inserted genes (Liu et al. 2008a). The plastid genome provides readily obtainable high protein concentrations and the possibility of expressing multiple proteins from polycistronic mRNAs from a single promoter (Maliga 2002). Importantly, uniparental plastid gene inheritance in most crop plants prevents pollen transmission of foreign DNA (Heifetz 2000). As transgenes integrate into the plastid genome via homologous recombination, this facilitates targeted gene replacement and precise transgene control, while sequestration of foreign proteins in plastids prevents adverse interactions with the cytoplasmic environment. Maliga (2004) and Verma and Daniell (2007) discussed the design of vectors for plastid transformation and the selection of transplastomic plants. To date, plastid transformation has been reported in cabbage, lettuce, oilseed rape, petunia, poplar, potato, tobacco, and tomato, with transplastomic plants being regenerated by organogenesis in these cases, or by somatic embryogenesis in carrot, cotton, rice, and soybean (Verma et al. 2008). Extension of plastid transformation to other major crop plants still necessitates reproducible explant, cell, or protoplast-to-plant regeneration systems.

1.3 Methods for Introducing Genes into Plants

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of developing into a whole transgenic plant (Sharma et al. 2005). Once a reliable shoot regeneration system is available, foreign DNA can be introduced into cells by either vector-mediated or direct transfer. Although the technology associated with the construction of chimeric genes is becoming more routine and simple, the transformation process itself remains a comparatively rare event. Consequently, the procedure must be robust and combine reproducible culture of recipient plant cells with efficient gene delivery. Gene transfer experiments focus mainly on maximizing the efficiency of recovery of stably transformed plants, and extending the range of species that can be engineered using a specific procedure.

Agrobacterium-mediated gene transfer and direct DNA transfer into cells by microprojectile bombardment (Fig. 1.1) are the most widely exploited methods for introducing genes into plants because of their ability to transform intact, regenerable tissues and organs. Although aspects of the precise molecular events of *Agrobacterium*-mediated gene delivery are still not fully understood, particularly the transfer and integration of the T-DNA (transferred DNA) from the bacterial tumor-inducing (Ti) plasmid of *Agrobacterium* into the nuclei of recipient plants, *Agrobacterium*-mediated gene delivery remains the preferred method of plant transformation in many laboratories. Lacroix et al. (2006a, 2006b) and Tzfira and Citovsky (2006) proposed mechanisms for the process. Knowledge of foreign gene integration into plant genomes is essential for precise gene targeting in the future.

Immersion of totipotent explants in a suspension of *Agrobacterium* is the main procedure for plant transformation. Several parameters affect transformation, including bacterial virulence, incubation temperature, age of the bacterial suspension, and the cocultivation period of the bacteria with the explants (Gelvin 2003; Wu et al. 2003). Sonication promotes gene delivery (Liu et al. 2006). In planta procedures have been developed to simplify the transformation procedure. Thus, the "floral dip" technique (Clough and Bent 1998) involves immersion of developing flowers in suspensions of *Agrobacterium*, followed by growth of the plants to maturity, the harvesting and germination of seeds, and the selection of transformed seedlings. This procedure, used routinely to transform *Arabidopsis thaliana*, has facilitated progress in understanding the genetics of this plant that is exploited extensively as a model in plant genetics and molecular biology. Chung et al. (2000) compared floral spraying with the floral dip procedure and reported comparable results with the two methods, enabling floral spraying to be used for transforming plants which are too large for the floral dip approach. Probably, in planta techniques will assume increasing importance for gene delivery.

Particle (microprojectile) bombardment has also been exploited extensively for plant transformation (Sharma et al. 2005; Davey et al. 2008) with instruments such as the helium driven HE-1000 device, facilitating technology transfer between laboratories. Microprojectile systems involve high-velocity particles penetrating cell walls and introducing DNA into cells, circumventing the host range limitations of *Agrobacterium*. This transformation procedure is versatile, independent of plant cell type and genotype, and has permitted the transformation of some of the most recalcitrant plants, such as cereals and legumes (Altpeter et al. 2005). Importantly, simple gene constructs, comprising only a promoter, the gene coding sequence and a terminator, may be used for transformation. A criticism of particle bombardment and *Agrobacterium*-mediated gene delivery is the complexity of patterns often associated with the integration of genes into recipient plants, especially with particle delivery. This necessitates detailed molecular analyses to select individuals carrying simple integration events, as such transformed plants are more applicable to longer-term breeding programs. Undoubtedly, the two procedures will continue

