PLANT BIOTECHNOLOGICAL APPROACHES FOR THE PRODUCTION AND COMMERCIALIZATION OF TRANSGENIC CROPS

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ABSTRACT

This review initially briefly introduces methods of plant transformation and then describes some of the major achievements concerning the production and commercialization of transgenic crops. It surveys the outlook of plant transformation for improvement of crop productivity by introducing genes of interest such as for enhancing soil nutrients utilization, resistance to insects, viral and bacterial diseases. It also deals with the high quality transgenic crops having improved nutritional and health characteristics as well as increased tolerance to drought, extreme temperature, high salinity and heavy metal soil contamination. Marketable biotechnological products like biodegradable plastics, metabolites with pharmaceutical properties and edible vaccines produced in genetically modified plants are also discussed, in this review.

Keywords: agriculture, biotechnology, crop improvement, cultivar improvement, plant transformation, transgenic systems

Introduction

Genetic modification of plants probably began through selection of novel types about 10 000 years ago when human agricultural activities were initiated and the useful results generated were often a product of random or selection of spontaneous mutations. Through elucidation of the laws of genetics, plant breeding became a deliberate and predictable activity with the result that tailor-made crops are now in place. Traditional plant breeding methods have been very successful, providing the volume of food required to allow the world population to grow to its present scale. Breeding efforts have led to remarkable diversity amongst various crop species. However, recent trends in crop productivity indicate that traditional methods alone will not be able to keep pace with the growing demands for food, fibre and fuel. A remarkable increase in the total grain production was noticed between 1950 and 1980, while only a marginal increase was realized during 1980-1990 (122). Much of the early increase in grain production resulted from an increase in an area under cultivation, irrigation, better agronomic practices and improved cultivars. Yields of several crops have already reached a plateau in the developed countries, and therefore, most of the productivity gains in the future will have to be achieved in developing countries through better natural resources management and crop improvement. Productivity gains are essential for long-term economic growth, but in the short-term, these are even more important for maintaining adequate food supplies for the growing world population.

Conventional plant breeding (65, 68), sometimes supported by marker assisted selection (36, 37, 70, 119), and wide hybridization coupled with manipulation of chromosome pairing (66, 67) have clearly been instrumental in producing superior crop cultivars. However, these procedures are time-consuming. It may take 10 or more years to transfer a trait from a donor species into a crop cultivar via conventional strategies. Wide hybridization is undoubtedly an effective means of incorporating desirable alien genes into crop cultivars, but it has several limitations like transmission of unwanted chromosomes and sterility due to adverse genetic interactions.

The basis of these breeding techniques lies on the modification of the genetic material, which in the past was not well understood. After the discovery of the structure of DNA molecule and the elucidation of its nature and replication during the 1950s, research into the precise modification of the genetic material got momentum. From the genetic point of view, one single cross between two plants used in conventional breeding puts two sets of about 15 000–25 000 genes together, i.e. a genetic modification at a massive level. In contrast, by means of modern biotechnological methods only few genes are modified leaving the rest of the genome unaltered. Plant biotechnology offers breeders access to an infinitely wide array of novel genes and traits, which can be inserted through a single event into high-yielding and locally adapted cultivars. This approach offers rapid introgression of novel genes and traits into elite agronomic backgrounds. Plant biotechnology, in particular genetic transformation, can bolster plant-breeding efforts to meet these new challenges in a sustainable way (55). Genetic improvement of the world's major crops has been notably expedited by adopting several measures, such as *Agrobacterium tumefaciens*, direct gene transfer, depending on plant species (6, 31, 33, 77, 98, 102, 112). The biotechnology-derived crops will better address current and future challenges in agriculture including the need for improved yields, tolerance to biotic and abiotic stresses, and improved nutrition.

Although the plant transformation technology has been developed in 1983, the first genetically transformed crop reached into markets during the mid-1990s. During the past

decades the development and adoption of the transgenic technology has progressed rapidly. In 2007, the global area of biotech crops increased for the twelfth consecutive year at an annual growth rate of 12%, with a total area of 114.3 million hectares in 23 countries (64). Transgenic crops have contributed more than US$ 23 billion to the economies of developing as well as developed countries (nearly 90% of the transgenic crops are planted by resource-poor farmers), exhibiting unprecedented advantages like reduction in the use of agro-chemicals, increased productivity and a positive influence on conservation of the environment and biodiversity (4**)**. All of these are ascribed to progress made in understanding of the developmental, physiological and metabolic processes responsible for plant growth and development. Significant progress has been made towards the elucidation of plant genes in improving crop yields (23, 113).

