Metabolic Engineering in Plants

26

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Abstract

Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering. Metabolic engineering in plants involves the modification of endogenous pathways to redirection of one or more enzymatic reactions to produce new compounds or improve/retard production of known compounds. Metabolic engineering is the redirection of one of more enzymatic reaction to produce new compounds in an organism, improve production of existing compounds or mediate the degradation of compounds. It reviews the current status of metabolic engineering.

Keywords

Metabolic engineering • Terpenoids • Alkaloids • Cellulose • Gene • Transcription regulation

26.1 Introduction

Significant advances have been made in metabolic engineering through the application of genomics and proteomics technologies to elucidate and characterise metabolic pathways and their regulation at all levels like genes, enzymes, compartmentation, transport and accumulation. Recently, the focus has been on metabolic pathways in the context of the entire cell rather than at the single pathway level. The various interactions between proteins, DNA and metabolites, called the interactome, are the key determinant of any cellular process. Proteins are the functional entities that carry out the actual metabolic process by interconverting metabolites (metabolome). It is necessary for the metabolic engineer to understand these relationships in order to accurately design and control biological systems (Vemuri and Aristidou 2005).

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26.2 Aims of Metabolic Engineering

The goal of metabolite engineering is to increase the production of certain compounds in the normal producing plant species or to transfer a pathway to other plant species or production of novel compounds not yet produced in nature by plants by gene manipulation. To increase the production of compounds, two general approaches have been followed. (1) Methods have been employed to change the expression of one or a few genes, thereby overcoming specific rate-limiting steps in the pathway, or to shut down competitive pathway and to decrease catabolism of the product of interest. (2) Attempts have been made to change the expression of regulatory genes.

26.3 Metabolic Engineering

Neumann et al. (2009) and Kumar (2015) (this volume) reviewed in detail the production of secondary metabolites, pharmaceutical and other biologically important compounds in plant cell and cultures. Ramawat et al. (2009) and Kumar and Sopory (2010) reviewed metabolic engineering in plants with respect to primary and secondary metabolites including terpenoids, carotenoids, phenolics and flavonoids. Plant molecular breeding and use of plant biotechnology for production of novel compounds have been described by Kumar et al. (2014). Several antitumor compounds have been isolated from plants. Kumar (2010), Kumar and Roy (2011) and Dar et al. (2013) reviewed breeding methods for plant tissue culture and under field conditions for medicinally important plants. Sujatha and Kumar (2008) and Tang and Newton (2010) described different plant tissue culture systems with improved efficiency using *Agrobacterium*- mediated genetic transformation. Various natural products like tropane alkaloids, atropine, hyoscyamine, scopolamine and steroidal precursors are mainly produced by roots (Ramawat and Merillon 2000; Kumar and Roy 2006; Kumar and Sopory 2008, 2010; Kumar and Shekhawat 2009). *Eclipta alba* (L.) Hassk. was transformed by *Agrobacterium rhizogenes* strain MTCC 532 (Bhansali and Kumar 2014). Plants of family Valerianaceae, e.g. *Nardostachys jatamansi*, have been induced in tissue culture to produce higher levels of compounds than in intact plants. Studies on medicinal uses of ferns and their biotechnology have been extensively covered by Fernadez et al. (2011).

26.3.1 Biofuels

The world's fossil fuel reserves are running out. Governments around the world are looking to diversify their energy sources with nuclear, hydroelectric, wind and biofuel energy. Recent advances in synthetic biology and metabolic engineering can engineer microorganisms to produce new fossil fuel replacements. Such products might include short-chain, branched-chain and cyclic alcohols as well as alkanes, alkenes, esters and aromatics.

Biofuels include bioethanol, biodiesel (methyl and ethyl esters), biohydrogen, alkanes and various other hydrocarbon mixtures (such as wood gas and syngas). Today, however, ethanol is by far the most widely available; in the United States, for example, it constitutes 99 % of all biofuel produced (a total of over four billion gallons in 2005 from the fermentation of sugars derived from corn). Ethanol is biodegradable and produces slightly less greenhouse emissions than fossil fuel (carbon dioxide is recycled from the atmosphere to produce biomass); it can replace harmful fuel additives (e.g. methyl tertiary butyl ether, MTBE).

Ethanol is derived from corn or wheat for bioethanol and soya bean for biodiesel. However, recently biotech innovations and metabolic engineering are making it possible to use cellulosic biomass (wood chips, corn stalks, willow trees and switchgrass) for biofuel production.