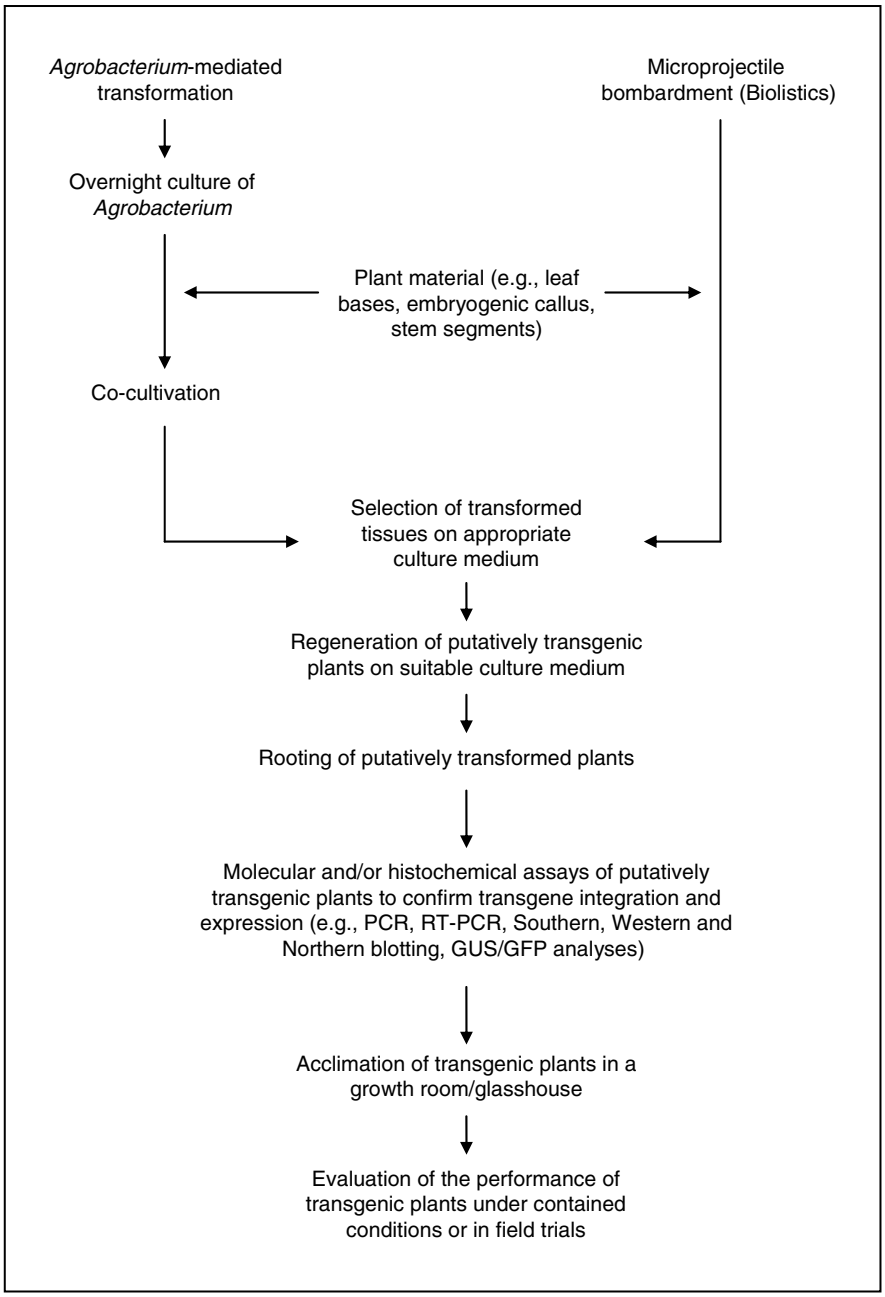

Fig. 1.1 A generalized flow chart depicting the steps involved in plant transformation

to be exploited routinely for gene delivery to plants, the procedure used depending upon the product required and the expertise of the personnel.

Other gene delivery procedures have been exploited, including uptake of DNA into isolated protoplasts, treatment with polyethylene glycol, and/or electroporation inducing DNA uptake. However, the development of robust protoplast-to-plant systems is a labor-intensive, specialized part of the procedure. Several parameters influence transformation, including the stage of the cell cycle of the recipient protoplasts, temperature, pH, and the intensity and duration of the electric field. Even with optimal conditions, the frequency of stable transformation is low and rarely exceeds one transformed cell in every 10^4 treated protoplasts. Protoplast transformation was the procedure of choice for monocotyledons, particularly cereals such as rice, but was superseded by particle bombardment and, more recently, by *Agrobacterium*-mediated gene delivery. Rakoczy-Trojanowska (2002) and Sharma et al. (2005) discussed transformation procedures involving micro- and macroinjection, the use of silicon carbon fibers, and pollen-tube-mediated DNA delivery. Virus-based DNA delivery methods have been reported (Chung et al. 2006). The real success and application of several transformation procedures remains unclear. Specific crops necessitate particular adaptation of techniques to generate transgenic plants, an excellent example being provided by some of the difficulties encountered in applying *Agrobacterium*-mediated gene delivery developed for rice to other cereals (Shrawat and Lörz 2006). However, gene sequencing, as in rice (Matsumoto et al. 2005), and general advances in plant bioinformatics, will facilitate broader application of transformation technology.

1.4 Vector Construction and Genes for Plant Transformation

Vector development has proceeded from the cointegration of foreign genes into the T-DNA region of Ti plasmids, to the construction of disarmed binary and superbinary vectors (Komori et al. 2007; Davey et al. 2008). As Tzfira et al. (2007) explained, although binary vectors were initially revolutionary, subsequent generations of vectors have had more versatility, often being designed for specific transformation purposes (Chung et al. 2005). Some vectors have incorporated recombinase-mediated gene cloning (Karimi et al. 2002). Importantly, advances in vector construction have enabled *Agrobacterium*-mediated transformation to be exploited for gene introduction into monocotyledons (Cheng et al. 2004), as well as dicotyledons. New gene expression technologies developed for nonplant systems rapidly become adapted and exploited in plant biology (Tzfira et al. 2007). This emphasizes the necessity for plant biologists to recognize and exploit developments in fields of research other than their own. A schematic representation of the steps involved in the construction of vectors for plant transformation is shown in Fig. 1.2.

