Engineering of Plants for the Production of Commercially Important Products: Approaches and Accomplishments

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Abstract

For centuries humans have used plants as a source of food, fiber, fuel, and medicine because they have the ability to synthesize a vast array of complex organic compounds using light, carbon dioxide, and water. Advances in recombinant DNA and transgenic technologies during the last several decades have opened many new avenues to further exploit plants for production of many novel products. The potential to use plants to synthesize diverse native and nonnative industrial and pharmaceutical products coupled with the depletion of fossil fuels that are the source of many commercially important products and the adverse effects of chemical synthesis of platform chemicals on the environment have renewed considerable interest in using plants for large-scale production of chemicals and value-added compounds. To accomplish this, different genetic engineering and transformation strategies have been developed for introducing multiple genes (gene stacking), modulating their expression with regulatable promoters, and targeting the products to a specific compartment in the cells. Successful metabolic engineering of plants should lead to sustained production of platform chemicals, pharmaceuticals, and biopolymers. In

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this chapter, we present an overview of different methods that are currently used to introduce and manipulate expression of one or more genes into plants and discuss some of the recent achievements in producing value-added products and pharmaceuticals in plants.

Keywords
Metabolic engineering • Pharmaceuticals • Platform chemicals • Value-added compounds • Biomass • Butanetriol • Bioenergetics • Bioplastics • Transformation • Molecular pharming

28.1 Introduction

Plants, as photoautotrophs, convert light energy into chemical energy and use it for their growth, development, and synthesis of many complex organic compounds. Humans have been using plants for centuries as a renewable source of food, fiber, fuel, pharmaceuticals, and other biomaterials. Since plants naturally produce a vast array of complex organic compounds and secondary metabolites, they have been used extensively to produce many commercially important industrial and consumer products (United States Department of Agriculture 2008; Saito and Matsuda 2010; Yoon et al. 2013). During the last century, petroleum-based chemicals were widely used to produce diverse products ranging from plastic polymers to cosmetics. However, increasing demand for petroleum and petroleum-derived products due to population growth (United Nations 2013), the diminishing supplies of fossil fuels, and the adverse impact of producing industrial chemicals from fossil fuels on the environment renewed the interest in using plants to produce industrial chemicals and other value-added products in a sustainable and environmentally safe manner. It is anticipated that plants will play an important role in bio-based economy in reducing reliance on petrochemicals and in producing many unconventional chemicals that are currently produced from petrochemical feedstock and also in producing biofuels (United States Department of Agriculture 2008). This bio-based approach offers several advantages for the production of industrial chemicals. These include sustainability as plants are renewable sources and the likelihood of being highly cost-effective as they use light energy and CO_2 to produce sugars and a vast repertoire of organic compounds that can serve as precursors for the synthesis of desirable carbon compounds. Furthermore, bio-based synthesis of industrial chemicals is environmentally friendly as compared to chemical synthesis. In addition, the development of technologies in the 1980s to stably introduce and express genes in plants has opened new avenues to use plants to produce novel platform chemicals and pharmaceutical products that are not normally produced in plants (Poirier et al. 1992, 1995; Bohmert-Tatarev et al. 2011; Golovkin 2011; Abdel-Ghany et al. 2013).

Advances in plant biotechnology have allowed engineering of complex biochemical pathways not only by manipulating expression of a single gene but also by introducing multiple genes from other plants and non-plant systems including bacteria. Coordinated expression of multi-subunit proteins and manipulation of metabolic pathways have been widely used for diverse applications (Table 28.1). These include improvement of food and feed quality (Fitzpatrick et al. 2012; Tieman et al. 2012), increased tolerance of crop plants to abiotic and biotic stresses (Rhodes 1994; Nanjo et al. 1999; Munns 2005), development of weed and insect resistant crops, and use of plants for production of polymers (bioplastics), biofuels, platform chemicals, and pharmaceuticals (Nikolau et al. 2008; Andrianov et al. 2010; Bohmert-Tatarev et al. 2011; Abdel-Ghany et al. 2013; Golovkin 2011).

This chapter will focus on production of commercially important products such as platform chemicals and pharmaceuticals in plants using

Table 28.1 Some examples of different applications of plant biotechnology in agriculture, medicine, and other areas

Agriculture	Molecular farming	Other areas
Herbicide resistance	Pharmaceutical proteins	Phytoremediation
Pathogen resistance	Medicinal compounds	Biofuels
		Energetic materials
Manipulate growth and development	Industrial chemicals/proteins	
Yield and product quality	Polymers	Cosmetics
Senescence and stress control		

plant biotechnological approaches. Introduction and manipulation of foreign genes in plants is fundamental to all biotechnological approaches; hence, we will first provide an overview of different methods that are currently used to introduce and manipulate expression of one or more genes in plants. We will then discuss how these methods are used to engineer plants to produce many valuable products by manipulating existing metabolic pathways or introducing novel pathways.

28.2 Commonly Used Methods to Introduce Genes into Plants

Prior to the 1980s, people used to introduce desired genes/traits into crop species through conventional breeding between the domesticated varieties and the wild relatives that have the traits of interest, and then new cultivars with desirable characteristics are obtained by backcrossing (Yan et al. 2010; Powell et al. 2012). This is very laborious, time consuming, and limits gene transfer between sexually compatible plant species. However, genes of many desirable traits may not be found in crossable relatives of the interested crop. The development of a method in the early 1980s to stably introduce and express a foreign gene from other organisms into plants has changed the way to introduce new traits into plants. As discussed below, it is now possible to introduce multiple genes (gene stacking) from any organism into the nuclear or chloroplast genome of plants; produce proteins constitutively, in a particular cell type or in response to a chemical or environmental cue; and target the proteins to a desired cellular location. These improvements allowed high level of expression of multiple genes and allowed engineering of plant metabolism to produce desired chemicals. In Table 28.2, we summarized various methods to introduce and manipulate expression of genes in plants and their advantages and disadvantages.

28.2.1 *Agrobacterium*-Mediated Transformation

The most commonly used method for introducing a gene into a plant genome is *Agrobacterium-mediated* transformation using the soilborne pathogen *Agrobacterium tumefaciens* (Tzfira and Citovsky 2003; Valentine 2003; Gelvin 2012). In *Agrobacterium*-mediated transformation, the bacterium represents the biological machinery that delivers the gene(s) of interest into the plant genome. This process involves proteins derived from both the bacterial pathogen and the host plant (reviewed in Gelvin 2012). In nature, *Agrobacterium* infects some plant species and causes crown gall tumors. During infection, the *Agrobacterium* transfers a portion of the DNA, called T-DNA, from the tumor-inducing (Ti) plasmid into the host where it subsequently integrates into the host genome. Wild-type T-DNA encodes genes involved in hormone and opine biosynthesis that are expressed in host cells and cause cell proliferation and tumor formation (Gelvin 2012). To introduce desired genes into plants, the T-DNA was engineered by replacing the hormone and opine biosynthetic genes with selectable marker genes and multiple cloning sites for insertion of gene(s) of interest flanked by the T-DNA left and right border sequences. The engineered T-DNA was also moved into a small plasmid for easy cloning of genes using an *Escherichia coli* system, and then this vector with

Table 28.2 Summary of current plant transformation and expression systems

Major system		Expression levels	Advantages	Disadvantages
Stable	*Nuclear*			
	Constitutive expression Transgenic plants and cell cultures	Modest (<1 % TSP) (Seeds >1 %)	Inheritable gene Broad host range Possible direct consumption Plant-specific PTM (glycosylation) High yield and scale-up	Low expression Lengthy procedure Positional effect and gene silencing Environmental risks
	Chloroplast			
	Constitutive expression Transplastomic plants	High (>5 % TSP)	High expression and accumulation Inheritable gene No gene transfer though pollen No position effect and gene silencing Scale-up capacity	Prokaryotic machinery No glycosylation Narrow host range Somatic propagation Lengthy procedure
Transient	*Plant viruses*			
	Transient expression/ modern systems/ wild-type plants	High (>10 % TSP)	High expression and accumulation Easy molecular manipulations Simple and quick transformation Short production times High scale-up capacity Less environmental risks	Not inheritable Restricted to some hosts Need of purification (endotoxins) Some environmental risks

PTM posttranslational modification, *TSP* total soluble protein

genes of interest is introduced back into an *Agrobacterium* strain (helper strain) containing a Ti plasmid harboring the virulence (*vir*) genes but without T-DNA (Anami et al. 2013). To stably introduce gene(s) into plants, the *Agrobacterium* containing the engineered plasmid is cocultivated with the explant. During the cocultivation, *Agrobacterium* transfers T-DNA into the host cell, which then integrates randomly into the nuclear genome by nonhomologous recombination. Transgenic calli or somatic embryos are selected and regenerated on selection medium. In Arabidopsis, transgenic lines can be generated by submerging or spraying the floral buds with *Agrobacterium* culture and then selecting the transgenic lines by germinating the seeds on selection plates (Clough and Bent 1998). In nature, *Agrobacterium* mainly infects dicotyledonous plants (De Cleene and De Ley 1976). However, under laboratory conditions, it is the method of choice for genetic manipulation of many plant species including monocots (Tzfira and Citovsky 2006). Some genotypes and plant species are not transformable with *Agrobacterium*, a barrier that limits the use of *Agrobacterium-mediated* transformation in these species.