Metabolic engineering for the production of biofuels has been reviewed by Kumar (2013). Bioethanol from bamboo is shown to be both technically and economically feasible, as well as competitive with petrol in China. Some measures may include improving sugar release with more

effective pretreatments and reduced enzyme usage, accessing low-cost bamboo feedstock or selecting feedstocks with higher/more accessible cellulose.

26.3.2 Lignocellulosic Biomass to Ethanol

Lignocellulosic biomass, such as agricultural residues and forestry wastes, has been widely recognised as a sustainable source for biofuel production (Gong et al. 2014). Roy and Kumar (2013) reviewed methods for lignocellulosic materials. However, cellulose and hemicellulose, two major sugar polymers of lignocelluloses, have to be depolymerised by hydrolysis to enable more efficient microbial utilisation (Acker et al. 2013b). Biomass hydrolysates usually contain monosaccharides as well as various amounts of oligosaccharides. Oligosac-charides are more challenging substrates than monosaccharides for microorganisms, because assimilation of oligo-saccharides may require additional hydrolytic enzymes and transportation systems.

26.4 Metabolic Engineering of Oilseed Crops

Recently, the demand for bioenergy produced from plant materials has increased. Transcription factors that control the biosynthesis of lignocellulose and lipids should be particularly investigated. Increase of the lipid content and modification of fatty acids in oil seeds have been required by the oleo-chemical industry.

The metabolic engineering of seed oils is attractive as the modification of lipid metabolism to change the quantity and quality of fatty acids in plants has important applications in the food industry, as well as in the production of detergents, fuels, lubricants, paints and plastics (Drexler et al. 2003).

It is possible to genetically manipulate the composition of long-chain and medium-chain fatty acid in some of common oil crops, such as *Glycine max* (soybean), *Brassica napus* (oilseed rape), *Helianthus annuus* (sunflower) and *Zea mays* (maize) (Drexler et al. 2003).

Recent notable studies include experiments in which oilseed rape (*Brassica napus*) was transformed with multiple cDNAs encoding enzymes for the desaturation and functional modification of long-chain fatty acids. Liu et al. (2003) expressed 18:1 D12 desaturase either alone or in combination with 18:2 D6 desaturase. With the 18:1 D12 desaturase alone, the seeds produced up to 46 % linoleic acid, whereas with both enzymes, the seeds produced up to 43 % linolenic acid. Recently, the a-linolenic acid content of rice grains has been increased tenfold by expressing the soybean FAD3 gene encoding O3 fatty acid desaturase (Anai et al. 2003).

26.5 Metabolic Engineering of Secondary Metabolites

26.5.1 Terpenoids

Terpenoids, also known as isoprenoids, are the largest family of natural compounds, consisting of more than 40,000 different molecules. All terpenoids are synthesised from the common precursors, dimethylallyl pyrophosphate and isopentenyl pyrophosphate. This occurs through two distinct pathways, the mevalonate-independent pathway and the mevalonate pathway, both of which have been targets for metabolic engineering (Broun 2004). Transgenic plants with modified terpenoid production help in fundamental studies of the biosynthesis and regulation of these compounds. Monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20) and tritrepenoids (30) are considered to be secondary metabolite. Following the recent discovery of the role of the 2-C-methyl-D- erythritol-4-phosphate (MEP) pathway in the biosynthesis of plastidial terpenoid, such as the carotenoids and monoterpenes and diterpenes, several genes of this pathway have been cloned (Broun 2004; Broun and Somerville 2001). Luecker et al. (2004) have reported the transformation of tobacco plants with three different monoterpene synthases from lemon. When the three transgenes

were stacked in one transgenic line, the tobacco plants produced more terpenoids and displayed a different terpenoid profile to wild-type plants.

26.5.2 Ornamentals

The global value of the ornamental sector of the horticulture industry is likely to be 250–400 billion USD (Chandler 2013). Value addition to ornamentals used in the floristry, gardening, landscaping and the environmental amenity industries can be done by metabolic engineering. The molecular breeding approaches represent vast opportunities as alternatives to chemical treatments. Genetic engineering has potential to create ornamental plants suitable for environmentally friendly production. Metabolic engineering approaches targeting elements of the ethylene signal transduction pathway and its perception (e.g. etr1-1 and IPT) serve as possible alternatives to avoid the use of chemical ethylene inhibitors. Over-expression of GA2ox genes and reduction of expression of GA20ox genes as well as modulation of regulatory genes involved in GA perception and signal transduction (GAI, SHI) and overall hormone homeostasis (KNOX) have successfully led to compact plants (see for details Lütken et al. 2012).

