Chapter 6
Next Generation Plant Biotechnology

M.R. Ahuja

Abstract Modern plant biotechnology began with the transfer of foreign chimeric genes into plants. Initially recombinant genes were derived from bacteria, animals and plants for gene transfer. Gene transfer was accomplished by *Agrobacterium*-mediated or biolistic methods into the plant genome. The first wave of transgenic plants that were monitored for transgene integration and expression, the second wave transgenic plants carried economically important genes for herbicide tolerance, pest resistance, drought and salt tolerance, growth traits, and flowering control. Subsequently, a number of genetically modified crops with several useful traits have been commercialized. Although relatively stable transgene expression has been observed in a number of plant species, there were also unintended unstable events in transgenic plants. This is due to the fact that transgene integration achieved by the two traditional methods (*Agrobacterium* or biolistic) of gene transfer in the plant genome is random, and one to several copies of the transgenes may be integrated at one or several locations in the genome. In order to overcome the problem of randomness of transgene integration, site-specific transgene integration strategies have been experimentally tested in plants, and offer prospects of stable gene integration and expression in transgenic plants. In order to broaden the scope of transgenic plants, biotechnologists started looking for other useful avenues for their utility. With finite reserves of fossil fuels and climate change, and growing demands for fuels, plastics, and pharmaceuticals, transgenic plants have been also explored as production platforms for these commodities. This paper is an overview of next generation transgenic plants that can serve as bioreactors or biofactories for the cost-effective production of biofuels, biopharmaceuticals, bioplastics, and as a resource for nutritional supplements to meet human demands in the future. New developments in nanobiotechnology offer prospects for improved production of crop plants.

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6.1 Introduction

The world population has been increasing at an accelerated rate during the last century. The world population was around 3.25 billion in the 1960s. By the year 2000 the world population reached the 6 billion mark. During the 40 years (1960–2000) it almost doubled. By the year 2010 it increased by another 1 billion to reach 7 billion. It is expected to reach the 9 billion mark by the year 2050 (http://www.fao.org/index-en.htm 2013). That is an increase of 2 billion from the current world population of 7 billion over the next 40 years. The bulk of the population increase has occurred in the developing countries; by the year 2050 the world population would be around 8 billion in the developing countries, and only 1 billion in the developed countries. How to feed the world by the year 2050 remains a daunting challenge for the food security. In order to feed this larger world population food production would necessarily have to increase by 70 % (Godfray et al. 2010; Bogdanski 2012). This explosion in world population requires an enormous increase in food production, improvement of nutritional quality of the staple food (biofortification), production of safe and natural pharmaceutical proteins (molecular farming), and increase in energy and plastics by alternatives routes (biofuels and bioplastics) in the future to meet the human demands. In addition to conventional breeding, plant biotechnology can play a significant role for the improvement of human resources in the future.

Modern plant biotechnology began with the transfer of chimeric genes, constituted by the DNA recombinant technology, into the plant genome in the1980s, and the first transgenic plants were produced by *Agrobacterium*-mediated gene transfer (Bevan et al. 1983; Herrera-Estrella et al. 1983; Fraley et al. 1983). Subsequently, transgenic plants from a number of different plant species, including agricultural crops and tress, were produced with novel genotypes by *Agrobacterium*-mediated and particle bombardment gene transfers (Peňa and Séguin 2001; Sharma et al. 2002; Boerjan 2005; Jauhar 2006; Herdt 2006; Dunwell 2010). The first wave of transgenic crops carried transgenes for resistance to fruit rotting, herbicide tolerance, and pest resistance.

Genetically engineered FlavrSavr tomatoes, developed by Calgene (Kramer and Radenbaugh 1994), carrying an antisense polygalacturonase (*PG*) transgene which makes tomatoes resistant to rotting, was released in the market in 1994. Under normal conditions, the enzyme polygalacturonase which degrades the pectin in the cells wall results in softening of the fruit and consequently makes it susceptible to fungal infection. FlavrSavr tomatoes, on the other hand, have a relatively longer shelf-life, and also can be allowed to ripen on the tomato vine. However, the FlavrSavr tomatoes turned out to be a disappointment in that the antisense *PG* gene,

Table 6.1 Global area of biotech crops in 2013. (Data from James (2013))