28.2.2 Direct Gene Transfer

Direct gene transfer by high-velocity microprojectiles (also called particle bombardment or biolistic method) and electroporation has also been widely used to introduce genes into plants, especially in those species that are not amenable for transformation with *Agrobacterium* (Altpeter et al. 2005; O'Kennedy et al. 2011). In case of particle bombardment, metal particles coated with plasmid DNA containing the gene(s) of interest are introduced into plant cells in a pressurized helium stream using a gene gun. Physical and chemical parameters such as rupture pressure, type of particles, particle travel distance, and DNA concentration can be optimized to different tissues and different species. In case of electroporation, plasmid DNA is introduced into protoplasts

by subjecting the cells to an electric shock (Anami et al. 2013). There are advantages and disadvantages of both *Agrobacterium-mediated* transformation and particle bombardment methods. The ability of Agrobacterium to introduce a single or a few copies of gene per genome is an advantage over particle bombardment that results in insertion of multiple copies, which might result in recombination and instability (Gao and Nielsen 2013). On the other hand, some advantages make particle bombardment a method of choice for engineering of recalcitrant plant species, different cell types, organelle transformation, and simultaneous transformation of multiple genes with no need for complex cloning or crossing of lines carrying different transgenes (Altpeter et al. 2005).

28.2.2.1 Stable Versus Transient Transformation

Once foreign DNA is integrated into the host genome (stable transformation) by either method, it is inherited and segregated in the progeny according to Mendelian laws (De Block et al. 1984; Horsch et al. 1984). However, during propagation of these lines, complete loss of function of the transgene or significant variation of gene expression among lines might occur as a result of gene silencing, multiple integration events, or complex genetic rearrangements (Slater et al. 2003; Schiermeyer et al. 2004). Analysis of a large number of independent transgenic lines for each gene is necessary to achieve the highest expression/accumulation of introduced gene product. In stable transformation, the effect of transgenes can be studied at the biochemical, physiological, morphological, genetic, and cell biological levels to gain insight into their functions and biological roles. This is different from transient transformation where gene expression, subcellular localization, or protein targeting can be tested within hours to days, thus avoiding *in vitro* plant regeneration procedures (Sheen 2001). Transient transformation can be performed by shooting DNA-coated beads into epidermal peels, cell cultures, and tissues, electroporation with protoplasts, or agroinfiltration of leaves (Kirienko et al. 2012; Mangano et al. 2014). Agroinfiltration of *Nicotiana benthamiana* leaves has been used widely to study protein-protein interactions by means of bimolecular fluorescence complementation technology (BiFC) and fluorescence resonance energy transfer (FRET) (Ali et al. 2008; Boruc et al. 2010). Recently, an Arabidopsis proteome chip prepared from thousands of transiently expressed Arabidopsis cDNAs in *Nicotiana benthamiana* leaves was used to perform protein-protein interaction studies, which resulted in identification of targets of calmodulin and MAP kinases (Popescu et al. 2007; Lee et al. 2011).

28.2.3 Organelle Transformation

There is a public concern about the insertion of a transgene in the nuclear genome as the transgenes could move – "transgene flow" – to wild species or to weedy relatives growing in natural or seminatural communities (Wilkinson et al. 2003; Singh et al. 2010). In recent years, researchers have begun to introduce genes into plastids as an alternative approach to nuclear transformation as it offers several advantages over transferring genes into nuclear genomes (Bock 2007; Wani et al. 2010). First, plastid transformation has the potential to eliminate the gene flow through pollen grains since plastids are maternally inherited in a majority of angiosperms (Wani et al. 2010). Second, plastid transformation allows high levels (up to 40 %) of protein expression compared to 0.5–3 % in case of nuclear transformation (Daniell et al. 2002). The high production of expressed protein in transplastomic plants (plants with transformed plastid genome) is possible due to the high copy number of plastids per plant cell, and also the expressed proteins are less affected by pre- and posttranscriptional gene silencing. Third, plastid transformation has the advantage of expressing multiple genes from polycistronic mRNA with the absence of epigenetic effect and gene silencing (Sidorov et al. 1999; Maliga 2001). However, there are a few drawbacks in introducing genes into the plastid genome. The expressed proteins in the plastids are not glycosylated because of the prokaryotic

nature of the plastids. This is important especially for production of pharmaceuticals (Golovkin 2011). Another problem is the lack of tissue-specific and developmentally regulated control mechanisms (Wang et al. 2009). This is necessary when expression of the transgene is desired to be limited to a specific tissue, organ, and/or developmental stage or the high level of foreign proteins in plastids may have deleterious pleiotropic effects (Magee et al. 2004). Recently, the toxic effects of transgene expression in plastids have been addressed by using an inducible expression system that requires coordination between nucleus and plastids. A T7 RNA polymerase gene (T7RNAP) fused to a chloroplast transit peptide at the N-terminus was cloned under the control of an alcohol-inducible promoter and introduced into the nuclear genome of tobacco. Induction of T7RNAP by ethanol application leads to its expression and transport to plastids where transcription of the plastid transgene under the control of the T7 promoter is turned on (Lossl et al. 2005). Also, an external control of plastid transgene expression was developed using modified promoters containing binding sites for bacterial *Lac* repressor. This allows chemical induction of plastid transgene in intact plants or after harvesting (Muhlbauer and Koop 2005).

Plastid transformation has been used to introduce new traits and also for basic research to understand processes in plastids such as RNA editing (Ruf and Bock 2011). Transgenic chloroplasts have been tested for the production of many therapeutic proteins. This method avoids the expensive fermentation procedures, and, in addition, the oral delivery of plastid-derived therapeutic proteins could eliminate the expensive purification, cold storage, and cold transportation (Singh et al. 2009). More than 20 vaccines were successfully expressed in chloroplasts (Golovkin 2011), suggesting that chloroplast contains the mechanisms that allow correct folding and disulfide bond formation that result in fully functional proteins. However, further studies are needed to establish a feasible production system for biopharmaceuticals in chloroplasts. If functional glycoproteins could be expressed in chloroplasts, then plastid engineering would be of great use in expressing many commercially important proteins. Plastid engineering was also used for enhancing the nutritional value of food crops such as producing fruits with elevated contents of β-carotene (Apel and Bock 2009), for phytoremediation (Ruiz et al. 2003), for reversible male sterility (Ruiz and Daniell 2005), for biotic and abiotic stress tolerance (Wani et al. 2010), and for production of platform chemicals such as the bioplastic polyhydroxybutyrate (PHB) (Nakashita et al. 2001; Lossl et al. 2003) (Sect. 28.7.4.2).

Although the first transgene delivery to plastids was achieved using the *Agrobacterium-mediated* method (Block et al. 1985), particle bombardment is the method of choice for plastid transformation. To obtain a genetically stable transplastomic line, all genome copies have to be uniformly transformed (homoplasmic), a process that takes a long time. Plastid transformation occurs through the following steps: First, introduction of the plasmid containing the selectable marker (e.g., an antibiotic resistance) and the gene of interest by the biolistic process or by polyethylene glycol; second, integration of the foreign DNA by homologous recombination into the plastid genome; third, gradual elimination of wild-type genome copies during repeated cell division on a selective medium; and fourth, regeneration of the transplastomic shoot. In some cases, the antibiotic resistance used in selection is not desirable in the final products; therefore, many strategies have been developed to eliminate the necessity of using these selectable markers (Maliga 2002, 2003; Wang et al. 2009).