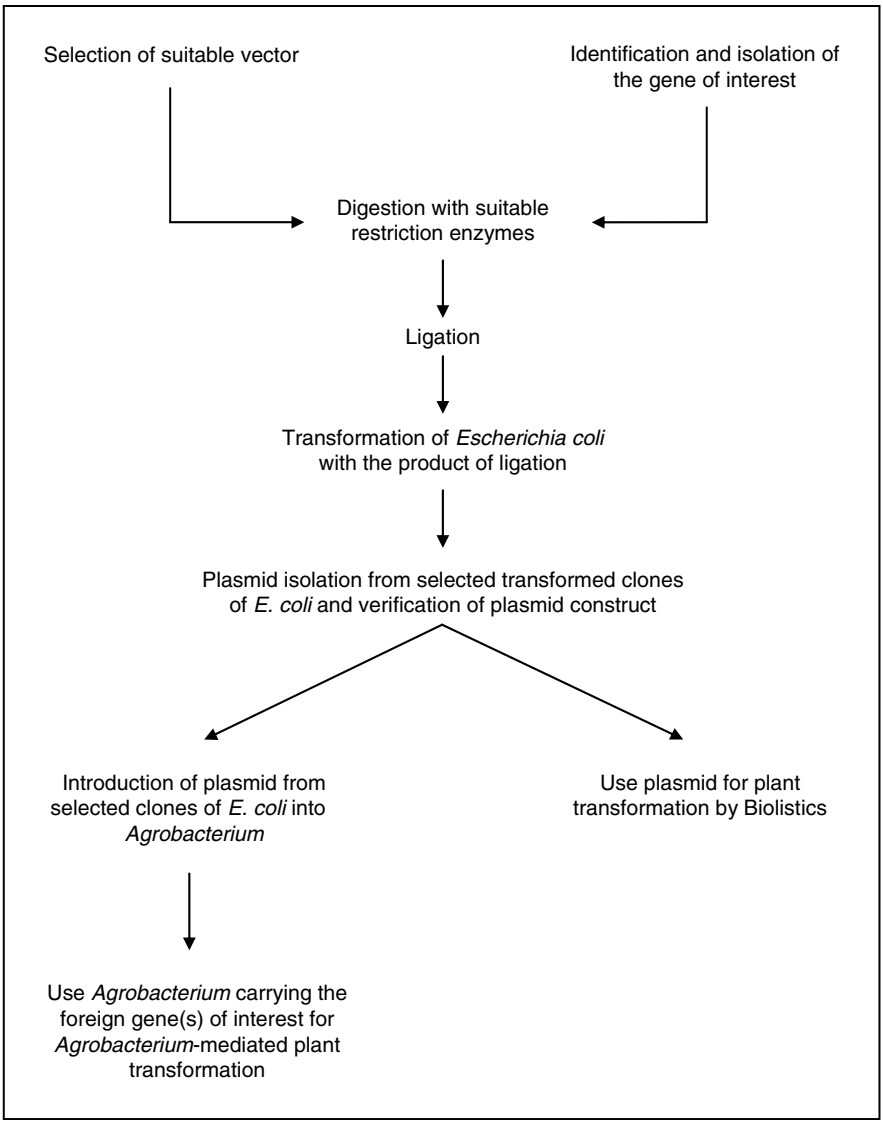

Fig. 1.2 Flow chart depicting the steps involved in the construction of vectors for plant transformation

1.4.1 Promoters for Plant Transformation

Efficient and reliable procedures are essential for constructing vector(s) for plant genetic engineering. Venter (2007) highlighted the importance of focusing attention on promoter construction, because the choice of promoter and its fine-tuning

determine constitutive, spatial, and/or temporal transgene expression. Considerable effort has focused on gene promoters. Efficient expression of genes is assured only when they are controlled by plant-derived promoters, or by promoters that are active in plant cells, such as the cauliflower mosaic virus 35S promoter (CaMV 35S). In early transformation assessments, the choice of promoter was governed by promoter availability. The *nos* promoter from the nopaline synthase gene of the T-DNA of the Ti plasmid of *A. tumefaciens* was one of the first to be used in plant genetic engineering, with the 35S promoter from CaMV also featuring in many of the early transformation assessments. Subsequently, other constitutively expressed viral promoters were evaluated, including those from cassava vein mosaic virus (CsVMV), sugarcane bacilliform badnavirus (ScBV), and figwort mosaic virus (Samac et al. 2004; Govindarajulu et al. 2008). The CaMV 35S promoter may have a negative effect on transgene expression in some plants (Yoo et al. 2005). A limitation of the promoters of viral origin is that host plants may recognize and inactivate these sequences (Potenza et al. 2004). However, this may be negated by using promoters of plant origin. Indeed, several promoters including those from *Medicago truncatula* (Xiao et al. 2005), *Vigna radiata* (Cazzonelli et al. 2005), and the tobacco EI1α together with the Cab promoters (Aida et al. 2005) have been evaluated.

Constitutive expression at the incorrect time may have a serious negative effect on plant development, emphasizing the need to refine the promoters for transgene expression. Tissue-specific promoters fulfill this requirement. Examples include a tissue-specific promoter driving a β-1, 3 gluconase gene in pea (Buchner et al. 2002), promoters from fruit-ripening and seed-specific genes (Zakharov et al. 2004) particularly seed storage glutelin genes (Qu et al. 2008) and promoters of glycoproteins in tubers and roots. Flower-specific promoters have application in the genetic manipulation of fruit trees and ornamental plants (Annadana et al. 2002; Sassa et al. 2002). Comparisons of promoter function are important, a cotton α-globulin promoter being evaluated in cotton, *Arabidopsis*, and tobacco (Sunilkumar et al. 2002). Potenza et al. (2004) provided a schematic representation of the sources of many promoters. Tissue-specific promoters have been combined with RNA interference (RNAi) technology to modify flower pigmentation (Nakatsuka et al. 2007a). Modification of promoters may result in changes in tissue and developmental specificities (Kluth et al. 2002). Promoters of considerable potential are those associated with the interaction of plants and microorganisms, such as root-specific promoters involved in nutrient uptake and legume-*Rhizobium* symbiotic associations. These promoters from green tissues confer light-inducible and tissue-specific expression. Cell-type-specific promoters are available, such as those from trichomes, guard cells and stomata, root hairs, phloem (Zhao et al. 2004; Guan and Zhou 2006), and cortical cells (Fruhling et al. 2000). Vectors for plastid transformation normally employ promoters from the plastid genomes of the target plants.

Some plant promoters are induced by biotic and abiotic stress (Pino et al. 2007), wounding (Yevtushenko et al. 2004; Luo et al. 2006), iron deficiency (Kobayashi et al. 2007), and exogenously applied chemicals. The latter include antibiotics,

steroids, copper, ethanol (Peebles et al. 2007), inducers of pathogen-related proteins, herbicide safeners and insecticides (Padidam 2003). Synthetic promoters have been assembled, such as a chimeric endosperm-specific promoter for cereal transformation (Oszvald et al. 2008). Liu et al. (2008b) constructed a novel pollen-stigma and carpel-specific promoter, which has potential in controlling pollen and seed-mediated gene flow from genetically manipulated plants. However, some synthetic promoters are unsuitable for plant transformation. For example, the (AocS)(3)AmasPmas promoter driving the *bar* gene for herbicide tolerance inhibited shoot regeneration (Song et al. 2008). Synthetic promoters, with the minimum of sequence similarity, could reduce homology-dependent gene silencing in transgenic plants during gene pyramiding experiments. Indeed, the availability of a broad spectrum of promoters that differ in their ability to regulate temporal and spatial expression patterns of transgenes could increase dramatically the success of transgenic technology (Potenza et al. 2004). Promoter development is still in its infancy. Major advances in transcriptomics, proteomics, and genome sequencing (Yu et al. 2007) will contribute to future development of promoters to drive gene expression in specific cells and tissues.