Rank	Country	Area (millions of hectares)	GM crops
1.	USA	70.1	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
2.	Brazil	40.3	Soybean, maize, cotton
3.	Argentina	24.4	Soybean, maize, cotton
4.	India	11.0	Cotton
5.	Canada	10.8	Canola, maize, soybean, sugar beet
6.	China	4.2	Cotton, papaya, poplar, tomato, sweet pepper
7.	Paraguay	3.6	Soybean, maize, cotton
8.	South Africa	2.9	Maize, soybean, cotton
9.	Pakistan	2.8	Cotton
10.	Uruguay	1.5	Soybean, maize
11.	Bolivia	1.0	Soybean
12.	Philippines	0.8	Maize
13.	Australia	0.6	Cotton, canola
14.	Burkina Faso	0.5	Cotton
15.	Myanmar	0.3	Cotton
16.	Spain	0.1	Maize
17.	Mexico	0.1	Cotton, soybean
18.	Columbia	0.1	Cotton, maize
19.	Sudan	0.1	Cotton
20.	Chile	<0.1	Maize, soybean, canola
21.	Honduras	<0.1	Maize
22.	Portugal	<0.1	Maize
23.	Cuba	<0.1	Maize
24.	Czech Republic	<0.1	Maize
25.	Costa Rica	<0.1	Cotton, soybean
26.	Romania	<0.1	Maize
27.	Slovakia	<0.1	Maize
Total area		175.2	

1 ha = 2.47 acres

which had a positive effect on the shelf-life, resulted in a negative effect on fruit firmness, and also had a very bland taste. Consequently, Calgene halted the production of FlavrSavr tomatoes in 1997.

In the next decade a number of genetically modified crops that were resistant to herbicide tolerance and insect and disease resistance were commercially released in the marketplace in many countries (Table 6.1). These included soybean, maize, cotton, canola, sugar beet, papaya, squash, tomato, poplar, and sweet pepper. Initially, transgenic crops were engineered for either herbicide or pest resistance. However, later on herbicide and insect resistant transgenes were stacked in some of the transgenic crops (James 2013). In spite of the commercially profitable transgenic crops, there are questions regarding the genetic stability of and mode of inheritance of transgenes in the subsequent generations, transgene containment to effectively prevent escape of transgenic pollen and seed, and impact of genetically modified crops on the ecosystem and human nutrition. In order to address some of these concerns and, at the same time, improve human nutrition and other utilities of biotech plants, new areas of biotechnology are being explored for the next generation of genetically modified plants. These include: (a) gene targeting and genome editing; (b) nanobiotechnology; (c) biofortification; (d) molecular farming; (e) biofuels; and (f) bioplastics.

6.2 Gene Targeting and Genome Editing

The established methods of gene transfer have so far led to unpredictable insertion and integration of transgenes in plants, including trees (Finnegan and McElroy 1994; Buteye et al. 2005; Filipecki and Malepszy 2006; Ahuja 1997, 2009, 2011). Gene transfer has been accomplished by *Agrobacterium*-mediated and particle bombardment methods in plants. Transgene integration in the plant genome is a complex process. Generally, *Agrobacterium*-mediated genetic transformation produces transgenic lines with a relatively low (1–3) transgene copy number (Kohli et al. 2003; Olhoft et al. 2004; Oltamanns et al. 2010; Fladung et al. 2013). Particle bombardment transformation method, on the other hand, typically integrates on average higher (1–10 or even up to 100) transgene copy number and complex integration in the genome (Svitashev and Somers 2001; Makarevitch et al. 2003; Kohli et al. 2003). A large number of diverse recombinant genes have been transferred in the genomes of agricultural crops and trees. Transgene integration occurs in plants by illegitimate recombination (Gheysen et al. 1991; Mayerhofer et al. 1991) between T-DNA and host genome. Integration of transgene is a random process, and transgenes may be integrated at one location or dispersed on different chromosomes in plants (Gelvin and Kim 2007; Kohli et al. 2010). One to a number of copies of a transgene may be generally integrated at one or several sites in the host genome. Transgene integration can occur throughout the plant genome (Alonso et al. 2003). Depending on the site of integration of a transgene in the genome, transgene expression may be fairly stable, or there may be variation in transgene expression, or instability/silencing of the transgene.