28.2.4 Viral-Based Transient Expression of Proteins

Modular transient expression systems using plant viral vectors are offering a robust industrial method to achieve high yields of heterologous proteins. The advantage with this method is that the transgene is not heritable. Two strategies have been used for transient expression using viral vectors. The first is called a "full virus strategy." The protein of interest, e.g., small protein fragment such as immunogenic epitope, is expressed as a

fusion to the viral coat protein usually in tobacco (Grevich and Daniell 2005). The major limitation of this strategy is that inserts larger than 1 kb cannot usually be expressed. In the second strategy that uses so-called deconstructed viral vectors, there is no "20–25 amino acid barrier" for coat protein fusion and longer proteins up to 80 kDa can be expressed. In this strategy, only the viral elements required for efficient expression of the interested gene were maintained, while the components needed for infectivity, amplification/replication, cell-to-cell movement, assembly of viral particles, and systemic spread are provided using nonviral components (Gleba et al. 2007). Depending on the vector used and the host organism, the process takes 4–16 days, and depending on the gene of interest, up to 5 g recombinant protein per kg fresh leaf biomass can be produced (Gleba et al. 2007). The viral-based expression system has already been used to produce functional platform proteins of different origin such as biologically active human growth hormone and antigens (reviewed in Gleba and Giritch 2011). Overall, this industrial process has a rapid scale-up and high-yield production of diverse heterologous proteins, thereby greatly reducing the cost and time.

28.3 Manipulation of Transgene Expression

Unlike in bacteria, in plant metabolic engineering great care must be taken to control transgene expression. Plants have organelles that affect resources compartmentalization. Also, plants have numerous specialized and differentiated organs in which gene expression may differ dramatically. In addition, temporal and developmental processes can significantly influence when and where the transgene will be active. Moreover, the aim of the engineering process also influences the design strategy. For example, is the engineering intended to impact native physiological processes or synthesis of metabolites or is the goal to overproduce new products or proteins? Such considerations mandate the type of promoters that drive the transgene and its level of expression.

The promoter, a portion of DNA upstream of a gene transcription start site, controls the gene expression levels and its specificity i.e., constitutive, inducible, tissue-specific, or developmentally regulated. In transgenic plants, promoters are used to regulate the level and specificity of the transgene expression (Anami et al. 2013). Various types of promoters that differ in strength and regulation have been used in plant metabolic engineering.

28.3.1 Constitutive Promoters

Constitutive promoters are used to produce large quantities of the transgene product in all plant parts and throughout the growth and development period. Several constitutive promoters have been used successfully to express transgenes in plants. The most commonly used are those of viral origin such as the cauliflower mosaic virus 35S promoter (35S CaMV) that confers a high expression in most cell types except pollen. The expression of these promoters is independent of environmental conditions, and they are active in various monocotyledonous and dicotyledonous plants (Odell et al. 1985; Benfey and Chua 1990). Although a very high level of expression is achieved by using the constitutive promoters of viral origin, gene silencing via co-suppression has been reported (Koosha et al. 1989). Co-suppression phenomena can be avoided by using constitutive promoters of plant origin such as maize ubiquitin (maize *Ubi-1*) (Shrawat and Lorz 2006), rice actins (*OsAct1* and *OsAct2*) (McElroy et al. 1990), *Brachypodium distachyon* elongation factor, ubiquitin (*BdEF1α* and *BdUBI1*) (Coussens et al. 2012), and switchgrass ubiquitin promoters (*PvUbi1* and *PvUbi2*) (Mann et al. 2011). Other constitutive promoters that are also widely used are nopaline synthase (*nos*), octopine synthase (*ocs*), mannopine synthase (*mas*) (von Lintig et al. 1994), and maize alcohol dehydrogenase 1 (*Adh1*) (Kyozuka et al. 1994). To introduce more than one gene in a transgenic plant, different promoters and terminators should be used to avoid the co-suppression and silencing of the introduced genes (Abdel-Ghany et al. 2013).

28.3.2 Inducible Promoters

In some cases, constitutive expression of an introduced gene can have adverse effects on plant growth and development when the overproduction occurs at the wrong time in development or in a tissue where it is not normally expressed. For instance, in metabolic engineering of Arabidopsis for cellulose production, constitutive overexpression of glucanases and cellulases (cellulose-degrading enzymes) reduced cellulose crystallinity and the glucose content in the cell wall (Takahashi et al. 2009), modified leaf shape and growth (Petersen and Bock 2011), and reduced environmental stress tolerance (Taylor et al. 2008). Therefore, under certain circumstances, it is desirable to use inducible promoters to regulate foreign gene expression to a distinct developmental stage or for a certain duration (Cai et al. 2007). Inducible systems are usually based on two components. The first component is a native or chimeric transcription factor (activator) that when induced specifically binds to a specific promoter. The second component contains the binding sites for the activator to activate transcription of a gene fused to this promoter (Borghi 2010). Inducible promoters are activated in response to various external stimuli, which can be chemical factors such as tetracycline, ethanol, steroids, metal ions, and herbicides or physical factors such as heat, cold, and light (Zuo and Chua 2000). Those promoters that respond to specific chemical compounds, not found naturally in the organism of interest, are of particular interest in metabolic engineering because they offer very stringent regulation of gene expression. Tetracycline inducers are particularly attractive because they are small lipophilic compounds that enter easily into eukaryotic cells by passive diffusion. They have been used successfully to produce valuable pharmaceuticals or industrial proteins in plant cell suspension culture (Bortesi et al. 2012). Another antibiotic, which has been used widely in bioengineering of cell culture, is pristinamycin. The use of a pristinamycin-responsive promoter to drive transgene expression has the advantage of controlling bacterial contamination as well as easy induction of the transgene within the bioreactor (Frey et al. 2001). The ethanol-inducible system (Roslan et al. 2001), derived from the filamentous fungus *Aspergillus nidulans*, has been optimized for the production of proteins in plants and studying the function of proteins (Van Hoewyk et al. 2007). Steroid-inducible systems have also been used in studying the function of plant transcription factors that control plant developmental pathways (Aoyama et al. 1995; Gunl et al. 2009). Also, the promoter of the Arabidopsis heat shock protein (HSP18.2) has been used successfully to induce expression of desired genes in several plants after heat shock (37 °C) (Matsuhara et al. 2000; Masclaux et al. 2004). In the absence of heat stress, the HSP18.2 promoter is repressed (Yoshida et al. 1995).

28.3.3 Organ-, Tissue-, Domain-, and Cell-Type-Specific Promoters

Rather than overexpressing the transgene in all tissues, another option is to target the transgene expression to a specific organ or tissue that is rich in precursors of a desired metabolic pathway or is easy to harvest, e.g., leaves, tubers, or fruits (Lessard et al. 2002). Several endosperm-specific promoters have been used to express single or multiple enzymes of biochemical pathways in this tissue either to dissect a metabolic pathway or to improve the nutritional value of seeds (Naqvi et al. 2009; Coussens et al. 2012). Expression of phytoene synthase (*psy*) in indicia rice under the control of seed-specific glutelin promoter (Gt-1 P) in combination with lycopene β-cyclase (*lcy*) and phytoene desaturase (*crtI*) fused to the transit peptide sequence of the pea-Rubisco small subunit and, driven by the CaMV35S promoter, caused the seeds to accumulate high levels of carotenoids and become yellow (Datta et al. 2003). Other tissue-specific promoters that have been used in plant biotechnology are the tapetum-specific promoters for generation of male sterility in canola, brassica, tobacco, Arabidopsis, rice, and others (Mariani et al. 1990; Tsuchiya et al. 1995; Mitsuda et al. 2006; Zhan et al. 1996), the green tissue-specific Rubisco small subunit promoter

(Rubisco SSU) (Bakhsh et al. 2011; Abdel-Ghany et al. 2013), and the floral whorl-specific AP1 promoter (Byzova et al. 2004).

28.4 Suppression of Gene Expression

In addition to overexpression of transgenes that confer desirable traits, suppression of the levels of endogenous gene(s) expression is also of interest for plant biotechnology. Studies in gene silencing-related mechanisms started in early the 1990s based on a surprising observation in which the introduction of a transgene into the genome led to the silencing of both the transgene and homologous native gene (Napoli et al. 1990; van der Krol et al. 1990a), a phenomena known as posttranscriptional gene silencing (PTGS). Different methods have been applied for gene silencing in plants.