The correct assembly of constructs for plant transformation is fundamental for maximum gene expression at the correct time in target tissues (Butaye et al. 2005). The merit of bidirectional as well as unidirectional promoters necessitates consideration. Undoubtedly, continued advances in plant genetics, bioinformatics, systems biology, and high through-put gene expression technology will be crucial in predicting coordinated gene expression and the design of synthetic promoters. Terminator sequences must also originate from plant sources or from plant pests such as the CaMV or *Agrobacterium*. Although most investigations are targeted to maximizing gene expression in transgenic plants, the ability to silence genes is equally important in some cases, virus-induced gene silencing (VIGS) being a way of down-regulating expression (Robertson 2004).

1.4.2 Reporter and Selectable Marker Genes

Transformation, being a rare event, requires an efficient selection system to distinguish between transformed and nontransformed plant cells. Reporter genes enable cells and tissues to be monitored soon after the transformation procedure to assess the success of a specific construct and/or protocol. Such genes may permit the manual or automated selection of transformed from nontransformed cells, but do not enable transformed cells to outgrow their nontransformed counterparts in culture. In contrast, selectable marker genes provide transformed cells with a competitive advantage, enabling them to outgrow nontransformed cells in vitro, usually in the presence of specific substrates in the culture medium.

Although more than 50 genes have been exploited in nuclear and plastid transformation strategies, only a limited number are used routinely (Miki and McHugh 2004). The *uidA* (*gusA*) gene for β-glucuronidase is a versatile reporter.

In fluorometric and histochemical assays, cleavage of the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) by β-glucuronidase results in an indigo compound that is readily observed in transformed cells. A disadvantage of the GUS assay is its destructive nature. Consequently, it has been superseded in many investigations by more versatile, nondestructive assays based on expression of the luciferase (*luc*) gene, or the green fluorescent protein (*gfp*) gene, the latter from the jellyfish, *Aequorea victoria*. Mutant versions of the *gfp* gene that emit blue, cyan, and yellow light are available. Novel proteins from reef coral organisms that fluoresce cyan, red, green, and yellow have also been developed as nondestructive reporters for plant transformation (Wenck et al. 2003). Importantly, significant differences in the excitation and emission wavelengths of some of these proteins permit simultaneous visualization of more than one of these fluorescent proteins in transformed cells. Dixit et al. (2006) emphasized the importance of fluorescent proteins to image dynamic processes within plant cells, highlighting some of the practical issues in exploiting these proteins for live cell imaging. Genes for anthocyanin and carotenoid biosynthesis have also been used to visualize transformed cells prior to their manual selection.

Selection systems have been reported that encourage the growth of transformed cells, although "escapes" may occur, with some nontransformed cells growing in the presence of a selective agent. Commonly used selection systems employ tolerance to antibiotics, particularly kanamycin, encoded by the neomycin phosphotransferase (*npt*II) gene, and to hygromycin through expression of hygromycin phosphotransferase (*hph*, *hpt*, *aph*IV) genes. Phosphinothricin and glyphosate have featured in selection systems based on herbicide resistance, tolerance to phosphothricin being encoded by expression of the *bar* (*pat*) gene, while the *aroA*, *cp4*, and *epsps* and *gox* genes confer tolerance to glyphosate. Streptomycin and spectinomycin have been used to select transplastomic plants. Recently, Pinkerton et al. (2008) introduced resistance based on the enzyme organophosphate hydrogenase, encoded by the bacterial *opd* gene, to generate a new scorable and selectable marker system for transgenic plants. Some investigators have focused on plant genes as selectable markers. For example, Yemets et al. (2008) based selection on a modified plant α-tubulin gene that conferred resistance to dinitroaniline herbicides, with trifluralin as the selective agent. Ogawa et al. (2008) used a mutated rice acetolactate synthase gene to select transgenic plants of wheat. Acetolactate synthase catalyzes the first step in the biosynthesis of the essential branched-chain amino-acids, isoleucine, leucine, and valine, and is a target enzyme for several herbicides. Other procedures have incorporated toxic drugs and metabolite analogs into the culture medium. Genes that stimulate cytokinin biosynthesis stimulated shoot regeneration from transformed cells without the need for selection based on toxic compounds (Zuo et al. 2002). The *Escherichia coli pmi* gene for phosphomannose isomerase converts mannose-6-phosphate, an inhibitor of glycolysis, to fructose-6-phosphate, an intermediate in glycolysis. Expression of *pmi* in plant cells allows transformed cells to grow on medium containing mannose, as in the case of transgenic flax, following *Agrobacterium*-mediated transformation (Lamblin et al. 2007). Future legislation will, almost certainly, demand the elimination of antibiotic resistance genes as

selectable markers. Although selectable markers are generally indispensable in plant transformation protocols, they are not required once transgenic plants have been generated. General strategies to eliminate selectable marker genes have been reported. Jia et al. (2006) exploited the Cre/*lox* site-specific recombination system, while Charng et al. (2008) developed an inducible transposon system to terminate selectable marker gene function in transgenic plants. More detailed description of selection systems is presented by Miki and McHugh (2004) and Davey et al. (2008).