In order to overcome possible problems of variable transgene expression arising from randomness and multicopy insertions of a transgene in the plant genome, gene targeting systems have been developed in the past decades for directing a single copy of a transgene, or its multiple copies thereof, in a predefined site in the host genome (Pazskowski et al. 1998; Lyznik et al. 2003; Kumar et al. 2006; Poczai et al. 2013; Puchta and Fauser 2013; Ahuja and Fladung 2014). Site-specific recombination systems developed from viruses, bacteria and yeast has been proposed as tools for gene targeting (Liu et al. 2000; Kumar and Fladung 2001; Srivastava and Ow 2004). Two components are needed for site-specific recombination: (1) a site-specific recombinase, and (2) its recognition site (that is a defined sequence). The recombinase systems include, the Cre-*lox* system of bacteriophage P1 (Sauer and Henderson 1990), the FLP-*FRT* (Golic and Lindquist 1989), the R-*RS* (Onouchi et al. 1991) system of yeast, and the Gin/*gix* system of the bacteriophage Mu (Odell and Russell 1994). Site-specific recombination takes place at a recognition site or a specific DNA sequence and involves cleavage, and reunion leading to integration of a recombinant gene, or deletion or inversion of a DNA fragment (Wang et al. 2011). Site-specific recombination systems, experimentally used for *in vivo* excision of donor DNA sequence, have been suggested as strategy to remove the antibiotic marker genes or even the whole transgene cassette from the genome of transgenic plants (De Buck et al. 2007; Luo et al. 2007; Gidoni et al. 2008; Wang et al. 2011). However, the same system, but in the reverse reaction, can be used for targeted integration of DNA (Kumar and Fladung 2001; Lyznik et al. 2003; Tzfira and White 2005). As a prerequisite, one copy of the recognition site must be present in the targeted region, and a second one is located in the DNA to be inserted (Fladung and Becker 2010). If the respective site-specific recombinase is temporally expressed, the desired DNA fragment can exactly be inserted in the targeted region.

A gene targeting approach routinely requires two rounds of transformation: in round one, the target site (e.g. *lox* or *FRT*), is randomly introduced into the plant genome, and in the second round, a *lox*- (or *FRT*) containing recombinant gene is inserted into the previously targeted genomic site (De Buck et al. 2007; Li et al. 2009). Cre-mediated site-specific gene integration has been demonstrated in rice (Srivastava et al. 2004; Srivastava 2013), *Arabidopsis* (Vergunst et al. 1998; Louwerse et al. 2007; De Buck et al. 2007), and hybrid aspen (Fladung and Becker 2010); while FLP-mediated site-specific gene insertion has been shown in soybean (Li et al. 2009), and hybrid aspen (Fladung et al. 2010). In addition, stacking multiple transgenes via repeated recombinase-mediated transformation, at selected genomic sites is experimentally feasible in plants (Li et al. 2010; Ow 2011). The advantage in stacking transgenes at the same site on a chromosome is that the linked-transgenes, following crossings, will most likely be transmitted as a single locus to the progeny. However, it still has to be demonstrated whether site-specific recombination is practically feasible for targeted transfer of numerous stacked transgenes to one genomic position.

A second gene targeting system utilizes synthetic recombinases or nucleases for site-specific insertion of transgenes in the plant genome (Carroll 2011; Curtin et al. 2012; Tzfira et al. 2012; Puchta and Fauser 2013). These nucleases are engineered proteins that are designed to break the double stranded DNA at a specific site, and then exploit homologous recombination to insert a gene at a predetermined location in the host genome. Three sequence-specific nuclease systems have been developed for site-specific integration of genes and mutagenesis in plants. These include: zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and LAGLIDADG homing nucleases, also known as “Meganucleases” (Carroll 2011; Bogdanove and Voytas 2011; Curtin et al. 2012). Targeted integration of herbicide tolerance genes by site-directed homologous recombination using ZFNs has been reported in maize (Shukla et al. 2009) and tobacco (Cai et al. 2009), and by TALENs in tobacco (Townsend et al. 2009). Both recombinase- and nuclease-mediated gene insertions also require transformation systems that include either *Agrobacterium* or particle bombardment.

Although recombinases and nucleases are promising avenues for gene targeting, alternative methods for plant genome editing are being developed because of the complicated designs and laborious assembly of specific binding proteins for each specific target site (Belhaj et al. 2013). Recently, new methods of gene editing/genome engineering have emerged that involve clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases (Cong et al. 2013; Mali et al. 2013; Belhaj et al. 2013; Gaj et al. 2013). The CRISPR/Cas system based targeted cleavage of genomic DNA is guided by a small customized non-coding RNA in gene targeting by both non-homologous and homology-directed repair mechanism.