28.4.1 Antisense RNA

The antisense RNA strategy is a powerful tool for the selective suppression of gene expression in plants (reviewed in Bird and Ray 1991). It has been used to study genes involved in a wide range of physiological and biochemical processes in a variety of tissue types such as flower pigmentation (van der Krol et al. 1990b), photosynthesis (Stockhaus et al. 1990), fruit-ripening, and viral resistance (Hemenway et al. 1988; Powell et al. 1989). The principle of antisense RNA is based on the introduction of a gene corresponding to a desired gene in a reverse orientation so that an antisense RNA molecule that is complementary to whole or part of the sequence of the mRNA of the target gene is produced. Inside the nucleus, a duplex is formed between the sense and antisense transcript, which interferes with several aspects of gene expression at the transcriptional, post-transcriptional, and translational levels.

Antisense RNA has several limitations. Its effectiveness varies between cells and organs depending on the promoter employed even in the case of the 35S CaMV constitutive promoter because its expression was found to vary in different cell types (Wilkinson et al. 1997; Tang et al. 1999). A further limitation is that gene silencing methods will potentially target all homologous genes, either members of a family or closely related genes (Thorneycroft et al. 2001). Also, it is not possible to eliminate an enzyme completely by antisense RNA, and in many cases the transcript is reduced to undetectable levels without apparent phenotype, making it difficult to derive a firm conclusion about its function as it is usually argued that a small amount of residual enzyme is sufficient to fulfill the required role *in vivo* (Takaha et al. 1998).

28.4.2 Insertional Mutagenesis

Gene knockout is one of the important tools in plant functional genomics. It is based on the insertion of a foreign DNA (either T-DNA or transposon) into the gene of interest. Insertion of foreign DNA into a structural gene (whether into an exon or intron) in most cases will disrupt gene expression completely and result in a null mutation. In some cases, insertion of foreign DNA in the promoter or in the 3′-UTR reduces the expression (knockdown) rather than producing null mutation. The foreign DNA not only disrupts the expression of the gene into which it is inserted but also acts as a marker for subsequent identification of the mutation. One advantage of such insertional mutagenesis is that it allows the recovery of mutations in essential genes, as the plants can be maintained in the heterozygous state. Another advantage of this method is that only a single gene within a family of closely related genes is affected, allowing functional analysis of individual members in a gene family. Using *Agrobacterium*-mediated transformation, a large number of T-DNA-transformed lines have been generated, and, therefore, one has a very high chance of finding a plant carrying a T-DNA insert within any gene of interest. Many T-DNA lines have been developed in the two most important model plant species, Arabidopsis and rice (Krysan et al. 1999; Jeon et al. 2000; Alonso et al. 2003; Sallaud et al. 2004). These T-DNA

stocks have served as a foundation for functional characterization of thousands of genes in these two species. Unfortunately, in many plant species, the *Agrobacterium*-mediated transformation is not developed yet or is not efficient enough to produce a sufficient population of insertion lines that cover the whole genome (Duangpan et al. 2013). In these species, the transposable elements (TEs) are the choice for insertional mutagenesis. Several TEs have been used in plants including the *Activator* and *Mutator* transposons in maize (Walbot 1992), the *Tam3* transposon in *Antirrhinum majus* (Luo et al. 1991), the *Tos17* retrotransposon in rice (Hirochika 2010), and *Tnt1* retrotransposon in tobacco, potato, soybean, and other species (Grandbastien et al. 1989; Cui et al. 2013; Duangpan et al. 2013).

28.4.3 Artificial microRNAs (amiRNAs)

Since insertional mutagenesis approaches are available for a limited number of model plants, targeted gene silencing using amiRNA technology is a valuable tool for gene manipulation and functional analysis in plants (Li et al. 2013). amiRNA technology exploits the endogenous miRNA biogenesis and silencing machineries to generate miRNAs that direct silencing of a desired gene. In plants, the miRNA precursor transcript is 80–250 nucleotides in length with some structural features such as mismatches, bulges, and one or several imperfect foldbacks and when processed produces an miRNA/miRNA* duplex. When both duplex sequences are altered without changing the structural features, a high-level accumulation of miRNAs of a desired gene can be produced in different plant species (Parizotto et al. 2004; Alvarez et al. 2006; Schwab et al. 2006; Ossowski et al. 2008; Sablok et al. 2011). Therefore, artificial miRNA is built on a native miRNA precursor by replacing the original miRNA/miRNA* duplex with a customized sequence to silence a gene of interest, thereby generating a loss-of-function phenotype for the targeted gene (Parizotto et al. 2004; Alvarez et al. 2006; Schwab et al. 2006; Ossowski et al. 2008). A Web-based miRNA designer (WMD) (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd), which works for >100 plant species, allows the design of gene-specific amiRNA candidates within a given transcriptome (Schwab et al. 2006; Ossowski et al. 2008). WMD computationally generates hundreds of amiRNAs for each target that are ranked based on the complementarity and hybridization energy without knowing the *in vivo* efficiency. The efficacy of amiRNAs has been tested in Arabidopsis (Schwab et al. 2006), rice (Warthmann et al. 2008; Schmidt et al. 2012), *Physcomitrella patens* (Khraiwesh et al. 2008), *Chlamydomonas reinhardtii* (Molnar et al. 2009), and other species. The advantages of amiRNA compared to other RNA silencing approaches such as RNA interference (RNAi) and virus-induced gene silencing (VIGS) are that it has minimal off-target effects and a unique capacity for multigene silencing (Alvarez et al. 2006; Niu et al. 2006; Schwab et al. 2006; Ossowski et al. 2008). Since miRNA silencing depends on many unpredictable *in vivo* factors such as target mRNA structure and mRNA binding proteins (Fabian et al. 2010; Pasquinelli 2012) and WMD prediction does not consider these factors, many amiRNAs should be tested to find an optimal amiRNA. Recently, an epitope-tagged protein-based amiRNA (ETPamir) screen was developed for efficient amiRNA gene silencing in plants, in which target mRNA encoding epitope-tagged proteins were coexpressed in protoplasts with amiRNA candidate targeting single or multiple genes (Li et al. 2013). These screens were applied to diverse plant species including Arabidopsis, tobacco, tomato, sunflower, maize, and rice with the potential for improving crop engineering with highly predictable and efficient amiRNAs (Li et al. 2013).

28.5 Metabolic Engineering Using Multiple Transgenes

In plants, metabolic pathways can be enhanced by overexpressing the rate-limiting enzymes or introducing enzymes that are insensitive to

negative feedback regulation. However, introduction of a novel pathway or metabolic engineering of a plant for production of platform chemicals often requires expression of multiple transgenes. In prokaryotes, multigene engineering is possible through coexpression of an operon as a polycistronic transcript (Steen et al. 2010). But in plant cells, except in chloroplasts, the polycistronic mRNA is not efficient in translation because of the scanning mode of the eukaryotic ribosome for recognizing the 5′ cap and the initiation codon in the transcript and the need for a proper sequence context in the 5′ UTR (Kozak 2005). Therefore, different strategies have been developed to introduce multiple transgenes into plants.

The common strategy for introducing more than one gene is stacking of expression cassettes in one vector. Each cassette consists of its own promoter, gene of interest, and a terminator. To avoid gene silencing through co-suppression, different promoters and terminators should be used and the cassettes are arranged in tandem and in the same transcriptional orientation (Hamilton and Baulcombe 1999; Kooter et al. 1999). For example, introduction of β-carotene pathway in rice was achieved by co-transformation with two plasmids, each with a two-gene cassette (Ye et al. 2000). One plasmid contained the phytoene synthase gene *PSY* under the control of the endosperm-specific glutelin promoter and the phytoene desaturase gene *crt1* under the control of the CaMV 35S promoter. The second vector contained the lycopene β-cyclase gene and the selectable marker gene. The three genes for carotenoid biosynthesis additionally contained an N-terminal transit peptide sequence to direct the proteins to the chloroplast, the sites for carotenoids biosynthesis. Another example is the introduction of the butanetriol metabolic pathway in Arabidopsis (Abdel-Ghany et al. 2013). Production of butanetriol from its precursor, xylose or arabinose, requires four enzymes. The genes for the first three enzymes in the pathway were cloned in binary vectors with each gene driven by a different promoter and terminator (Fig. 28.1). Each gene was cloned into a promoter vector, and then the gene with the promoter/terminator was moved sequentially into a binary vector that was used for *Agrobacterium-mediated* transformation (Abdel-Ghany et al. 2013).