Transformation Systems

Development and deployment of transgenic plants in an effective manner are important pre-requisites for sustainable and economic use of genetic transformation for crop improvement (101). According to Hansen and Wright (1999) the main requirements for efficient genetic transformation systems are favorable target tissues, competent for propagation or regeneration, an efficient DNA delivery method, appropriate agents to select transgenic tissues, the ability to recover fertile transgenic plants at a reasonable frequency, a simple, efficient, reproducible, genotype-independent and cost-effective process and a tight time frame in culture to avoid somaclonal variation and possible sterility (53).

Although several systems of transformation ranging from acupuncture (108) to shotgun using real gunpowder (99) have been reported, so far three principal methods have been extensively used for crop improvement, namely *Agrobacterium tumefaciens*-mediated gene transfer, direct DNA delivery into protoplasts by osmotic or electric shock and high velocity bombardment of DNA coated microprojectiles (the biolistic procedure).

***Agrobacterium tumefaciens*-mediated transformation**

Agrobacterium tumefaciens, a soil-borne, gram-negative bacterium, is capable to genetically colonize susceptible host plants. It is suitable for transferring DNA inserted in its T-DNA between a pair of direct repeats called border sequences, with the help of a site and strand-specific endonuclease. *Agrobacterium tumefaciens-mediated* transformation is now considered one of the most efficient methods of plant biotechnology. Very often T-DNA integration occurs in transcriptionally active regions of the plant genome and hence the expression of the transgene becomes a routine phenomenon. A detailed insight into the *Agrobacterium*-mediated DNA transfer process in plants is given in a previous report (127).

Agrobacterium tumefaciens has played a major role in the development of plant genetic engineering and the basic research in molecular biology. It accounts for about 80% transgenic plants produced so far (51). Initially, it was believed that only dicots, and a few monocot species could be transformed by this bacterium; but recent achievements changed in general this view by showing that many "recalcitrant" species not included in its natural host-range can now be transformed (21, 25). In addition, the transformed cells usually carry single or low copy number of T-DNA integrated in their genome with less rearrangement, and very large DNA segments can be transformed into the plants (52).

The *Agrobacterium* system has several advantages over other transformation methods and it is considered as the first option to transform plants. These advantages include the following aspects: (a) In a significant percentage of the transformation events, a single copy of the T-DNA is integrated into the chromosomes of the transformed cell. (b) Numerous vector systems are now available containing the T-DNA borders and various reporter and selectable marker genes, allowing researchers to choose the most appropriate combination to insert heterologous genes. (c) It is possible to transfer large fragments of DNA, including bacterial artificial chromosomes (52). (d) Transformation *in planta*, without the necessity of tissue culture, is possible in some species such as *Arabidopsis thaliana* and *Medicago trunculata* (111).

However, it has to be pointed out here that this system is dependent on the transformation ability of a certain species by *Agrobacterium,* some monocot species have still shown recalcitrance, while others are now routinely being transformed by this method (105). Another drawback of this system is that the site of integration is random and the integrated T-DNA can be rearranged or truncated or occasionally may comprise vector DNA backbone sequences from outside the T-DNA borders (27). It is also possible that the carrier *Agrobacterium* itself may persist internally or externally in transformed plants.

Protoplast transformation

Methods for the direct delivery of DNA into protoplasts were developed during the early 1980s (103), especially for the economically important cereal crops as they were considered at the time to be outside the host range of *Agrobacterium*, and therefore not amenable to *Agrobacterium*- mediated transformation (see 112). In this system osmotic or electric shock is delivered to protoplasts suspended in solutions containing DNA, followed by plating on selection media for the preferential growth of transformed colonies, and eventually plants. Transformation of protoplasts isolated from embryogenic cell suspension cultures led to the production of the first transgenic cereals (95). The use of protoplasts for genetic transformation became less attractive once it was shown that monocots, including the cereals, could be transformed by co-cultivation of embryonic tissues or embryogenic cultures and super-virulent strains of *Agrobacterium* in the presence of acetosyringone, a potent virulence genes inducer (56, 69).