The real power of the engineered nucleases, recombinase, and CRISPR/Cas systems lies in their ability to precisely engineer not only foreign genes, but also native plant genes for the production of transgenic plants. Another utility of synthetic gene editing systems lies in their potential for activating/editing native plant genes for herbicide tolerance and disease resistance, drought resistance and other qualitative and quantitative traits, rather than engineering exogenous genes for these traits. Such innovations will pave the way for next generation biotech crops to be less regulated or not regulated by federal oversights, as these novel genotypes will be, more or less, substantially equivalent to genetically unmodified plants. Although, site-specific gene integration by recombinases and nucleases, and CRISPR/Cas systems is a promising avenue for stable integration of transgenes in plants, it is still in experimental stages and further research is necessary for their application to next generation crop plants and trees.

6.3 Nanobiotechnology

Nanobiotechnology is a promising field of interdisciplinary research in life sciences. It has enormous potential in the fields of medicine and agriculture. Although, nanobiotechnology is considered to become an important technology in the twenty-first century, it is still in experimental stages in plants. Nanoparticles are extremely small with dimension ranging between 1 and 100 nanometer (nm). Nanoparticles used in agricultural plants generally range in size-dimension from 5 to 200 nm (Ghormade et al. 2011). A wide variety of physical and chemical methods have been used to fabricate nanoparticles, including iron, silver, gold, silicates, and polymers (Mohanraj and Chen 2006; Hayashi et al. 2008; Barman et al. 2014). However, use of toxic compounds used in producing nanoparticles by these methods limit their application. More recently eco-friendly biological methods using plants as biofactories for the production of metallic nanoparticles are being used (Nair et al. 2010; Hasna et al. 2012; Burris et al. 2012; Kavitha et al. 2013; Rai and Yadav 2013; Marchiol et al. 2014; Vadlapudi and Kaladhar 2014; Barman et al. 2014). Potential applications of nanobiotechnology in agriculture include: (1) nano-encapsulated agrochemicals, including fertilizers, for controlled release in the soil; (2) nano-encapsulated herbicides and insecticides for weed and pest control; and (3) nanoparticle-mediated genetic material delivery in plants (Nair et al. 2010; Ghormade et al. 2011; Rai and

Ingle 2012). Mesoporous nanoparticle-mediated DNA delivery has been demonstrated in plants (Torney et al. 2007; Fu et al. 2012; Xia et al. 2013). More recently, mesoporous nanoparticle-mediated site-specific co-delivery of DNA and proteins also been demonstrated in plants (Martin-Ortigosa et al. 2012, 2014). These studies on gene and protein transfer mediated by nanoparticles open up new avenues for loading of nanoparticles with multiple DNA, protein, and chemical complexes for delivery in the plant cells (Martin-Ortigosa et al. 2014). Further experimental studies in nanobiotechnology offer promising prospects not only for the production of genetically targeted transgenic plants, but also for novel applications in improving production and management agricultural crops.

6.4 Biofortification

Recent research in biotechnology has also been focussed on the nutritional enhancement of micronutrients and vitamins in genetically modified crops. Genetic engineering has been employed for fortification of minerals, amino acids, anti-oxidants, vitamins for improving the nutritional quality of the staple crops. Biofortified crops can alleviate essential micronutrient malnutrition in the human population, particularly in the developing countries (Mayer et al. 2008; Hirschi 2009; Beyer 2010; Bashir et al. 2013; Murgia et al. 2013; Pérez-Masscot et al. 2013; Saltzman et al. 2013; Zhu et al. 2007, 2013). More than 50 % of the human population worldwide has little or no access to healthy staple fresh foods (Christou and Twyman 2004). Malnutrition of humans, particularly children, is rampant in underdeveloped countries. Strategies to develop genetically modified plants as a resource for nutritionally enhanced crop plants for food security have been developed by plant biotechnologists in the past decades. Of course, some of the transgenic crops for food security are still in experimental stages, while others have moved to field trials and may become available in the market place in the future. In this direction, the next generation biofortified transgenic crops include:

- Rice that produces β-carotene (provitamin A) in the endosperm (Golden Rice) (Ye et al. 2000; Beyer 2010), and has increased amounts of folate, and mineral (iron and zinc) in the seed (Beyer 2010; Lee et al. 2009, 2012; Yang et al. 2013);
- Wheat grain with enhanced levels of iron and zinc (Borg et al. 2012; Sui et al. 2012; Borrill et al. 2014);
- Potatoes that are protein-rich (Chakraborthy et al. 2010), have better aroma and less browning (Llorente et al. 2010), and exhibit reduced cold-induced sweetening and increased carotenoid content (Giuliano et al. 2006; Chen et al. 2008; Bhaskar et al. 2010; Barrell et al. 2013);
- Tomatoes with increased lycopene and β-carotene (Guo et al. 2012; Liu et al. 2014);
- Bananas that are resistant to fungal wilt (Panama wilt) and black leaf streak diseases, and exhibit increased β-carotene and iron (Aravanityonnis et al. 2008; Kovács et al. 2013; Cressey 2013);
- Corn with enhanced levels of multivitamins, including β-carotene (provitamin A), ascorbate (vitamin C), and folate (vitamin B9) (Naqvi et al. 2009);