Vectors containing different cassettes can also be introduced into plants by co-transformation, serial transformation, or crossing. Co-transformation with direct gene transfer is a straightforward method but more challenging if *Agrobacterium*-mediated transformation is to be used due to the requirement to maintain two plasmids in one *Agrobacterium* cell or the need to infect a single cell with two *Agrobacterium* strains (Ebinuma et al. 2001). In serial transformation, referred to as super transformation, a transgenic plant will be transformed successively with additional transgenes (Bock 2013). This method is more time consuming and requires a new selectable marker gene for each super transformation step unless the selectable marker gene is recycled using marker excision techniques (Hare and Chua 2002). Crossing is also a time-consuming process especially if the plant generation time is long, and this is not an option in crop plants that propagate vegetatively such as potato and sugarcane.

Another strategy for multigene transformation is combinatorial transformation (Zhu et al. 2008). This method takes advantage of the particle bombardment method, which induces a few double-stranded DNA breaks into which multiple copies of the transforming plasmid's DNA can co-integrate before DNA repair (Altpeter et al. 2005). Individual transgenes required for a metabolic pathway as well as the selectable marker are cloned in expression cassettes, mixed, and loaded onto gold or tungsten particles. Particle bombardment with these microprojectiles sometimes introduces copies of all constructs present in the mix. How many of them are introduced into the genome depends on the efficiency of the integration process as well as on the stability of the DNA released from the particles in the host cells. Following the selection, transgenes co-transformed with the selection marker can be analyzed, and transgenic lines can be generated. The most attractive feature of combinatorial transformation is that there is no theoretical limit to the number of transgene cassettes that can be co-transformed. Combinatorial transformation

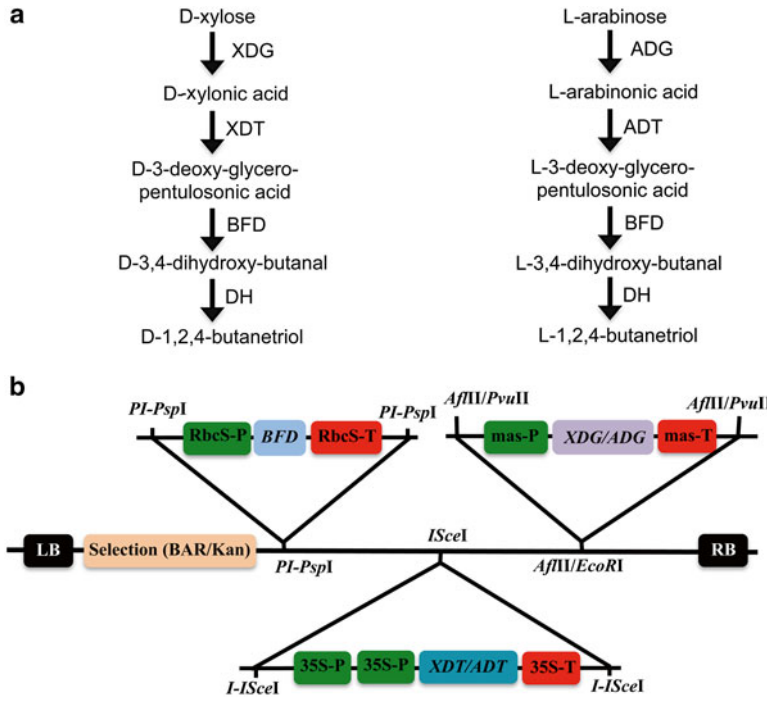

Fig. 28.1 Schematic diagrams of 1,2,4-butanetriol pathways and design of gene constructs for plant transformation. (**a**) Schematic representation of the biochemical pathways showing the synthesis of D-1,2,4-butanetriol and L-1,2,4-butanetriol from xylose and arabinose. XDG, D-xylose dehydrogenase; ADG, L-arabinose dehydrogenase; XDT, D-xylonate dehydratase; ADT, L-arabinonate dehydratase; BFD, benzoylformate decarboxylase; DH, dehydrogenase. (**b**) Binary vector construct containing the three genes encoding the first three enzymes used in the butanetriol synthesis pathways from xylose and arabinose. Each vector had a set of genes amplified from bacteria or codon-optimized synthetic genes for the xylose pathway or the set of codon-optimized synthetic genes for the arabinose pathway. *RbcS-P and RbcS-T* Rubisco small subunit promoter and terminator, respectively, *mas-P and mas-T* mannopine synthase promoter and terminator, respectively; and *35S-P and 35S-T* cauliflower mosaic virus promoter and terminator, respectively (From Abdel-Ghany et al. 2013)

has been used to improve the nutritional value of maize by introducing the biosynthetic pathways for three vitamins (vitamin A, C, and B9) (Naqvi et al. 2009).

28.6 Targeted Genome Engineering

Although the co-transformation, re-transformation, and sexual crossing methods have been proven to be useful for metabolic engineering of plants, these methods suffer from several flaws (reviewed in Dafny-Yelin and Tzfira 2007; Naqvi et al. 2010). For instance, the integration of genes in the genome is completely random, which can have marked effects on the host, such as inactivation of an endogenous gene, different levels of transgene expression among lines, and even silencing of the transgene when inserted into a heterochromatic region. To overcome these issues, a technology that allows stacking of a number of genes in a single multigene array, introducing it at a precisely identified chromosomal locus, and then manipulating the array by genome editing is needed. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) offer potential for efficient targeted plant genome engineering (Bogdanove and Voytas 2011; Zeevi et al. 2012; Zhang et al. 2013). ZFNs and TALENs are sequence-specific nucleases that create *in vivo* double-stranded breaks at target loci followed by nonhomologous end joining (NHEJ) and homologous recombination, resulting

in targeted gene insertion or gene replacement (Wright et al. 2005; Zhang et al. 2013). Using these systems, binary vectors that can carry up to nine genes and integrate them in the genome were developed (Zeevi et al. 2012). In plants, the use of these sequence-specific nucleases for targeted genome modifications has many applications, ranging from dissecting gene function (Curtin et al. 2012) to creating plants with new traits such as disease resistance, altered metabolite profile, and tolerance to herbicides (Shukla et al. 2009; Townsend et al. 2009).

28.7 Applications of Metabolic Engineering

28.7.1 Improving the Nutritional Value of Crops

Multigene transformation for metabolic engineering helped to generate plants with high nutritional values. For example, to develop golden rice, the entire β-carotene (pro-vitamin A) biosynthetic pathway was introduced into rice endosperm (Ye et al. 2000). The transgenic rice produced seeds with yellow endosperm due to the accumulation of β-carotene (Ye et al. 2000). Substantial improvements of β-carotene were achieved by testing enzymes from different sources, some of which turned out to have superior catalytic activities (Paine et al. 2005). A similar technology has been used to improve the content of other vitamins that are limiting in diet and important in maintaining optimal health (DellaPenna 2007).

Iron biofortification is another application of metabolic engineering. Iron is the most commonly deficient micronutrient in the human diet and about 25 % of the world population suffers from iron deficiency. To increase the iron content in seeds, the iron storage protein ferritin and *Aspergillus* phytase were expressed in the endosperm of rice and maize, respectively, which resulted in significant increase in the levels of bioavailable iron (Goto et al. 2000; Lucca et al. 2002; Drakakaki et al. 2005; Aung et al. 2013). Similar technology was applied for lettuce (Goto et al. 2000). Other nutritionally improved traits that are introduced into agricultural crops and intended to provide health benefits for human and animals include protein quality and level, essential amino acids, oil and fatty acids, fibers, vitamins, and minerals (reviewed in Newell-McGloughlin 2008).

28.7.2 Enhancement of Biomass and Photosynthetic Efficiency

Metabolic engineering has also been employed to improve the photosynthetic efficiency of crop plants (Kebeish et al. 2007). Phosphoglycolate produced from the oxygenation reaction of Rubisco is recycled into phosphoglycerate during photorespiration, which is then used in the Calvin cycle. This recycling process results in net loss of fixed carbon, thereby reducing the yield of biomass and sugars (Kebeish et al. 2007). Introduction of a glycolate catabolic pathway in the chloroplast enabled the release of CO_2 in close proximity of the Rubisco enzyme, which in turn suppresses the unwanted oxygenation reaction and increases the CO_2 fixation capacity (Kebeish et al. 2007). The five transgenes required for the glycolate metabolic pathway were introduced into Arabidopsis chloroplasts by the re-transformation strategy (Kebeish et al. 2007). Transgenic lines showed reduced photorespiration rate and increased biomass production (Kebeish et al. 2007), a potential for improving the efficiency of photosynthesis in crop plants. Also, in a green house simulation experiment using antisense technology, it was possible to produce transgenic plants with reduced Rubisco activity (Makino et al. 1997). These plants showed 15 % higher photosynthetic rates and efficient nitrogen use in an elevated partial pressure of carbon dioxide and high irradiation, although 65 % reduction was observed under normal condition (Makino et al. 1997). Also, overexpression of other enzymes such as maize phosphoenolpyruvate carboxylase in rice and cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhanced photosynthesis and growth (Ku et al. 1999; Miyagawa et al. 2001).