1.5 Methods for Screening of Genes Introduced into Putatively Transformed Plants

The strategies used for screening transformed plants usually depend on the type of selectable marker and/or reporter gene used. When an antibiotic resistance gene is employed as a selectable marker, screening is performed by culturing the transformed cells on a medium containing that particular antibiotic (Soneji et al., 2007b, 2007a). In the case of reporter genes, screening is for the distinctive phenotype (Chalfie et al. 1994). However, putative transgenic plants selected by scoring for the presence of selectable marker and/or reporter genes need to be evaluated for the integration and expression of the transgene(s) to minimize escapes. Polymerase chain reaction (PCR)-based screening techniques are used to assess the presence of a specific DNA sequence of the foreign gene of interest, or the selectable marker/reporter gene by screening putative transgenic plants with primers specific to these gene(s) (Xu et al. 2005; Soneji et al. 2007b, 2007a). Southern hybridization confirms the presence of transgenes and their copy number (Bhat and Srinivasan 2002). Enzyme-linked immunosorbent assay (ELISA) is the preferred method to detect the presence of a specific protein produced by a transgene in a recipient plant. Real-time polymerase chain reaction (RT-PCR) is utilized when more than one gene needs to be analyzed by PCR, along with the detection of the copy number of the desired gene(s) (Yuan et al. 2007).

1.6 Gene Expression in Transgenic Plants

Integration of transgenes into the genomes of plants is a random process, necessitating investigations of their expression in transformed plants. Expression is influenced by several parameters, including the site and pattern of integration, the location of heterochromatic regions, the presence of enhancer elements, the nature of the promoter, gene copy number, truncation, rearrangement, silencing and the presence of any DNA sequences from the vector into which the foreign DNA has been cloned. Although some of these factors can be circumvented by experimental design, it is still necessary to correlate phenotypic differences between transgenic

and control plants with transgene expression (Page and Minocha 2005). Thus, transgenic plants require detailed phenotypic, physiological, and molecular analyses to complete their characterization. Techniques such as Western blotting, Northern blotting, ELISA, and quantification and localization of mRNA transcripts are used to analyze transgene expression. These assessments are essential, especially when transgenic material is incorporated into breeding programs.

The use of genetic manipulation in crop improvement also requires transgenes to be expressed either constitutively or in specific cell or tissue types, often at definite stages of plant development (Perret et al. 2003). Although individual transgenic plants within a population may be phenotypically identical, generally they all differ in some subtle way at the molecular level. This emphasizes the requirement to generate as many transgenic plants as possible from an individual experiment and to analyze the maximum number of the regenerants at the phenotypic and molecular levels (Bhat and Srinivasan 2002). Currently, there is no reliable procedure to target foreign genes to specific regions of the genome of plants. It may also be necessary to test individual promoters to establish their expression patterns in different species (Perret et al. 2003). While gene targeting by homologous recombination is potentially extremely important, the development of a routine procedure that incorporates this process remains a major challenge (Cotsaftis and Guiderdoni 2005).

In order to determine the value and application of transformed plants, it is important to understand the inheritance and stability of introduced gene(s). Transmission and segregation analyses of the transgene(s) in subsequent progenies allow insight into transgene inheritance (Yin et al. 2004). *Agrobacterium*-mediated transformation, as well as direct DNA uptake, enables foreign genes to be integrated at a single Mendelian locus, regardless of copy number (Spencer et al. 1992). Stably integrated transgenes are usually inherited in a dominant, Mendelian fashion. However, in subsequent generations, some instability may be observed probably due to rearrangements or methylation of the T-DNA region, and/or to homologous recombination between copies of the transgene inserted into the same nucleus. A non-Mendelian segregation pattern is usually associated with unstable transformation or poor transgene expression (Limanton-Grevet and Jullien 2001).

1.7 Target Genes for Genetic Transformation

Major advances in gene isolation, vector construction, and DNA delivery enable plants to be modified for specific traits, providing an important underpin to conventional breeding. Although genetic engineering reduces the time to integrate desired genes into target plants, it will not replace gene manipulation by sexual hybridization. It has been emphasized that many of the constraints associated with conventional breeding can be overcome by advances at the molecular level (Dalal et al. 2006). Transgenes to be introduced into plants are selected on the basis of their economic/agronomic importance. Recent advances in DNA array technology allow

researchers to detect sets of genes that function co-ordinately in the biological processes of interest (Gachon et al. 2005). Several constructs have been developed for use in gene transfer to facilitate the generation of herbicide-, insect-, viral-, fungal-, bacterial-, and nematode-resistant plants (Gubba et al. 2002; Hsieh et al. 2002; Jeanneau et al. 2002; Dasgupta et al. 2003; Grover and Gowthaman 2003; Ranjekar et al. 2003; Prins et al. 2008). Transgenes that may affect quality traits of important crops (Paine et al. 2005), and those for antigens and proteins of pharmaceutical importance, have been introduced into transformation vectors.

Agronomically important genes for biotic and abiotic stresses and quality attributes have been the major focus of research on genetic manipulation, with an extensive range of chimeric genes being introduced into plants (Babu et al. 2003). The majority of transgenes introduced express enzymes that confer novel traits on the respective plants. Proteins lacking enzymatic activity have also been expressed. About 50 important genetically manipulated crops are cultivated in more than 25 countries (Wenzel 2006; James 2008).

1.7.1 Resistance to Biotic and Abiotic Stresses

Biotic and abiotic stresses have a considerable impact on crop growth, development, and productivity throughout the world (Zhao and Zhang 2007). Plant genetic engineering holds the promise of circumventing the problems faced in wide hybridization programs, especially when sources of resistance are not available in taxonomically related species. During the past decade, understanding of the complex molecular events that occur in plant-pathogen interactions has progressed considerably and has provided the opportunity for exploiting the theoretical knowledge and practical skills to generate transgenic plants resistant to pathogens (Grover and Gowthaman 2003). The discovery of abiotic stress-related novel genes, determination of their expression patterns and their roles in adaptation to stress have also provided the foundation for efficient transgenic strategies (Zhao and Zhang 2007).

It is not unexpected that since major crop losses are incited by weeds, insects, viruses, and fungi, increased tolerance to these agents will continue to be a focus of genetic manipulation technology. Transformation of crop plants for increased herbicide tolerance dominated the initial stages of the application of genetic manipulation technology to crop plants. Castle et al. (2006) discussed the ways in which technological advances have been incorporated into agricultural practice and traits introduced into crops such as alfalfa, cotton, maize, oilseed rape, papaya, soybean, and squash, together with the first year of commercialization of the products. Importantly, it is possible to stack transgenes in target plants, conferring tolerance simultaneously to more than one agent.