Lilies, *Lilium* spp., are of importance in floriculture. New lily cultivars with improved qualities such as resistance to insects and diseases, longevity and novel flower colour are desirable for both producers and consumers. Genetic modification has been proven to be an alternative to conventional breeding for the introduction of resistance to insects and disease in ornamental crops (Chandler and Brugliera 2011). Wang et al. (2012) provided information on factors affecting lily regeneration in a broad range of cultivars and also on gene transfer for further studying the transformation in the commercially most interesting lily hybrid cultivars, i.e. Orientals and OTs.

26.5.3 Beta-carotene

'Golden rice' contains up to 200 mg β-carotene per 100 g grain. Engineering high levels of β-carotene in the endosperm of 'golden rice' is a major breakthrough. Rice endosperm accumulates geranylgeranyl diphosphate. Therefore, four novel enzymes are required for β-carotene synthesis in this tissue: phytoene synthase, phytoene desaturase and z-carotene desaturase and lycopene b-cyclase. The researchers found that phytoene synthase and crtI alone were sufficient not only for the synthesis of lycopene but also for that of β-carotene and zeaxanthin.

Brassica napus seeds and oil contain negligible amounts of β-carotene. In this case, the simple over-expression of a bacterial phytoene synthase, crtB, fused to a plastidic transit peptide and under the control of a seed-specific napin promoter, is sufficient to boost β-carotene 300-fold, to a level similar to that found in oil palm. The β-carotene (provitamin A) levels have been increased in rice and canola seeds and in tomato fruits (Roemer et al. 2002). Astaxanthin, a non-plant carotenoid, has been produced in tobacco flowers (Mann et al. 2000). For several reasons, the carotenoid biosynthetic pathway is a very interesting target for genetic engineering. The carotenoids are important colour compounds in flowers, fruits and food products, and they are also good antioxidants. Vitamin A is formed from β-carotene. Vitamin A deficiency is a widespread problem in many areas like Asian countries. The introduction of β-carotene biosynthesis into the major staple food rice, by over-expression of phytoene synthase, phytoene desaturase and lycopene-cyclase, is thus an important achievement.

26.5.4 Insect Repellents

In one study, petunia was transformed with the (S)-limonene synthase gene from *Clarkia breweri* resulting in the production of linalool, which repels aphids (Lucker et al. 2001).

26.5.5 Flavonoids

Flavonoids have a wide range of colours from pale yellow to red, purple and blue. The flavonoid

biosynthetic pathway has been the choice to genetically engineer flower colours to obtain novel colours. Because of their antioxidant activity, higher levels of anthocyanin and flavonoids in food are an interesting objective. Much work has focused on tomato. Chalcone isomerase (CHI), an early enzyme of the flavonoid pathway, was found to be the key to increase flavonol production (Muir et al. 2001). Over-expression of petunia CHI gene led to a 78-fold increase of flavonoid levels in the tomato peel. Upon processing such tomatoes, a 21-fold increase of flavonols in tomato paste was achieved, if compared with non-transgenic controls.

26.5.6 Alkaloids

The terpenoid indole alkaloid pathway has been the target of numerous genetic engineering attempts, owing to the fact that about 15 terpenoid indole alkaloids are industrially important, including the antitumour alkaloids vinblastine, vincristine and camptothecin. Most efforts have been concentrated on the early part of the pathway and on over-expression of early genes, aiming to increase the metabolite flux into the alkaloid pathway. In particular, the genes encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR) have been studied extensively in *Catharanthus roseus* cell cultures (Verpoorte et al. 1994; Verpoorte et al. 2000; Verpoorte and Memelink 2002).

26.6 Alkaloid Production in Tryptophan Decarboxylase Silenced Lines

Tryptophan decarboxylase, a pyridoxal phosphate–dependent enzyme that produces tryptamine **1** from tryptophan **2** (Facchini et al. 2000), was targeted for gene silencing (Helliwell and Waterhouse 2005). The plasmid designed to suppress tryptophan decarboxylase (pTDCi) was transformed into *Agrobacterium rhizogenes*, which was then used to infect *C. roseus* seedlings to generate hairy root culture as described (Hughes et al. 2002; Runguphan and O'Connor 2009). Hairy root lines harbouring the pTDCi silencing plasmid were cultured in liquid media, and alkaloids were extracted from the plant tissue according to standard protocols. Gratifyingly, the production of all major tryptamine-derived alkaloids ajmalicine **3**, serpentine **4**, catharanthine **5** and tabersonine **6** was substantially decreased in the five silenced lines that were examined. Suppression of alkaloid production appears to be stable, with little change in alkaloid production levels after seven subcultures performed thus far.