Biolistic transformation

The biolistic transformation, a universal method of plant transformation, was developed by Sanford et al. (99). It involves the high velocity bombardment of DNA-coated gold

or tungsten microprojectiles into intact cells or tissues. The term 'biolistic' was derived from 'biological ballistic'. Molecular analysis of plants transformed biolistically in general reveals a complex pattern of transgene, indicating the integration of multiple copies of the bombarded DNA. However, it has been demonstrated that in most cases, these multiple copies are arranged as a single locus and segregate in a Mendelian pattern.

Due to the genotype-independent physical nature of DNA delivery, the methodologies employed are simple, efficient and essentially identical regardless of the nature of the target cells and DNA used. Compared to other techniques this method is a highly versatile and adaptable technique, which can be applied to a wide range of cells and tissues (107). During the last two decades, micro-projectile bombardment has become a routine and reliable method for the production of transgenic plants bypassing *Agrobacterium* host-specificity and tissue culture-related regeneration difficulties encountered with several important crops. The complex pattern of transgene integration, generally revealed by molecular analysis of plants obtained by biolistic transformation, can be circumvented by the use of 'agrolistic' approach (53).

Other systems of transformation

There is a perpetual quest to find more efficacious and economical methods for plant transformation. Some of these are chloroplast transformation*, in planta* transformation-floral dip method, and pollen-mediated transformation. Although they have been tried in some plants, their use is still limited due to their impracticability at present. A review of several interesting methods of plant transformation has recently been published (see 4).

Prospects and achievements of transgenic technology

With the development of plant transformation methods, knowledge of the structure and function of certain genes, and the desire to resolve some of the classic problems in the traditional agriculture and the race to obtain better plants by genetic engineering, began with satisfactory results. Initial strategies considered the introduction of single genes into plants of interest; now, however, strategies involving multiple genes from a single metabolic pathway can be used. The main approaches used to produce improved transgenic plants with commercial or agricultural applications are mentioned in the following sub-headings.

1. Improvement of crop productivity

A considerable proportion of the crop yields are lost by many biotic and abiotic factors that affect plants' growth and development and restrict their geographical distribution. Genetic transformation has now been shown to be a key tool to circumvent these barriers and can bring a new wave of revolution for crop enhancement. The following sections highlight some of the applications of transgenic crop plants that may address these constraints and/or mitigate negative consequences of the conventional solutions.

Disease resistance

A variety of diseases pose a serious threat to global food security owing to reduced crop productivity (12). In naturally occurring ecosystems, elaborate networks of defences function at many levels to protect plants from diseases (2). Elucidation of these defence pathways has recently become a particularly active area of research in plant molecular biology, and has led to growing appreciation for the complex interplay between basal defences and specific disease resistance (38). Cultivation of plant lines bred for resistance to one or a few pathogens, often conferred by so-called *R* genes, can lead to the emergence of pathogens that have undergone natural selection to overcome the resistance (50). Despite the potentially short-sighted nature of such agricultural practices, identification of *R* genes has been the focus of considerable effort over the past decade (12, 30). At least one such gene, the *Bs2* gene from pepper, has been used successfully to engineer durable resistance to the agronomically significant bacterial spot disease in tomatoes (109). The *Xa21* resistance gene from rice, which provides wide-spectrum resistance to the devastating bacterial blight caused by *Xanthomonas oryzae*, has been introduced into a variety of rice cultivars using *Agrobacterium*-mediated gene delivery (115). Likewise, one of four *R* genes cloned from a wild, highly resistant, potato species (106) confers broad-spectrum resistance to potato late blight.