Behrens et al. (2007) indicated that there has been a rapid increase in the weeds that are tolerant or resistant to the herbicides used with genetically manipulated crops, indicating that such economically important weed management traits may have a finite life. In order to prolong the durability of genetically manipulated

herbicide tolerance, these workers developed a nuclear and chloroplast-encoded herbicide balance strategy based on the inexpensive, widely used, and ecologically safe herbicide, dicamba. Similarly, Soberón et al. (2007) discussed the ways in which the evolution of insecticide resistance by insects threatens the application of effective *Bt* toxins from the soil bacterium *Bacillus thuringiensis* that are employed as bacterial sprays, and *Bt* genes that are introduced into genetically manipulated crops. The natural resistance of insects to insecticides will probably necessitate the use of modified *Bt* toxins in the future. Likewise, Gatehouse (2008) stressed the fact that not all pests are targeted adequately by the *Bt* toxins currently in use. *Bt* toxin expression needs to confer adequate protection against target insects, with plastid transformation being superior to nuclear transformation in this respect. Other approaches for maximizing gene expression include the use of novel *Bt* toxins, gene stacking to effect multiple *Bt* toxin expression and protein engineering.

The exploitation of plant defense proteins, such as α-amylase inhibitors and lectins, is also a possibility; novel approaches include the exploitation of new insecticidal proteins such as those from nematodes, the use of bacterial cholesterol oxidase, and the strong insecticidal effect of avidin. Engineering secondary metabolism of plant defense compounds and of the volatiles emitted by plants, and an RNAi approach to generate double-stranded RNAs are also possibilities. Dudareva and Pichersky (2008) discussed the importance of enhancing plant defense by metabolic engineering of volatile compounds, and suggested that priming crops by planting transgenic plants, that constantly emit defense volatiles, among their nontransgenic counterparts, may provide efficient protection. More needs to be known about the properties of specific plant volatiles in terms of their ability to attract or inhibit insect pests.

The status of virus resistance in transgenic plants has advanced considerably since the initial studies involving coat protein-mediated resistance (Prins et al. 2008). The precise mechanism of coat-protein-mediated resistance is not fully understood. It varies with different viruses, but the procedure has been successful in a range of target plants. Other approaches include replicase-mediated resistance and resistance based on movement proteins. RNA-mediated resistance against RNA and DNA viruses is also discussed, as are nonviral sources of resistance using genetic manipulation, particularly an antibody strategy to induce plants to synthesize similar compounds (plantibodies). Transgene-mediated resistances against viroids have been investigated, a promising approach being the expression of recombinant dsRNA-specific RNases by transgenic plants. Several strategies for virus and viroid resistance have been described in the literature, but only a limited number have progressed past the "proof-of-principle" stage, or small-scale field trials (Prins et al. 2008).

In a critique of the deliverables from genetic manipulation technology, Collinge et al. (2008) emphasized the fact that, to date, very few genetically manipulated disease resistant cultivars have been generated, in contrast to plants tolerant to insect pests using a *Bt* approach, and plants that are herbicide tolerant. Indeed, insect- and herbicide-tolerant plants represent more than 90% of all genetically

manipulated crops generated to date. Weed control exploiting genetic manipulation technology has been facilitated by understanding the biology of herbicide tolerance and the specificity of synthetic herbicides. Similarly, success in the genetic manipulation of insect resistance was based, at least initially, on knowledge arising from the extensive use of the soil bacterium *B. thuringiensis* as a natural insecticide. Since the organisms that cause disease are taxonomically and physiologically diverse with complex life cycles, Collinge et al. (2008) advocated a balance between classical plant breeding and genetic manipulation to generate disease-resistant plants. They concluded that transgenic fungal and bacterial resistances will probably not be introduced into commercial crops in the near future, although progress in the introduction of a barley class II chitinase gene into wheat to confer resistance to *Fusarium graminearum* represents an advancement in engineering fugal resistance (Shin et al. 2008). Plants experience considerable environmental stresses, with drought posing one of the most important constraints for agriculture on a global scale in the near future (Umezawa et al. 2006; Bhatnagar-Mathur et al. 2008). Tolerance to drought, cold, and salinity are often linked, which may facilitate genetic manipulation to combat these natural agents. Mutasa-Gottgens et al. (2009) showed that genetic modification of gibberellin signaling and metabolism significantly delays bolting in crops such as sugar beet, that are vulnerable to vernalization-induced premature bolting and flowering, reducing crop yield and quality. This approach confirms the potential in genetically modifying plants to minimize yield losses due to unfavorable environmental conditions.

1.7.2 Improvement of Quality

Nutritional value, being one of the most important traits for improvement of crop quality, involves enhancement of the content of amino acids and proteins, micronutrients, vitamins, minerals, dietary fiber, sugars, carbohydrates, starch, lipids and oils, which are essential for a healthy diet (Singh et al. 2008). Staple crops, such as cereals, are low in lysine, while proteins of legumes, roots, tubers, and most vegetables are deficient in sulfur-containing amino acids (Sun 2008). Engineering complex synthetic pathways may not be a simple task, as changing one biosynthetic route may have a detrimental effect on other aspects of metabolism.

Attempts have been made to enhance the essential amino acid and protein content of crops (Sun and Liu 2004). Transgenic technology will continue to be used to biofortify crops to increase vitamins and minerals. Engineering of provitamin A to generate “Golden Rice” and “Golden Rice 2” represents a major technological advance in this respect (Ye et al. 2000; Paine et al. 2005). As vegetables and fruits contribute significantly to human nutrition, they represent another important target for genetic modification in terms of tolerance to abiotic stress, nutritional quality, storage products, aromas and, in certain cases, seedlessness (Fraser et al. 2002; Dalal et al. 2006). Larkin and Harrigan (2007) discussed the attempts made to improve the nutritional value of maize and cotton seed, while others focused on

vitamins C (Agius et al. 2003) and E (Chen et al. 2006), particularly on oilseeds (Hunter and Cahoon 2007). Volatiles determine the aromas of fruits, vegetables, and herbs, with genetic engineering being able to ameliorate some of the deficiencies of classical breeding (Dudareva and Pickersky 2008). Tomatoes have been engineered for tolerance to chilling damage (Park et al. 2004), this being of relevance during growth of the plants and during transport of harvested fruit. Delay of fruit ripening and increased shelf-life are also targets for genetic manipulation.