- Soybean with lower levels of saturated fat and higher levels of unsaturated oleic acid, and higher levels of omega-3-fatty acids (Herschi 2009);
- Citrus with enhanced levels of β-carotene (provitamin A) (Cao et al. 2012), and antioxidants in the fruit (Pons et al. 2014);
- Cassava with improved nutritional quality, including starch (Ihemere et al. 2006; Zeeman et al. 2010), provitamin A, and other micronutrients (Montagnac et al. 2009; Welsch et al. 2010; Sayre et al. 2011; Adenle et al. 2012);
- Apple resistant to apple scab, fire blight, and improved growth (Malnoy et al. 2008; Borejsza-Wysocka et al. 2010; Joshi et al. 2011; Xu 2013; Schäfer et al. 2012; Krens et al. 2012); early flowering to reduce generation time for breeding to create new cultivars, and earlier yield (Flachowsky et al. 2011; Yamagishi et al. 2014);
- Crop plants with improved nutrition (McGloughlin 2010; Winkler 2011; Murgia et al. 2013; Pérez-Masscot et al. 2013; Saltzman et al. 2013), fortified micronutrients (Mayer et al. 2008; White and Broadley 2009), antioxidants (Zhu et al. 2013), vitamin A (Giuliano et al. 2008), vitamin B1 (Pourcel et al. 2013),vitamin C (Locato et al. 2013), vitamin E (Yabuta et al. 2013), amino acid lysine (Galili and Amir 2013), polyunsaturated fatty acids (omega-3-fatty acid) (Rogalski and Carrer 2011; Petrie et al. 2012; Haslam et al. 2013;), and multivitamins (Hirschi 2009; Fitzpatrick et al. 2012);
- Microalgae as a resource for fatty acids, such as omega-3-fatty acid (Adame-Vega et al. 2012; Vaezi et al. 2013; Martins et al. 2013).

6.5 Molecular Farming

Plants have been used for medicinal purposes for thousands of years by mankind. Molecular farming, or biopharming, is a recent development using transgenic plants, including algae, for the production of high-value pharmaceuticals, including recombinant proteins (vaccines, cytokines, growth hormones) and other secondary metabolites (Daniell et al. 2001; Fischer et al. 2004, 2009; Karg and Kallio 2009; Obeme et al. 2011). Plants offer great potential as production platforms for important pharmaceuticals for safe and effective use by consuming edible plant tissues and seed (Hefferon 2013). Although biopharmaceuticals are predominantly produced in transgenic animal and microbial bioreactor systems, transgenic plants also offer alternatives to large scale biopharmaceutical production in plant tissues and plant cell bioreactors. Plants cells are capable of full post-translational modification of recombinant proteins to fold properly and maintain their structural and functional integrity, with simple growth factor requirement, minerals and light, and essentially unlimited biosynthetic capacity and scalability of biopharmaceuticals in leaves, stems, tubers, seeds, or whole plant, whether grown in the field or in bioreactors. Besides, plants do not harbour human or animal pathogens, including prions, human viruses and oncogenes, making them as safe hosts for the production of biopharmaceuticals (Ma et al. 2003; Davies 2010). Although in earlier works,

Table 6.2 A short list of biopharmaceuticals expressed in transgenic plant systems

Plant	Product	Disease	Reference
Algae	Human papilloma virus Type 16	Human papilloma virus	Giorgi et al. 2010
Tobacco	VLP	Influenza	D'Aoust et al. 2010
Tomato	Hepatitis B surface antigen	Hepatitis B	Gao et al. 2003
Lettuce	Hepatitis B antigen	Hepatitis B	Stearfield 2006
Tomato	Cholera Clox A and B	Cholera	Sharma et al. 2008
Algae	*Plasmodium falciparum* specific protein	Malaria	Jones et al. 2013
Tobacco	CTB-EsAT6 fusion protein	Tuberculosis	Lakshmi et al. 2013
Tobacco	Cytokines	Cancer, immune disorders	Sirko et al. 2011
Tobacco	E-glycoprotein	Dengue	Kim et al. 2009
Maize	Heat labile toxin B	Diarrhea	Tacket 2005