28.7.3 Resistance to Biotic and Abiotic Stresses

Several biotic and abiotic stresses such as pathogens, insects, salinity, drought, high temperature, and freezing severely limit plant growth and development. Plant biotechnology has allowed the development of transgenics that are more resistant to stresses. In response to osmotic stress, plants accumulate compatible solutes or osmolytes such as glycine betaine, proline, and sugar alcohols, suggesting that overproduction of these solutes in transgenic plants may result in improved stress tolerance. Overexpression of bacterial choline monooxygenase (CMO), the first enzyme in the betaine biosynthesis pathway, in plants resulted in increased resistance to various stresses such as salt and extreme temperature (Sakamoto and Murata 2001). *P5CS* ($\Delta^{1\text{-pyrroline}}$-5-carboxylate) is the rate-limiting enzyme in the proline biosynthetic pathway, and transgenic tobacco plants overexpressing the *P5CS* gene produced 10- to 18-fold more proline and exhibited better performance under salt and freezing stresses (Kishor et al. 1995; Konstantinova et al. 2002). Another way to increase the proline content is to downregulate its catabolism. Arabidopsis plants expressing the antisense mRNA for proline dehydrogenase (*AtProDH*), the first enzyme in the proline degradation pathway, constitutively accumulated proline and showed tolerance to freezing (−7 °C) and to NaCl (600 mM) (Nanjo et al. 1999). Rice plants engineered for trehalose overexpression showed higher capacity for photosynthesis, decrease in photooxidative damage, and tolerance to multiple abiotic stresses (Garget al. 2002).

The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) are conserved transcription factors that are induced in response to stresses and induce the expression of stress-regulated genes. Increased expression of Arabidopsis *CBF1*, *CBF2*, and *CBF3* induced the expression of many cold-regulated genes and increased the freezing tolerance of non-acclimated plants (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). ABA signaling plays a vital role in drought response as many of the drought-inducible genes are induced by ABA (Wang et al. 2003). Constitutive overexpression of ABA-responsive element-binding proteins ABF3 and ABF4 in Arabidopsis demonstrated enhanced drought tolerance with altered expression of ABA- and stress-responsive genes (Kang et al. 2002). Similarly, overexpression of heat shock transcription factors *HSF1* and *HSF3* led to the expression of several *hsp* genes, conferring thermotolerance in transgenic plants (Lee et al. 1995), while the tomato plants expressing *HsfA1* antisense mRNA were extremely sensitive to elevated temperature (Mishra et al. 2002). Engineered plants overexpressing antioxidants such as catalases, ascorbate oxidases, and superoxide dismutases showed increased resistance to oxidative stress caused by high light, low temperatures, and UV radiation (Gupta et al. 1993; McKersie et al. 2000).

Application of plant biotechnology led to improved resistance to insects, diseases, and herbicides (reviewed in Muthurajan and Balasubramanian 2010). For example, transgenic plants that express both *Bt* and *cry* endotoxins were found to exhibit resistance against insects that feed by chewing or biting. Also, simultaneous introduction of three genes expressing insecticidal proteins *Cry1Ac*, *Cry2A*, and *gna* into indica rice controlled three of the major rice pests (Maqbool et al. 2001). Coexpression of two PR-protein genes (chitinase and glucanase) was shown to confer resistance to several fungal diseases (Muthurajan and Balasubramanian 2010).

28.7.4 Production of Platform Chemicals

According to the US Department of Energy, 12 chemicals and chemical classes could be produced from plants, and these chemicals can function as building blocks or platform chemicals from which many value-added compounds such as biopolymers, biofuels, energetic materials, and pharmaceuticals can be produced (United States Department of Agriculture 2008).

28.7.4.1 Energetic Materials

1,2,4-Butanetriol (butanetriol) can be nitrated to produce butanetrioltrinitrate (BTTN), an energetic material and a useful building block for the synthesis of various drugs and important chemicals (Niu et al. 2003; Sato et al. 2003; Yamada-Onodera et al. 2007; United States Department of Agriculture 2008). BTTN is used as a propellant and as an energetic plasticizer in mining and in a variety of Department of Defense and Department of Energy applications. In addition, it is anticipated that BTTN could replace nitroglycerin as a vasodilator for the treatment of angina. Advantages of BTTN over nitroglycerin are that it is less hazardous, less shock sensitive, less volatile, more thermally stable, and resistant to degradation by nitrate reductase, minimizing the number of metabolites generated from degradation.

Chemical synthesis of D, L-butanetriol needs petroleum-derived substrates and requires expensive metal catalysts that adversely impact the environment (Niu et al. 2003). On the other hand, bacterial synthesis of butanetriol from precursors D-xylose or L-arabinose requires continuous supply of these sugars (Niu et al. 2003), a process that limits the bacterial production. Four enzymes are necessary for synthesis of butanetriol from either xylose or arabinose: The first two enzymes D-xylose dehydrogenase (XDG) and D-xylonate dehydratase (XDT) for xylose pathway or L-arabinose dehydrogenase (ADG) and L-arabinonate dehydratase (ADT) for arabinose pathway are substrate specific, whereas the last two enzymes benzoylformate decarboxylase (BFD) and dehydrogenase (DH) are common for both pathways (Fig. 28.1).

Plants synthesize the pentose sugars (xylose and arabinose), which can be converted into butanetriol by expressing microbial enzymes allowing plant-based production of this platform chemical. To produce butanetriol in plants, the native bacterial genes or the codon-optimized ones for both xylose and arabinose pathways were cloned under different promoters and introduced into Arabidopsis plants (Abdel-Ghany et al. 2013). Stable transgenic lines accumulated butanetriol (Fig. 28.2) and showed an altered metabolic profile. Manipulation of the growth medium significantly increased the butanetriol production.

Another energetic bio-based intermediate with potential for production of propellants/explosives is phloroglucinol. In addition to being the starting material for biosynthesis of 1,3,5-triamino-2,4,6-trinitrobenzene (TATB), a stable energetic material used by the US military, phloroglucinol has medical, agricultural, and industrial applications. Chemical synthesis of phloroglucinol involves oxidation of 2,4,6-trinitrotoluene (TNT), a process that presents an explosion hazard and generates carcinogenic chromates as well as other waste streams. On the other hand, bacterial synthesis of phloroglucinol requires a continuous supply of glucose to generate the precursor malonyl-CoA. Plants have malonyl-CoA as the precursor for fatty acid, flavonoids, and phytoalexins biosynthesis (Baud et al. 2003) and, therefore, can be used for sustainable production of phloroglucinol. We generated transgenic plants expressing the bacterial *PhlD* gene targeted to either chloroplast or to cytosol for phloroglucinol production (Abdel-Ghany, Day, Heuberger, Broeckling, and Reddy, MS in preparation).