Flavonoids and carotenoids play an important role in human nutrition and health, particularly anticancer activity, and understanding flavonoid and carotenoid biosynthetic pathways has enabled anthocyanins and carotenoids to be up- and down-regulated (Tanaka and Ohmiya 2008). Schijlen et al. (2004) also reviewed the modification of flavonoid biosynthesis in crop plants, while Enfissi et al. (2006) concentrated their attention on the genetic engineering of carotenoids in tomato. Plants have been engineered to produce unusual fatty acids, particularly very long-chain polyunsaturated fatty acids normally found in fish oils and marine organisms (Napier 2007). The longer-term result of engineering complex pathways will be influenced not only by the pathways *per se*, but also by the host plant and physical and chemical parameters. Food allergy is a prevalent medical problem in the western world. Allergen reduction is an important topic for genetic engineering, with RNAi technology being applied to reduce allergens in plants such as apple, peanut, rice, soybean, and tomato (Herman et al. 2003; Gilissen et al. 2005; Le et al. 2006; Chu et al. 2008).

1.7.3 Biopharmaceuticals

Vaccines and antibodies play a major role in human healthcare. The majority of drugs used by humans are derived from plants and have resulted in pharmaceutical companies initiating chemical synthesis of medicinally important compounds (Sharma et al. 1999). However, the full potential of synthesizing compounds has been hampered by production costs and maintaining distribution. The progress in plant transformation has attracted attention in exploiting plants as potential bioreactors or biofactories for the synthesis of immunotherapeutic molecules and recombinant proteins. Plants offer several options for transgene targeting and modification (Warzecha 2008). Indeed, as health care becomes an increasing global issue, the longer-term focus of plant genetic manipulation will be towards the biosynthesis of pharmaceuticals (Zhou and Wu 2006) and other specialty compounds (Fischer et al. 2004, 2007; Yonekura-Sakakibara and Saito 2006). Biofortification of crops with micronutrients is another target for genetic manipulation (Poletti and Sautter 2005). Linked to these goals are issues of biosafety, especially the use of marker genes for antibiotic resistance that are common to many transformation procedures. Davey et al. (2008) presented some of the merits and disadvantages of marker gene technology in the transformation of food crops.

Vaccines such as Hepatitis B surface antigen, Norwalk virus capsid protein, cholera toxin B subunit, Rabies virus glycoprotein, and insulin have been expressed in transgenic plants (Mason et al. 1998; Srinivas et al. 2008), as have immunotherapeutic molecules and industrial proteins, including serum albumin, human α-interferon, human erythropoetin, and murine IgG and IgA immunoglobulins. Oral vaccines synthesized in plants may circumvent some of the limitations of traditional vaccines (Robert and Kirk 2006), especially if vaccines can be synthesized in leafy vegetables that are consumed in the raw state. They will also be cost effective, easy to administer and store, and socioculturally readily acceptable (Lal et al. 2007).

1.7.4 Phytoremediation

Activities, such as intensive mining, agriculture, and military operations, release considerable amounts of toxic heavy metals and organic pollutants, posing a serious threat to living organisms (Cherian and Oliveira 2005). Consequently, there is an urgent requirement to decontaminate polluted environments. Phytoremediation, involving the use of plants and microbes to remove pollutants from contaminated soils, sludge, sediments, groundwater, surface water and waste water, is emerging as a cost-effective and environment-friendly technology compared with conventional methods of remediation (Czako et al. 2006).

Plants harbor highly versatile enzymes such as cytochrome P450 monoxygenases, glutathione S-transferases, glycosyltransferases, laccases, peroxidases, and transporters that detoxify pollutants. Although these enzymes may not completely degrade pollutants, they may form complexes, which can be harvested. In recent years, genetic engineering has been used to introduce key genes to increase the remediation ability of several species. Several genes, such as *merApe9, merB, MT1, MT2, CUP1, gshI, ZAT1, ZntA, arsC* (for heavy metal tolerance), mammalian cytochrome P450 2E1 (*CYP2E1*), *cbn4* (for chlorinated solvents), *CYP1A1, CYP2B6, CYP2C9, CYP2C18, CYP2C19* (for herbicide tolerance), and genes encoding rhamnolipid biosynthesis (for oil degradation), have been overexpressed in transgenic plants (Doty et al. 2000; Dhankher et al. 2002; Lee et al. 2003; Thomas et al. 2003; Cherian and Oliveira 2005; Czako et al. 2006), providing a basis for plant-based phytoremediation.

1.7.5 Floriculture

While food crops will continue to be prime targets for genetic manipulation, ornamentals have featured extensively in genetic manipulation strategies because of the significant contribution of the horticultural industry to the economy of many countries (Tanaka et al. 2005). Ornamentals, especially flower species, are well suited to genetic manipulation. As the end product is not food, it does not

necessitate food safety studies, removing major obstacles for commercialization and reducing the cost of production. Chandler and Lu (2005) tabulated the floriculture crops that have been transformed and those with modified characteristics. The latter include disease resistance, herbicide and freezing tolerances (Pennycooke et al. 2003) and, most importantly, modification of pigmentation following manipulation of the genes for pigment biosynthesis (Lu et al. 2003; Tsuda et al. 2004; Suzuki et al. 2007). Attempts have been made to increase the number of flowers produced and extending the life of cut flowers (Shaw et al. 2002). Early and delayed flowering traits have also been introduced (Baker et al. 2002), together with modification of plant architecture (Zheng et al. 2001) and stature (Aswath et al. 2004). The importance of gibberellic acids in controlling plant height in agriculture, horticulture, and silviculture is well recognized (Radi et al. 2006). Dwarf plants may be preferred in amenity planting because of their resistance to unfavorable weather conditions. In this respect, ectopic expression of a gibberellin 2-oxidase from oleander (*NoGA2ox3*) in *Nicotiana tabacum* resulted in dwarf plants (Ubeda-Tomás et al. 2006). Subsequently, Agharkar et al. (2007) demonstrated that genetic manipulation of gibberellin biosynthesis genes can improve the quality of turf grass by increasing the number of vegetative tillers, enhancing turf density under field conditions. Likewise, in order to demonstrate proof of principle and the application of a genetic engineering approach, Dijkstra et al. (2008) overexpressed a gibberellin 2-oxidase gene (*PcGA2ox1*) from *Phaseolus coccineus* to enhance gibberellin inactivation and to induce dwarfism in *Solanum* species. The ability to engineer plant stature through a genetic engineering approach should be of interest to the ornamental industry.