transgenic plants or their cell cultures were used for the expression of recombinant proteins, more recently transient expression systems, involving the agroinfiltration methods (Kapila et al. 1997; Pouge et al. 2010; Chen and Lai. 2013), the virus infection method (Porta and Lomonossoff 2002; Varsani et al. 2006; McCormick et al. 2008), and the magnifection technology (Gleba et al. 2005), have been developed in plants for the production of biopharmaceuticals. Transient expression platforms, perhaps the most convenient and efficient platforms, allows the cultivation of plants under stringent controlled conditions, without stable genetic transformation, for the rapid production of high grade pharmaceutical proteins on a large competitively commercial scale (Rybicki 2010; Komarova et al. 2010; Tremblay et al. 2010; Circelli et al. 2010).

The first recombinant proteins (human growth hormone) and with therapeutic potential was successfully expressed in transgenic plants (Barta et al. 1986). A few years later, the use of transgenic plants producing edible vaccines was reported (Mason et al. 1992). It was not until 1997 that a recombinant protein, avidin (an egg protein) was produced for commercial purposes in transgenic maize (Hood et al. 1997). Subsequently, it was shown that transgenic plants have the ability and capacity for the expression of a number of functional mammalian proteins with therapeutic value, such as human serum proteins, growth hormones, antibodies, vaccines, cytokines, and enzymes (Daniell et al. 2009; Karg and Kallio 2009; Obeme et al. 2011; Franconi et al. 2010; Penney et al. 2011; Sirko et al. 2011; Kumar et al. 2013; Rigano et al. 2013; Da Cunha et al. 2014; Specth and Mayfield 2014). Recombinant biopharmaceutical production is moving at a very fast pace since 2007 when it captured about 10% of the pharmaceutical market (Lowe and Jones 2007). By the year 2010, there were more than 200 bio-drugs approved biopharmaceuticals on the global market, generating more than $ 100 billion in the global pharmaceutical market (Walsh 2010). It is expected that the biopharmaceutical market will continue to expand in the future and the bio-drug sales may reach up to $ 240 billion by 2015 (Stewart 2010). Some of the biotech plants, including microalgae, used for the production platforms of a number of biopharmaceuticals are listed in Table 6.2. For more detailed listings of plant-based biopharmaceuticals see reviews by Daniell et al. (2009), Walsh (2010), Obeme et al. (2011), Da Cunha et al. (2014), and Specht and Mayfield (2014).

6.6 Biofuels

Finite reserves of petroleum-based fossil fuels, accompanied with global warming and depletion of fossil fuels at a rapid pace, have provided a strong impetus to search for alternative and sustainable sources of renewable energy. Plant cell wall carbohydrates are the most abundant renewable resource on our planet Earth. The major components of plant cell walls are cellulose, hemicellulose, and lignin that comprise up to 90% of its dry biomass (Harris and Stone 2008; Pauly and Keegstra 2008; Gibson 2012; Hadar 2013). The biofuels from renewable plant-based biomass could alleviate the world-wide dependence on the fossil fuels. The biofuel that would partially sustain the global energy demands is the liquid fuel that can be produced from abundant and freely accessible fatty acids and sucrose in plants. Biodiesel is presently produced from the fatty acids in palm oil, soybean oil, and oilseed rape. Bioethanol is currently produced mainly from sugars from sugar cane, and digested starch from the seeds of maize. However, there is an ongoing debate about the "food versus fuel" regarding the use of staple crop grains for biofuel production (Kullander 2010; Valentine et al. 2012; Zhang 2013).

In order to secure food for the rapidly growing world population, food-plant residues and non-food plants are also being used to produce biofuels. These include agricultural crop residues, grasses, and forest trees. Lignocellulosic feedstock from wood, straw and grasses is currently the focus of biofuel production from plants. The abundant resource for lignocellulosic feedstock can be found in short-rotation fast-growing trees species as, poplars (*Populus* spp. and hybrids), salix (*Salix*), eucalypts (*Eucalyptus*), and grasses, switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus* x *giganteus*) (Yuan et al. 2008; Capita and McCann 2008; Jørgensen 2011; Mizrachi et al. 2012; Nieminen et al. 2012; Joyce and Stewart 2012). In spite of the abundant lignocellulosic biomass, the costs incurred for extraction of biofuelic ethanol by routinely used methods are at least two to threefolds higher than sugar or starch based ethanol production (Sticklen 2008). Although breeding and mutation induction in plants for reduction of lignin content in biomass feedstock remains a viable alternative (Bourton 2007), it will take a long time to achieve this goal in plants, particularly trees. Therefore, the current focus on these non-food plants is on the reduction of lignin content by genetic manipulation for cost-effective ethanol production from the lignocellulosic feedstock (Gressel 2008; Hisano et al. 2009; Simmons et al. 2010; Mansfield et al. 2012). Next generation biotech plants for increased content and improved quality of biofuel production include:

- Maize with altered lignin biosynthesis by suppression of CAD activity to improve bioethanol production (Fornale et al. 2012);
- Switchgrass with down-regulation of *CAD*, or *COMT*, or silencing of *4CL* genes in the biosynthetic pathway to reduce lignin content, or use of transcription factor PvMYB-4 as potential for developing lignocellulosic feedstock for improved fermentable sugar for biofuel production; (Fu et al. 2011; Xu et al. 2011; Ye et al. 2011, Yee et al. 2012; Shen et al. 2012, 2013);

- Poplar with down regulation of *CCR* gene to reduce lignin content that resulted in increased saccharification and high ethanol yield (Van Acker et al. 2014); or suppression of other genes (*CAD*; *4CL*. *C3H*,*COMT*) involved in the biosynthesis of lignin to enhance biofuel production (Coleman et al. 2008; Mansfield et al. 2012; Voelker et al. 2011; Ye et al. 2011)
- Microalgae as a resource for production ofbiodiesel and bioethanol (Schenk et al. 2008; Beer et al. 2009; Brennan and Owende 2010; Gouveia and Oliveira 2009; Mata et al. 2009; Bajhaiya et al. 2010; Dragone et al. 2010; Huang et al. 2010; Khola and Ghazala 2012; Wu et al. 20123; Harun et al. 2014);
- Industrial waste agricultural residue biomass as a potential source of biofuel (Mwithiga 2013);
- Forest trees (especially poplars, eucalypts, and salix) as a resource of lignocellulosic feedstock, and lignin modification for improved production of biofuels (Rockwood et al. 2008; Simmons et al. 2010; Seguin 2011; Mizrachi et al. 2012; Nieminen et al. 2012; Pilate et al. 2012).

6.7 Bioplastics

In addition to current focus on using plants for biofuels, plants also produce a large number of useful chemicals and biopolymers. Plants naturally produce a large number of biodegradable polymers, which include starch, cellulose, proteins and rubber (Kulkarni et al. 2012). Starch and cellulose play a major role in food and fibre production for mankind. However, plants do not produce bioplastics, but many bacteria including, *Ralstonia*, *Pseudomonas*, *Azobacter*, *and Rhizobium* do (Dalton et al. 2011). Bacteria (e.g. *Ralstonia*) accumulate the polyester polyhydroxyalkanoate (PHA) as a bioplastic for carbon and energy reserve in response to nutritional stress (Anderson and Dawes 1990). Polyhydroxybutyrate (PHB), a short side-chain polymer of PHA, in produced in bacteria from acetyl-coA via a three enzymes biosynthesis pathway. These enzymes are encoded by three genes, *phaA*, *phaB*, and *phaC* respectively (Slater et al. 1988). Currently, the PHA bioplastics are being commercially produced in bacterial fermentation systems, with renewable resources as sucrose, glucose, fatty acids, or plant oils, or waste effluents (molasses, whey), and glycerol as carbon substrates (Chee et al. 2010; Chen 2009, 2010; Du et al. 2012). Wild type bacterial strain of *Ralstonia eutropha* (formerly known as *Alcaligenes eutropha*) has been most commonly used for industrial production of bioplastic (Chen 2009). However, the bacterially produced biodegradable bioplastic polymers are not cost-competitive with non-biodegradable petroleum-based plastic polymers. Alterative platforms to bacterial fermentation are being explored for production of bioplastics using transgenic plants that might be more cost-effective than bacterial fermentation and petroleum-based plastics (van Beilen and Poirier 2008; Mooney 2009; Somleva et al. 2013; Petrasovits et al. 2013).