28.7.4.2 Polyhydroxyalkanoates (PHAs), Bioplastics

For a long time, there has been an interest in the development and production of biodegradable plastics to address problems associated with plastic waste and its harmful effect on the environment (Poirier et al. 1995). In 1992 Somerville's group demonstrated the production of polyhydroxybutyrate (PHB), a 100 % biodegradable polymer, in Arabidopsis by expressing two bacterial proteins required to convert acetoacetyl-CoA to PHB (Poirier et al. 1992). PHB is the most common and best-characterized member in a group of polyhydroxyalkanoates (PHAs), which are biopolymers made entirely from bacterial fermentation. In bacteria, three enzymes are necessary for PHB synthesis: first, a β-ketothiolase that catalyzes the reversible condensation of two acetyl-CoA molecules to form acetoacetyl-CoA; second, an acetoacetyl-CoA reductase

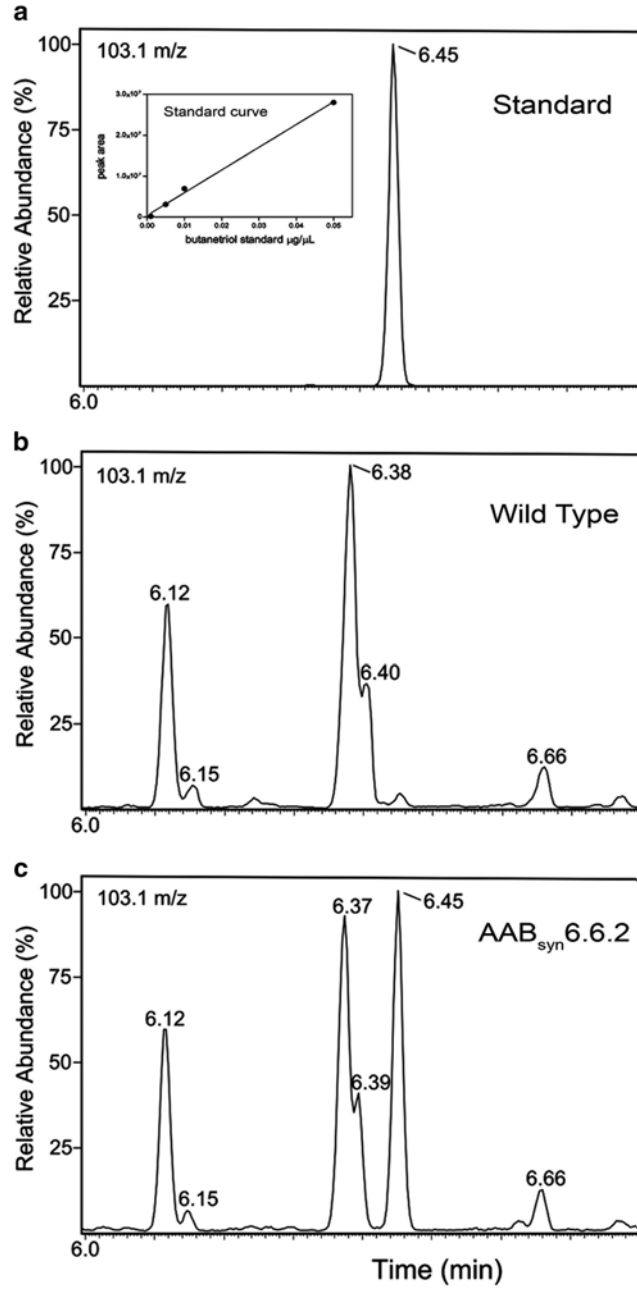

Fig. 28.2 Detection of 1,2,4-butanetriol in a transgenic line. Metabolites from vacuum-dried tissues of wild-type and transgenic lines were extracted and detected by GC-MS. (**a**) Extracted ion chromatogram (EIC, 103.1 m/z) of 1,2,4-butanetriol standard solution and calibration curve. Butanetriol eluted at 6.45 min. (**b**) EIC of wild type. (**c**) EIC chromatogram of AAB_{syn} 6.6.2 transgenic line showed large peak at 6.45 min with a mass spectrum that matches the commercial butanetriol standard (From Abdel-Ghany et al. 2013)

which reduces acetoacetyl-CoA to R-(−)-3-hydroxybutyryl-CoA; and third, a PHA synthase which subsequently polymerizes R-(−)-3-hydroxybutyryl-CoA to form PHB. By changing the carbon source and the bacterial strain used in the fermentation process, it was possible to produce biopolymers having physical properties ranging from stiff and brittle plastic to rubbery polymers (Anderson and Dawes 1990).

Despite its basic attractiveness as a substitute for petroleum-derived polymers, the major drawback of bacterial PHA synthesis is the high cost of bacterial fermentation, making bacterial PHAs five to ten times more expensive than the

synthetic polymer (Poirier et al. 1995). In this respect, crop plants were seen as an attractive system for the sustained production of large amounts of polymers at low cost as plants have the precursor acetyl-CoA and the enzyme β-ketothiolase in the cytoplasm. Through creation of novel metabolic pathways targeted to the cytoplasm, plastid, or peroxisome, varieties of PHAs with different physical properties have been produced in a number of transgenic plants. To date, the highest levels of PHB have been achieved in plastids, likely due to the high flux of the PHB pathway substrate acetyl-CoA through this organelle during fatty acid metabolism (Suriyamongkol et al. 2007; Van Beilen and Poirier 2008; Bohmert-Tatarev et al. 2011). In Arabidopsis plants engineered with plastid-targeted enzymes, PHB has been produced as granules at levels up to 40 % dry weight but with significant growth penalties such that plants were dwarf and produced no seeds (Bohmert et al. 2000). These deleterious effects might be caused by the depletion of acetyl-CoA and acetoacetyl-CoA from the endogenous flavonoid and isoprenoid pathways (Van Beilen and Poirier 2008). To circumvent the growth penalties associated with constitutive expression of the PHA biosynthetic pathway, the introduced genes were fused to an inducible promoter that can be activated at later stages of growth when the plant accumulates sufficient biomass for PHB production (Kourtz et al. 2007). PHAs synthesis in biomass crops of industrial interest such as switchgrass, sugarcane, maize, tobacco, cotton, flax, and others was also reported (reviewed in Bornke and Broer 2010), although more investigation is needed to channel the precursors toward PHA synthesis without deleterious effects on plant growth. In addition to being bioplastics, PHAs can also be converted through simple processes to a range of platform chemical intermediates such as acrylic acid, crotonic acid, propylene, and butanol (Zhong and Whitehouse 2005; Peterson and Fischer 2010), which have valuable industrial applications.

28.7.4.3 Cyanophycin, a Poly-Amino Acid Polymer

Cyanophycin is another important water-soluble and biodegradable polymer that contains only two amino acids, L-arginine and L-aspartate, in a ratio of 1:1 (Simon 1987; Elbahloul et al. 2005). It is synthesized via non-ribosomal polypeptide synthesis in many cyanobacteria in a polymerization reaction catalyzed by an enzyme cyanophycin synthetase (*cphA*). Cyanophycin is a major source of several platform chemicals that can be further processed into many other chemicals of industrial, agricultural, and medical applications (Joentgen et al. 2001) complementing the application ranges of PHB. Mild hydrolysis of cyanophycin results in homopolymers and copolymers of polyaspartate and L-arginine, which serve as the starting point for the syntheses of a range of compounds such as 1,4-butanediamine that can be used for the synthesis of nylon-4,6; 3-aminotetrahydrofuran that is used heavily in the polymer industry; fumaric acid that is used for polyester resin; and acrylamide which is used in papermaking and manufacturing dyes (Bornke and Broer 2010). Polyaspartate polymer could also replace the nondegradable synthetic polyacrylates in various processes such as water treatment and plastics (reviewed in Bornke and Broer 2010). For sustainable production of this important polymer in plants, the cyanophycin synthetase gene from cyanobacteria was introduced into tobacco and potato under the control of the 35S CaMV promoter with modest accumulation (0.24–1.14 % per unit DW) and deleterious effects on plants (Neumann et al. 2005). Targeting the cyanophycin synthesis to the plastids or to tubers significantly enhanced its accumulation up to 7.5 % DW without noticeable effect on plant growth (Huhns et al. 2008, 2009). Similar biotechnological approaches have been applied for bio-production of other important poly-amino acids such as poly-γ-glutamate and poly-lysine using bacterial operons (Oppermann-Sanio et al. 1999; Oppermann-Sanio and Steinbuchel 2002).

28.7.5 Production of Pharmaceuticals in Plants

The growing need for safer and cheaper drugs and the availability of tools to express foreign proteins of interest in plants led to the idea of using plants to produce vaccines and other pharmaceuticals. A schematic representation of the concept is shown in Fig. 28.3. Various pharmaceutical proteins can be produced *in planta* in their original or genetically modified form. The molecular design of the correspondent expression cassettes is largely dependent on the choices of expression system (Schiermeyer et al. 2004). The key considerations are the size, solubility, folding parameters, as well as possible glycosylation and other posttranslational modifications. Recombinant products can also be designed as protein fusions or chimeras with affinity tags that can aid in detection and purification (Gomord et al. 2004; Benchabane et al. 2008). Many proteins (>150) of interest to the pharma industry have already been tested for production *in planta*. The advantages of using plants for pharmaceutical production are that it is easy to scale up and is expected to incur low production costs. Furthermore, plants are considered as a safe and pathogen-free biomass source.