Fragrance will receive more attention (Xiang et al. 2007), since many plants have lost their traditional perfumes through classical breeding. Several approaches have been evaluated to alter scent by genetic modification, as in petunia (Lücker et al. 2001) and carnation (Lavy et al. 2002). However, even though the transgenic plants synthesized more volatiles, the latter could not be detected by humans. In contrast, Zuker et al. (2002) generated carnations with altered floral scent that could be detected by humans, but the resulting plants also had severe alteration in flower color. More recently, Lücker et al. (2004) demonstrated the possibility of modifying the flower fragrance profile by metabolic engineering of tobacco plants using three monoterpene synthases from lemon. These investigators stressed the difficulty of genetically modifying scent because of the need for multigene engineering. Flavonoids and carotenoids are important not only in nutrition and healthcare, as already discussed, but also in flower pigmentation (Nakatsuka et al. 2007b; Tanaka and Ohmiya 2008). Modification of flower color has always been one of the greatest challenges in floricultural plant breeding, since certain colors are difficult to achieve in some species. However, in some cases, genetic manipulation has enabled changes to be made to pigmentation, where classical breeding has failed, by introducing genes from other species and modifying the anthocyanin, carotenoid, or flavonoid biosynthetic pathways. This approach has enabled the generation of purple carnations (Fukui et al. 2003) and blue roses (Potera 2007).

1.8 Risks and Concerns

As with any new technology, there are uncertainties regarding the deployment of genetically engineered plants. There is an increasing concern that insect pests have the capacity to develop resistance against transgenes introduced into plants, or that transgenic properties may be transferred to insects, viruses, and bacteria. Apprehension has also been raised concerning the introgression of transgenes into wild relatives of genetically modified plants and the development of superweeds resulting from introgression of herbicide resistance from transgenic plants to weeds (Sharma et al. 2001, 2002). Transgenic plants may also affect nontarget species and the environment. Food biosafety research has also focused on toxicity and allergenicity of transgenic products.

Although concerns for ecological safety and the human well-being have led to mistrust over the application of genetic manipulation technology, many of these fears appear unsubstantiated or based on misinformation (Stewart et al. 2000). A concerted effort must be made to identify valid concerns and risks, and to provide reliable information to the public. The advent of plant genetic manipulation in vaccine production and quality improvement will increase the emphasis on consumer health benefits, which may facilitate, in turn, acceptance of the use of genetically engineered foods. Active participation of researchers from the fields of biotechnology, ecology, and nutritional sciences may be essential to better determine the biosafety of transgenic plants (Stewart et al. 2000).

1.9 General Conclusions

Modern agricultural biotechnology has been one of the most promising developments in recent years (Sharma et al. 2002). Major advances in understanding gene structure and expression have made significant contributions to the assembly of genes and their regulatory elements for plant genetic engineering. Likewise, progress in DNA delivery technologies has facilitated the introduction of novel genes into a wide range of plants. A common restriction to gene introgression into many crops is the recalcitrance of these plants to express their totipotency in culture. However, the exploitation of procedures that by-pass the requirement for extensive in vitro manipulations should eliminate some of these difficulties. Currently, genetic engineering is not a routine plant breeding tool (Arias et al. 2006), but is an important adjunct to classical breeding (Shewry et al. 2008).

World food supplies will demand more intensive crop production, despite a reduction in available agricultural land because of deterioration of soil quality, drought, climatic change, disease, and political unrest. Farmers will demand more value per unit of agricultural land. Genetic engineering, when used in collaboration with traditional or conventional breeding methods, will be able to increase crop production, increase resistance to major pests and diseases, develop tolerance to adverse weather conditions, improve the nutritional value of some foods, and

enhance the durability of products during harvesting or shipping (Sharma et al. 2002). Reduced use of agrochemicals will have less environmental impact. In the future, agriculturally important traits must satisfy not only the requirements of farmers, but also the availability of materials from researchers, governments, distributors, processors, and the opinions of the public (Castle et al. 2006).

Discussions on transgenic crops have placed undue stress on risk assessment, overshadowing potential advantages (Sharma et al. 2002). The issues relating to genetically modified plants, especially food crops, have been analyzed from a scientist's perspective (Lemaux 2008). These issues are not only complex, but are often aggravated by personal opinions, especially by those members of the public who have limited understanding of plant breeding and gene technology. The rapid escalation of increasingly stringent biosafety regulations regarding transgenic plants or food, in the absence of any scientifically proven genetic risk, is most likely to limit application of transgenic research to meet either the production of sustainable staple foods or the alleviation of poverty (Sharma et al. 2002). Moving crop production from one region to another will influence global trade patterns; legislation and the perceived risks of genetically engineered crops will also affect exploitation of these crops (Singh et al. 2006).

What remains clear is that changes in the genetic complement of those plants that contribute to our food supplies are primarily the result, to date, of sexual hybridization. Genetic engineering provides a precise approach to effect genetic modification over a much reduced time-scale. The safety of genetically engineered plants and those generated by conventional breeding needs to be evaluated on a case-by-case basis (Lemaux 2008). Condemning biotechnology for its potential risks without considering the risks associated with prolonging human misery caused by hunger, malnutrition, and infant mortality is unwise and unethical. The global community must endeavor to remain focused on the target of assuring food for all, and cannot afford to be philosophical and elitist regarding any part of a possible solution, including agricultural biotechnology (Sharma et al. 2002).

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