A variety of antibacterial proteins from sources other than plants have been used to confer resistance to bacterial diseases in several transgenic plants (reviewed in 80). Plants engineered to produce elevated levels of salicylic acid also exhibit enhanced disease resistance (114). The transgenic papaya that has been commercialized produces 98% of the world's papaya crop (44). Although this particular application made use of the particle bombardment rather than *Agrobacterium* to deliver the transgene, it serves as a convincing illustration of the potential for achieving virus resistance in other highly susceptible crops. In several cases, *Agrobacterium*-mediated expressions of viral replicase genes (16) or virus movement proteins (17), rather than the viral coat proteins, effectively conferred resistance. *Agrobacterium*-mediated delivery of a ribosome, a small RNA molecule capable of cleaving RNA, has been successful in conferring at least partial resistance to viruses and viroids in tobacco and potatoes (35, 121). A recent report shows that artificial small or micro RNAs can confer complete virus resistance in plants (84). An increasing number of miRNAs have been identified and deposited in major miRNA databases. Among them, 863 are plant miRNAs (118). They are involved in plant development, signal transduction, protein degradation, and response to environmental stress and pathogen invasion. Roles of miRNA in plant defense and virus offense interaction has been recently reviewed (73).

Herbicide tolerance

Herbicide tolerance has been engineered into many crop species, such as oilseed rape, maize, soybeans, sugar beet, fodder beet, cotton and rice. The first herbicide-tolerant GM plants grown commercially were glyphosate-tolerant soybeans

(86). The gene that confers tolerance of the herbicide is from the soil bacterium *Agrobacterium tumefaciens* and encodes an EPSPS that is not affected by glyphosate. Glufosinate and bromoxynil tolerant varieties of oilseed rape have been particularly successful in the USA and Canada (34, 41). In the USA in 2002, 81% of the US soybean crop, 59% of the upland cotton and 15% of the maize was GM herbicide tolerant (18, 40). In the same year, 95% of Argentine soybean and 66% of Canadian oilseed rape (canola) was GM herbicide tolerant. In 2007, herbicide-tolerant soybean, maize, canola, cotton and alfalfa occupied 63% or 72.2 million hectares of the global biotech crops (64). Herbicide tolerant crop species enable farmers to use a single herbicide instead of many, reducing application costs. Their use has led to a significant increase in the adoption of no-till farming, in which weeds and stubble are left undisturbed over winter. This reduces soil erosion and nitrate run-off.

Insect pest resistance

Genetically engineered inherent crop resistance to insect pests offers the potential of environment- and consumer-friendly method of crop protection. Numerous insect pests attack crop plants and cause enormous losses, threatening global food security. European corn borer (ECB) (*Ostrinia nubilalis*), for example, brings about a loss of up to 2000 million dollars annually in the USA alone (63). A gene from a soil-borne bacterium, *Bacillus thuringiensis* (*Bt*), has been bioengineered into the corn genome, which conferred almost complete resistance to ECB. China has been able to reduce its use of chemical pesticides on insect resistant (IR) cotton, with important environmental and farmer health benefits (62). Because of its higher productivity and positive health effects due to reduced pesticide use, *Bt* cotton has been increasingly commercialized in the world, especially in Asian countries like China (60) and India (116).

Transgenic rice varieties resistant to yellow stem borer (*Scirpophaga incertulas* Walker) have been produced in India (94). *Bt* rice has the potential to eliminate yield losses caused by lepidopteran insects, estimated at 2 to 10% of Asia's annual rice yield of 523 million tons (57). Field trials of transgenic rice suggested high tolerance of transgenic rice against yellow stem borer (15). In 2007, 20.3 million hectares (18%) were planted with *Bt* crops, making it the third most important transgenic trait.

Abiotic stress tolerance

Growing global demand for food continues to force farmers onto marginally arable land where soil salinity, water deficits, and climatic challenges such as low or high temperatures limit cultivation (13). These abiotic stresses are estimated to cause more than 50% of crop yield losses worldwide (20). Transgenic approaches offer an option to enhance abiotic stress tolerance (1, 7, 14, 39, 72, 124, 125). Strategies to engineer enhanced tolerance to such adverse conditions fall into at least two categories: direct protection from the stress(s), and enhanced resistance to the physiological damage caused by the stress. One strategy to increase the tolerance to such stresses is the production of osmoprotective compounds (osmolytes), such as sugars, alcohols, amino acids, and glycine betaine, which raise the osmotic potential of the cell, allowing the influx of water, stabilize the membranes and/or macro-molecular structures (59) and scavenging reactive oxygen species (126). Very recently, a comprehensive review on mechanisms of abiotic stress tolerance and on engineering tolerance to stress has been published (104).