Plants have been tested for vaccine production, often referred to as "edible," "oral," or "green vaccines" (Mor et al. 1998; Streatfield and Howard 2003). The main idea is to express immunogenic antigens in edible parts of plants and feed animals or humans to immunize them. These antigens are capable of inducing strong and protective immune responses sufficient to neutralize the pathogens (Bae et al. 2009), especially the ones against the reemerging infectious diseases, cancer, autoimmune diseases, and others (Ulmer et al. 2006). The pioneering work of Dr. Charles Arntzen and his colleague Dr. Mason in this area has led to many studies and establishment of a novel branch of plant biotechnology (Goldstein and Thomas 2004; Schiermeyer et al. 2004; Ma et al. 2005a). Successful oral feeding experiments with some strong antigenic proteins such as *E. coli* enterotoxin B (LTB), *Cholera* toxin B (CT-B), and Hepatitis B surface antigen (HBsAg) (Tiwari et al. 2009) that do not require immune stimulators (adjuvants) (Reed et al. 2009) confirm the potential for using plant-based vaccines. Plant vaccines together with plant-derived functional

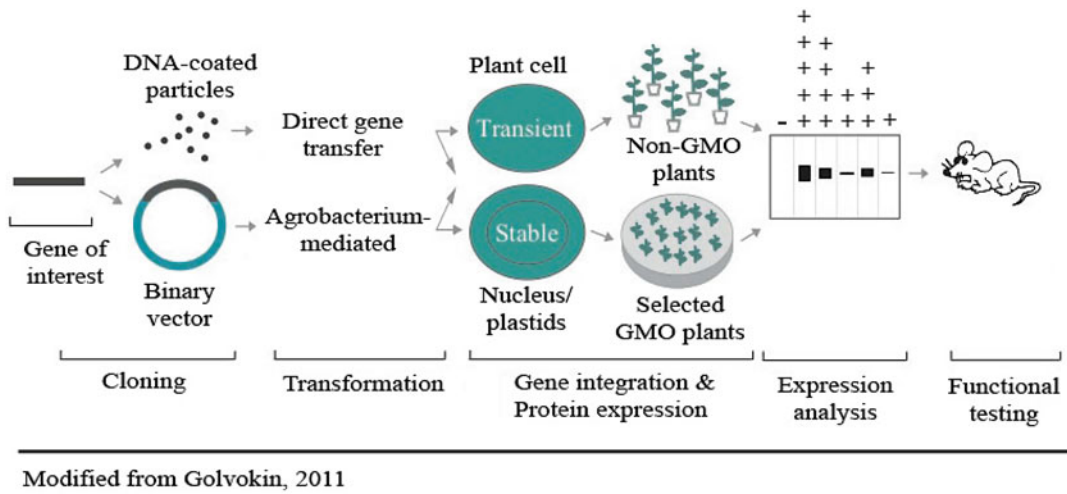

Fig. 28.3 Schematic representation of the key stages for production of pharmaceuticals in plants. The gene(s) of interest is cloned in a binary vector, which is then transferred into plant cells by either *Agrobacterium*-mediated transformation or direct gene transfer. In the case of stable transformation, the transgenic lines are selected and used for production of the recombinant protein. Production of recombinant protein is usually followed with functional testing in lab animals (Modified from Golovkin 2011)

antibodies (Hood et al. 2002; Stoger et al. 2002; Fischer et al. 2003) have reached the initial stages of testing in animals and humans (Table 28.3). The first officially approved trial was conducted in 1997 with LTB in potato tubers (Tacket et al. 1998). Some products can be used as a simple processed material directly in needle-free immunization (Ma et al. 2005b; Streatfield 2006; Floss et al. 2007; Tiwari et al. 2009). These groundbreaking studies and many other studies that followed suggest the potential for engineered plants in providing safe and, probably, cheap therapeutic products. The technology is now transitioning from academic labs to industrial production of valuable recombinant proteins like subunit vaccines, antibodies, and other therapeutic proteins.

The "edible" pharmaceutical concept has some issues that need to be addressed. For instance, the dosage control is difficult because of variable quantities of recombinant protein in plant tissues (fruits, vegetables, grains, etc.). Another concern is a potential environmental risk (Mascia and Flavell 2004; Peterson and Arntzen 2004). The release and use of edible crops, like vegetables, fruits, and grains, may lead to possible genetic cross-contamination of other agriculturally important crops (Sparrow and Twyman 2009). Special regulatory measures called "*c*urrent *g*ood *m*anufacturing *p*ractice" (cGMP) are currently in place (FDA 2002) to guide the entire research/production process. These regulatory measures are applicable to the plant-based pharmaceutical products also. A recent approval of the first US plant-based (tobacco) recombinant vaccine against Newcastle disease in chickens in 2006 (Vermij and Waltz 2006) indicates that plant vaccines are safe and efficacious at least for veterinary use. Simple downstream processing of biomass (dry or dry/freezing) could also help to normalize the active recombinant components, e.g., for low-concentration microbicides. The final products could be extracts, powders, capsules, tablets, and ointments (Daniell et al. 2005; Scott 2005; Yusibov and Rabindran 2008; Rybicki 2009; Tiwari et al. 2009). Scientists remain cautiously optimistic that the latest developments in this area will garner the attention of the pharma industry and bring needed support to establish plant biopharming.

28.8 Conclusions

As discussed above, significant progress has been made in recent years in engineering plants to produce commercially important products. This field has moved from a single-gene manipulation to introduction of whole new pathways from

Table 28.3 Plant-derived pharmaceuticals in clinical trials

Product	Targeted disease	Plant source
Vaccines		
E. coli enterotoxin B (LTB)	Diarrhea	Potato and maize
Hepatitis B surface antigen (HBsAg)	Hepatitis B	Potato and lettuce
Norwalk virus antigen	Sickness/diarrhea	Potato
Rabies glycoprotein G	Rabies	Spinach
Antibodies		
LSBC scFVs	Non-Hodgkin's lymphoma	Tobacco
Avicidin	Colorectal cancer	Maize
CaroRx	Dental caries	Tobacco
Other products		
Gastric lipase	Cystic fibrosis, pancreatitis	Maize
Human intrinsic factor	Vitamin B12 deficiency	Arabidopsis
Lactoferrin	Gastrointestinal infections	Maize
Human glucocerebrosidase	Gaucher disease	Carrot
Enzyme		

LTB Heat-labile enterotoxin B subunit, *LSBC* Lifesaver for Babies and Children, *scFVs* single-chain Fv fragments

heterologous systems. The current toolbox allows stacking of a large number of genes at the same chromosomal locus as a multigene array and its manipulation by genome editing. The choice of the tool used depends on several factors such as plant species, number of genes to be engineered into the plant, the desired expression level, cellular compartmentalization of the pathway of interest, and the available information about the metabolic pools, fluxes, and regulation of the metabolic pathway. Reduction in gene synthesis costs and progress in assembling larger pieces of synthetic DNA in recent years have made engineering complex networks possible.

Although much progress has been made in engineering plants to produce several commercially important products, there are still significant challenges in bringing these plant-based products to market. The main strategy that has been used for engineering plants is based on introduction of one or more genes under constitutive, regulatable, or tissue-specific promoters. This approach works well in producing mRNA from the introduced gene, but it does not always result in the required amount of protein due to many post-transcriptional and posttranslational regulatory mechanisms (e.g., mRNA stability, translatability of mRNAs, stability of proteins) that cells employ to modulate the levels of proteins and their function. Because of this, often-desired compounds are produced at very low levels. Incorporating elements that impact expression of a gene after mRNA biogenesis into gene construction design may lead to increased production of desired proteins. Also, production of native or novel chemicals and compounds in plants at high levels often is detrimental to plant growth and development. This is mainly due to lack of our understanding of the interconnections between metabolic pathways and insufficient knowledge about the multilevel regulation of metabolic pathways. These limitations must be addressed in order to make the outcome of metabolic pathway engineering more predictable and at the same time avoid unwelcome surprises from adverse phenotypes of transgenic plants. Comprehensive global studies on transgenic lines using "omics" approaches together with the knowledge obtained from systems biology should help in addressing some of the current issues associated with plant metabolic engineering. Furthermore, application of emerging tools in synthetic biology to manipulate existing gene circuits and create novel circuits to plant metabolic engineering is also expected to mitigate some of these issues.

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