26.7 C3 and C4 Metabolism and Plant Tissue Culture Studies

The majority of terrestrial plants, including many important crops such as rice, wheat, soybean and potato, are classified as C3 plants that assimilate atmospheric CO2 directly through the C3 photosynthetic pathway. C4 plants such as maize and sugarcane evolved from C3 plants, acquiring the C4 photosynthetic pathway to achieve high photosynthetic performance and high water- and nitrogen-use efficiencies. So, there is great scope to study the metabolic pathway and try to engineer this in another plant, which is having less photosynthetic performance and high water and nitrogen efficiencies. The good news is that technology continues to hold great promise for the future of plant metabolite engineering.

26.8 Conversion of Cellulose into Microbial Lipids by *Cryptococcus curvatus*

The oleaginous yeast *C. curvatus* can produce microbial lipids using a mixture of glucose and xylose as well as cellulosic biomass as feedstocks. Gong et al. (2014) has developed the simultaneous saccharification and lipid production (SSLP) process for direct conversion of cellulose into lipids by oleaginous species in the presence of cellulase and β-glucosidase.

26.9 Tocopherols

Higher vitamin E doses of 100–1,000 IU have been associated with cancer reduction, improved immune response and cardiovascular benefits. Such high doses are currently realised only through supplemental tocopherol intake. Tocopherols are also used in the pharmaceutical and cosmetics industry and as animal feed additives to improve the quality and shelf life of meat (Bramley et al. 2000). Natural vitamin E originates almost exclusively from soybean oil and is used predominantly for human applications due to its premium price and limited availability.

26.10 Role of Plant Tissue Culture Studies on Secondary Metabolite Production and Metabolic Engineering

Plant cell walls determine the shape and architecture of the plant body and play major roles during cell differentiation, defence against pathogens and cellular growth. Cellulose, the main polysaccharide of plant cell walls, is made of β-1,4-linked glucan chains, which form crystalline microfibrils and act as a framework for the deposition of other wall components. The matrix polymers, such as hemicelluloses and pectins, are structurally diverse polysaccharides, and most of them harbour heavily substituted side chains. Second-generation biofuels are generally produced from the polysaccharides in the lignocellulosic plant biomass, mainly cellulose. However, because cellulose is embedded in a matrix of other polysaccharides and lignin, its hydrolysis into the fermentable glucose is hampered.

26.11 Cell Wall Polysaccharides Are Synthesised in Different Cellular Compartments

Cell wall polysaccharides are synthesised in different cellular compartments. Cellulose is synthesised at the plasma membrane by hexameric cellulose synthase (CESA) complexes, which are believed to hold 36 individual CESA proteins (Somerville et al. 2004). In contrast to cellulose, the synthesis of the matrix cell wall polymers, that is, hemicelluloses and pectins, is carried out in the Golgi apparatus (see Liepman et al. 2005; Sterling et al. 2006; Jensen et al. 2008). These polymers are synthesised by a plethora of glycosyltransferases and are subsequently modified by different hydrolases, esterases and lyases (Henrissat et al. 2001; Somerville et al. 2004). Modification and secretion of cell wall components can depend on the demand for cell wall biosynthetic events that may be transduced by cell surface signalling devices needs to be transformed into cellular responses. These may include post-translational modifications of enzymes, modulation of the rate of secretion and transcriptional activations or repressions.

Although the lignin content had the main effect on saccharification, also other cell wall factors could be engineered to potentially increase the cell wall processability, such as the galactose content. A better understanding of the effect of lignin perturbations on plant cell wall composition and its influence on saccharification yield provide new potential targets for genetic improvement (Acker et al. 2013a).

In contrast to common perception, a reduction in lignin was not compensated for by an increase in cellulose, but rather by an increase in matrix polysaccharides.

Cell wall components to the cell surface are dependent on the secretory shuttling system (Taylor 2008). The endomembrane system also contains V-ATPase complexes which acidify the vesicle lumen, a process required for certain aspects of membrane trafficking. The vesicles containing the CESAs are transported to the plasma membrane with the help of actin cables via interactions with actin-associated proteins, possibly KAM1/MUR3 (Tamura et al. 2005; Geisler et al. 2008). After the delivery of the CESA complexes to the plasma membrane, vesicle components are recycled back to the endomembrane system. Microfibrils are produced by CESA complexes and are co-aligned with the

microtubules beneath the plasma membrane via microtubule-associated proteins (see review Geisler et al. 2008).