Some plants adapted to stress conditions naturally produce these osmolytes; however, many important crop plants do not accumulate sufficient osmoprotective compounds to be stress tolerant. Advances have been made to achieve or increase the production of osmolytes in transgenic plants. A detailed insight of glycine betaine and its role in enhancing abiotic stress tolerance in plants has been recently discussed (26). Glycine betaine has been produced in tobacco plants by the expression of a bacterial gene; these plants show an enhanced salt tolerance (71). The non-reducing disaccharide trehalose can stabilize biological structures upon desiccation in many bacteria, fungi, and invertebrates, but apparently does not accumulate naturally in plants (89). Transgenic tobacco and rice engineered to produce trehalose exhibit enhanced resistance to drought (97), salt, and low-temperature stress (43). More recently, an abscisic acid (ABA) inducible promoter to express a bi-functional enzyme that synthesizes trehalose was used to produce transgenic rice plants that exhibited sustained plant growth, less photo-oxidative damage under salt, drought and low temperature conditions (43).

A family of aldose-aldehyde reductases is activated in response to a wide variety of stresses (13). Ectopic expression of an alfalfa aldose-aldehyde reductase gene via *Agrobacterium*-mediated transformation results in reduced damage upon oxidative stress, apparently by eliminating reactive aldehydes, and increased tolerance to salt, dehydration, or heavy metal stress (85). Tall fescue (*Festuca arundinacea* Schreb.) exhibited enhanced tolerance to drought and stress after successful introduction of *AtHDG11* gene via *Agrobacterium*-mediated transformation (22). The dramatic role of *AtHDG11* in enhancing drought tolerance was identified via activation tagging and subsequent analyses in *Arabidopsis* and tobacco (123). Successful transgenic approaches have been described in several reviews (10, 120).

Enhanced nutrients utilization

Crop productivity is also limited by inadequate soil fertility, and regular use of inorganic, petroleum-based fertilizers that compromise plants' growth and development. Among the approaches to mitigating these constraints are some that involve modifying genetically the crop plants (45). Transgenic plants with enhanced capabilities to absorb micronutrients from the soil, by over-expressing nitrogen, potassium and phosphorus transporters and/or manipulating their regulation could decrease the need for fertilizers (58). For some nutrients, such as iron and phosphorus, the limiting factor is often

solubility rather than abundance in the soil. Plants synthesize and secrete a variety of organic acids that can chelate insoluble compounds, allowing uptake of the complex (49). Several important grain crops such as rice, maize and sorghum are particularly sensitive to low iron availability in alkaline soils, where iron is less soluble. *Agrobacterium*-mediated introduction of genes conferring enhanced biosynthesis of an iron chelator in rice resulted in improved growth and four-fold higher grain yields under conditions of low iron availability (110).

2. Improvement of crop nutritional qualities

Genetic modification of food crops offers the possibility of enhancing the nutritional content of the food (60). One example is the development of 'Golden Rice', genetically engineered to produce β-carotene (pro-vitamin A) in the seeds, in order to combat human vitamin A deficiency (32, 87, 88, 122). An iron-storage protein gene, *ferritin*, driven by the constitutive CaMV35S promoter, was transferred into tobacco, where the leaves of transgenic plants had a maximum of 30% more iron than the non-transformed plants (48). In lettuce, the transgenic plants had 1.2-1.7 times more iron and enhanced early developmental growth and superior photosynthesis than the control plants (47). Lucca et al. also observed increased iron content in rice seeds transformed with a *Phaseolus ferritin* gene (74).

Several food crops are now being developed with enhanced vitamin E, vitamin C, modified starch and amino acid profiles (11, 24, 42). Potato is the most important non-cereal food crop for human consumption and, therefore, the need to improve its nutritional quality cannot be overemphasized. Chakraborty et al. demonstrated that expression of the *AmAl* gene isolated from amaranth (*Amaranthus hypochondriacus* L.) in potato led to a significant increase in most essential amino acids as well as in higher protein content in tubers compared with non transgenic plants (24).

Successful production of health promoting polyunsaturated fatty acids in transgenic oilseed crops has recently been reported (117). Transgene- driven biosynthesis of naturally occurring or modified sulfur-rich proteins had been achieved in canola (5). Production of potent anti-oxidants including lycopene has been increased through transgenic over expression of relevant enzymes in tomatoes (78, 81, 83).