A publication entitled ‘In search of plastic potato’ by Pool (1989) generated great expectation in the scientific community that bioplastic PBA could be produced by

Table 6.3 Bioplastic production in some transgenic plants

Plant	Polymer	Tissue	Yield DW%	Reference
Arabidopsis	PHB	Leaves	13.2–40	Bohmert et al. 2000, 2002
Tobacco	PHB	Leaves	18.8	Bohmert-Tatarev et al. 2011
Switchgrass	PHB	Leaves	7.3	Somalev et al. 2013
Corn	PHB	Leaves	5.73	Poirier and Guys 2002
Sugar beet	PHB	Hairy root culture	5.5	Menzel et al. 2003
Sugarcane	PHB	Leaves	4.8	Petrasovits et al. 2012
Poplar	PHB	Leaves	1–2	Dalton et al. 2011

genetic engineering on plants. A few years later, PHB (0.01 % fresh weight; FW) was first produced in the model plant *Arabidopsis thaliana* transgenics (Poirier et al. 1992), which initiated a continuing wave of research to optimize the production of PHB in transgenic plants (Somleva et al. 2013). Some improvement in the PHB yield (14 % dry weight; DW) was reported in transgenic *Arabidopsis*, with no obvious effect on the growth and fertility of the transgenic plants (Nawrath et al. 1994). Subsequently, a later study on transgenic *Arabidopsis* showed efficient production of PHB (40 % DW) in the chloroplasts of the leaves, but that was accompanied by severe growth reduction of the transgenic plants (Bohmert et al. 2000). Since then a large number of studies with many plant species have been conducted that show variable production levels of the bioplastic PHB (0.005–40 % DW) in transgenic plants (van Beilen and Poirier 2008; Somleva et al. 2013). Different tissues, including whole plant, shoot, stem, leaves, and cell suspension were employed for PHB production, which mostly accumulated in the plastids, but also in the cytoplasm (Van Beilen and Poirier 2008; Somleva et al. 2013). In addition, transgenic microalgae, such as green algae *Chlamydomonas reinhardtii*, and diatoms*Phaeodactylum tricornutum* engineered with PHB pathway genes from *Ralstonia eutropha* have also been explored as bioreactors for bioplastic production (Chaogang et al. 2010; Hempel et al. 2011). A large number of transgenic plants, both crops and non-crops, have been employed, and show different amounts of biodegradable bioplastics (van Beilen and Poirier 2008; Someleva et al. 2013). Some of transgenic plants for bioplastic production are listed in Table 6.3.

6.8 Future Prospects

A lot of progress has been made in different areas plant biotechnology in the last two decades. Initially crop plants were engineered with foreign genes derived mostly from bacteria for herbicide and pest resistance to improve crop yields. Later on, other transgenes for lignin modification, early flowering, male sterility, and abiotic stresses were experimentally tested in crop plants. Earlier studies in plants used alien genes from bacteria, animals and plants for genetic engineering. Recent trend has been towards development of cisgenic and intragenic transgenic crop plants (Holme et al. 2013; Espinoza et al. 2013). Cisgenic plants are derived from transformation with identical copy of a gene from sexually compatible pool, including promoter, intron and terminator regions that are derived from the donor plant. On the other hand, intragenic plants are derived by transformation with combinations of different genes from the same or sexually compatible species. While both cisgenic and intragenics plants are guided by their own genes, they both require genetic transformation by *Agrobacterium* or biolistic methods. Recent research in gene targeting and directed genome engineering promises site-specific integration of transgenes in predetermines regions of the host genome, or tinkering of the endogenous genes of economic importance for their stable transgene expression and inheritance in the next generation crop plants. Next generation genome sequencing is already providing useful information regarding gene discovery and molecular markers associated with a number of diverse economic traits (Edwards and Batley 2010; Hamilton and Buell 2012). DNA sequencing is providing insight information genes that would be useful for plant improvement through plant biotechnology.

While these promising investigations are progressing at a rapid pace for the commercialization transgenic/biotech crops, plant biotechnology has also harvesting other useful plant products. These include next generation transgenic plants as future production platforms for biopharmaceuticals, biofuels and bioplastics, and nutritional supplements. In a sense, plants are becoming biofactories/bioreactors for useful bioproducts for mankind. Nevertheless, transgenic plants are subject to regulatory oversights. Containment of transgenes must be in place to effectively prevent escape of transgenic pollen seed, and vegetative propagules from the transgenic plants. In addition, these new areas in plant biotechnology must take into account risk assessment and biosafety considerations (Shama and Peterson 2008; Breyer et al. 2009; Sparrow and Twyman 2009; Domingo and Bardonaba 2011; Snell et al. 2012; Buiatti et al. 2013; Jouzani and Tohidar 2013), including the impact of these new developments on human health (new allergies), ecosystem, plant biodiversity, and sustainable agriculture. New developments in nanobiotechnology offer prospects for precise delivery of genetic material and enhanced production of agricultural crops. It would be necessary to maintain genetic biodiversity in the next generation biotech plants (Sharma and Sharma 2013) used for the bioproduction platforms as well as for crop improvement.

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