This system encompasses protein synthesis and folding in the ER, glycan modification and synthesis in the Golgi apparatus and endosomal vesicle loading and shuttling to different cellular locations, including the plasma membrane and the vacuole. Brux et al. (2008) reported that the cellulose content is dependent on V-ATPase activity. The pH difference across the vesicle membrane is established by endosomal-localised vacuola ATPases (V-ATPases). There is also evidence that these differently located isoforms may carry out different biological functions in the cell. RNA interference of the TGN-localised, but not of the tonoplast-located, VHA-a isoform leads to reduced cell expansion (Brux et al. 2008). The VHA-a1 protein is located at the trans-Golgi network (TGN), whereas two other isoforms, VHA-a2 and VHAa3, are specific for the tonoplast (Dettmer et al. 2006). This is consistent with phenotypes observed in other mutants for V-ATPase subunits, which also display altered morphology of Golgi stacks (Strompen et al. 2005; Dettmer et al. 2010).

It is therefore possible that the defects in TGN trafficking caused by blocking V-ATPase activity result in retention of the CESAs in the TGN. Thus, the activity of TGN-localised V-ATPases appears crucial for vesicle trafficking during cellulose synthesis and cell expansion in plant cells. As in other eukaryotic cells, small GTPases and guanine nucleotide exchange factors (GEFs) are involved in the regulation of vesicle formation and trafficking in plants and also in the definition of the membrane identity of endomembrane compartments (Strompen et al. 2005; Hurtado-Lorenzo et al. 2006). This suggests that the V-ATPase could act both as a pH modulator and sensor that governs the recruitment of proteins from the cytosol to the endosomal membrane (Paredez et al. 2006; Dettmer et al. 2005, 2006). At least one component that is directly involved in the synthesis of cell wall polymers also affects vesicle trafficking (Madson et al. 2003; Tamura et al. 2005). The importance of the actin filaments for cell wall deposition was further underscored in a recent study performed in xylem cells (Wightman and Turner 2008). They suggested that not only microtubules, which may guide the CESAs (Paredez et al. 2006), but also actin cables may be crucial for cellulose deposition (Wightman and Turner 2008).

26.12 Compounds of Pharmaceutical Importance

In the 1950s, Charles Pfizer Co. (USA) tried unsuccessfully to produce pharmaceutical compounds through the generation of large-scale cultures of plant cells. Such an attempt was however well received abroad, i.e. Germany and Japan, and resulted in the development of feasible industrial applications of cultured cells in the late 1970s (see Kumar and Roy 2006, 2011; Thorpe 2012; Neumann et al. 2009; Kumar and Sopory 2010). By using radioimmunoassay techniques and enzyme-linked immunosorbent assays, as well as cell cloning and repeat selection of high-yielding strains from heterologous cell populations, improvements in production have been attempted.

Cell immobilisation techniques have also been used. The utilisation of bioreactors allowing the large-scale growth of cells has also contributed to the field. Stirred tank reactors and several types of air-driven reactors have been employed. Such methods involve a two-step process: biomass accumulation through active cell growth followed by product synthesis coupled with reduced cell proliferation (Sharma et al. 2011).

Enhancement of secondary product formation has also been achieved by selecting mutant lines overproducing the desired product and by using abiotic factors, including ultraviolet treatments and exposure to heat or to cold and heavy metals (Neumann et al. 2009; Kumar and Sopory 2010; Kumar and Roy 2011).

26.13 Conclusions

Metabolite engineering is generally defined as the redirection of one or more enzymatic reaction to produce new compounds in an organism,

improve the production of existing compounds or mediate the degradation of compounds. As presented above, several points in a given metabolic pathway can be controlled simultaneously either by over-expressing and/or suppressing several enzymes or through the use of transcriptional regulators to control several endogenous genes. Over the past few years, there has been a growing realisation that metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level and that even the simplest modifications can send ripples throughout the entire system. Metabolic engineering in plants involves the modification of endogenous pathways to increase flux towards particular desirable molecules or redirection of one or more enzymatic reaction to produce new compounds in an organism or mediate the degradation of compounds. Recently, significant advances have been made in metabolic engineering through the application of genomics and proteomics technologies to elucidate and characterise metabolic pathways. The major challenge for the coming years is to obtain more information about regulation at all levels like genes, enzymes, compartmentation, transport and accumulation. This will open the way for successful strategies for altering the accumulation of certain compounds. In this review, efforts are made to look at recent examples of single- and multiple-gene metabolic engineering in primary and secondary metabolism groups.

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