3. Genetic modifications of plants to generate useful products

Biodegradable plastics

One of the potential benefits of transgenic crops is the production of biodegradable plastics (91), particularly PHB and PHV. Neumann et al. reported the synthesis of cyanophycin in transgenic tobacco and potato plants, which can be hydrolyzed to yield the soluble, non-toxic, biodegradable plastic-like compound poly-aspartate (82). Although these transgenic plants exhibit morphological alterations in chloroplast structure and growth rate, additional engineering of the amino-acid biosynthesis pathways may give rise to biodegradable plastics at economically viable levels (28). If successful, the substitution of a renewable process (solar-driven carbon fixation) for conventional petrochemically derived plastic production technologies would have substantial positive environmental consequences, decreasing our reliance on finite petroleum resources and reducing the accumulation of indestructible plastics (28, 90).

Primary and secondary metabolites with desirable properties

Considerable effort has been dedicated to metabolic engineering of terpenoids in plants. Some of the primary metabolites produced by the terpenoid biosynthetic pathway include phytohormones, pigments involved in photosynthesis, and the ubiquinones required for respiration (3). Some function as antimicrobial agents, thus contributing to plant disease resistance, while other terpenoid compounds serve to repel pests, attract pollinators or inhibit stunted growth, loss of fertility and significant alterations in the levels of various amino acids, organic acids, sugars, and sugar alcohols' growth of neighboring competitor plant species. The terpenoid biosynthetic pathway and strategies for its manipulation have been reviewed recently (3, 75). A comprehensive listing of transgenic plants with altered terpenoid biosynthetic properties is available elsewhere (3, 19). Examples include expression of heterologous syntheses in tomato, leading to enhanced aroma in ripening fruit and the introduction of bacterial genes directing the production of keto- carotenoids, thought to have medicinal value into tomato and tobacco (93).

Biopharmaceuticals/edible vaccines

Using *Agrobacterium*-mediated transformation, transgenic plants have been engineered to express a wide variety of exogenous proteins, from spider dragline silk (a fiber with high tensile strength and elasticity) (100) to vaccines, antibodies and other life-saving biopharmaceuticals such as anti-coagulants, human epidermal growth factor and interferon (46). To date, most such clinically relevant proteins have been produced in tobacco, although potato, alfalfa, soybean, rice and wheat have also been used successfully. Edible vaccines may hold considerable promise for the developing world, where refrigeration, sterile syringes and needles, and trained health care personnel are frequently in short supply (9). Oral immunization has been achieved using transgenic potatoes expressing antigens including the heat-labile enterotoxin from *E. coli* (54, 76) and the hepatitis B surface antigen (96).

Conclusions

The information and critical analyses provided in this review can only give a glimpse of the role of modern biotechnology in genetic improvement of both crop plants and trees. Plant biotechnology offers opportunities to improve the production and composition of crops with benefits to the environment and consumers. Crop productivity would be increased by the development of built-in disease and insect resistances crops. The successful deployment of transgenic approaches to combat

insect pests and diseases of important crops is a remarkable accomplishment. Pest-resistant crops contribute to increased yields and agricultural growth in many developing countries and benefit small-scale farmers (92).Transgenic crops would have significant impacts on increasing the food supply, thereby helping to reduce food prices for poor farmers. Biofortification of crops to reduce or alleviate malnutrition among the poor masses constitutes another exciting development. Thus, the development of Golden Rice, which is genetically enriched with vitamin A and iron, is a major milestone in tackling the problem of global hunger. Yet another application of the transgenic technology is in the production of edible vaccines for immunization against deadly diseases like hepatitis B or tuberculosis, two of the serious diseases of the poor masses in Africa and Asia. Biotechnology can help feed the billions of poor people who constantly struggle for a better life (29, 61).

Application of molecular plant breeding is now focusing to discover new genes and their functions opening new avenues for basic plant biology research (79). When carefully deployed, modern biotechnology will become an integral supplement to conventional plant breeding and its enormous potential should be harnessed to the best advantage of the entire human race, rich or poor. The combination of traditional crop improvement techniques and modern biotechnological techniques can contribute substantially to human well-being by providing renewable sources of food, feed, chemicals, pharmaceuticals, energy and for the creation of a sustainable environment for the developed as well as for the developing countries of the world.

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