Genetic Manipulation and Its Contribution to Pharmaceuticals: Past and Future Perspectives

25

Ashwell R. Ndhlala, Bhekumthetho Ncube, Phatu W. Mashela, Ian du Plooy, Hafiz A. Abdelgadir, and Johannes van Staden

Abstract

Genetic manipulation or metabolic engineering in plants has resulted in the ability to control plants with the aim to synthesize desired plant secondary metabolites for the pharmaceutical and nutraceutical industry. The evolution of "omics" has further provided advanced resources that allow for elucidation of uncharacterized biosynthetic pathways, enabling novel pharming approaches. Enhanced production levels of alkaloids with anticancer activities in *C. roseus*, vinblastine and vincristine through genetic manipulation, have been achieved. Various natural antioxidants in the form of phenolic compounds including flavonoids and proantocyanidins have not been spared. This chapter discusses the exciting topic of metabolites in transgenic plants and the investigation into the large-

A.R. Ndhlala (✉)
Division Crop Science, Agricultural Research Council, Pretoria, South Africa

Green Technologies Research Centre, University of Limpopo, Sovenga, South Africa
e-mail: NdhlalaA@arc.agric.za; ashwelln@yahoo.com

B. Ncube (✉) • J. van Staden (✉)
Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal Pietermaritzburg, Scottsville, South Africa
e-mail: Ncubeb@ukzn.ac.za; bbkncube@yahoo.com; rcpgd@ukzn.ac.za

P.W. Mashela (✉)
Green Technologies Research Centre, University of Limpopo, Sovenga, South Africa
e-mail: Phatu.Mashela@ul.ac.za

I. du Plooy (✉)
Agricultural Research Council, Vegetable and Ornamental Plants (VOP), Pretoria, South Africa
e-mail: IduPlooy@arc.agric.za

H.A. Abdelgadir (✉)
Agricultural Research Council, Vegetable and Ornamental Plants (VOP), Pretoria, South Africa

University of Limpopo, Sovenga, South Africa
e-mail: AbdelgadirH@arc.agric.za

S. Jha (ed.), *Transgenesis and Secondary Metabolism*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-28669-3_27

scale production of pharmaceutical ingredients in a number of heterologous systems including plant cell and organ cultures. The chapter also reveals the unknown and unexpected results in metabolic pathway engineering which still need to be researched and understood including the behavior of transgenes in the environment. In an attempt to map the future of transgenic plants, next generation technologies are put on the spotlight.

Keywords
Genetic engineering • Hairy root cultures • Pharming • Pharmaceuticals

Contents

1 Introduction

Genetic engineering in pharmaceuticals, also known as "pharming", involves the use of genetically modified organisms including plants (transgenic plants) as factories for pharmaceutical production. The purpose of genetic manipulation or metabolic engineering in plants is to improve by increasing the production or expression of an existing compound, reduce the production of a specific compound, or introduce the production of novel compounds through the modulation of a group or single sets of enzymatic reactions in a biosynthetic pathway [1]. Pharming is achieved by the use of cells, organs, and/or organisms to manipulate the genetic make of a plant so as to hijack the biochemical pathways to diverge metabolic fluxes towards the upregulation or downregulation of desired compounds [1]. To complement this, the fast evolving "omics" era provides advanced resources that allow for elucidation of uncharacterized biosynthetic pathways, enabling novel pharming approaches [2].

The ability presented by the "omics" to genetically manipulate plants has led to changes in the course of plant metabolism and the potential for enhancing the content and nature of plant secondary metabolites of commercial value. This makes plants potential factories for the production of a variety of useful compounds [1]. By

definition, secondary metabolites are compounds produced by specialized biosynthetic pathways in plants, often counseling their functions as defenses against invasion by pathogens, pests and herbivores while regarded as nonessential for normal growth and development. More often than not, secondary metabolites confer beneficiary effects for the survival and/or behavior of plants, microbes, insects, mammals, and most importantly as useful pharmacological agents in humans and animals. Usually the pharmacological compounds can only be obtained as extracts from medicinal plants which in most cases grow slowly and are difficult to cultivate. Once pharmacological agents are identified, chemical synthesis, which is often impractical or uneconomical due to the complexity of their molecular structures, is used for industrial production. This opens up a whole new and exciting field of metabolites in transgenic plants and the investigation into the large-scale production of pharmaceutical ingredients in a number of heterologous systems including microbes, plant cell/organ cultures, and intact plants. In this chapter, the biosynthetic platforms for pharmacological compounds in transgenic plants are discussed. We highlight bottlenecks that remain to be overcome for a successful next generation metabolic bioengineering for the benefit of humans.

2 Gene Manipulation and Plant Transformation

Under natural circumstances, secondary metabolic pathways result in the production of tens of thousands of compounds and intermediary components involved in various biological responding processes, under stimuli of specific external environmental stress elicitors as well as signal molecules of normal growth and development [3, 4]. However, in some instances, depending on compound demand, enhancing production of specific compounds using different methods is called for. Since the successful introduction and expression of diverse foreign genes in tobacco during the early 1980s, gene manipulation and plant transformation became the core technological tool in plant biology and a practical tool for cultivar improvement and enhancement of specific compounds for both plant basic research and for agricultural biotechnology applications [5, 6]. The successes of the gene manipulation tools have now been extended to include most major economic crops, vegetables, ornamental, fruit, tree, pasture plants [5], and most recently medicinal plants. However, the rapid and simultaneous developments in the evolution of technology and information technology make tabulations of transformed species quickly out of date; thus it is advisable to always locate current transformation methods for species of interest. The fast evolution of gene manipulation process is aimed for higher diversification and refinement of transformation techniques for greater convenience, higher efficiency, broader genotype range, and desired molecular characteristics of transformants.

2.1 Secondary Metabolites and Pharmaceuticals

Because of their photoautotrophic nature, plants have developed an unlimited capacity for growth and development which is driven by a relatively plastic

metabolism that allows them to rapidly adapt to changing environmental factors, pathogens, and other biotic and abiotic challenges [1]. This phenomenon is reflected in the wide variety of secondary metabolites accumulated by plants in their organs. Be that as it may, the biosynthetic origin and roles of the secondary metabolites in the plant is still poorly understood, yet they are of considerable interest because of their potential industrial, pharmacological, and medicinal value [1].

Humans are in the process of mastering the use of natural products derived from plants growing wild in nature or cultivated as crops as the prime ingredients in many drugs, beverages, flavors, and cosmetics. Pharmanutraceuticals are positioned between food and pharmaceuticals (functional foods). The nutraceuticals comprise foods and substances and/or combinations of substances that consist of molecules or elements found in nature for the purpose of maintaining or improving health and treating or preventing diseases. Major food and pharmaceutical companies are currently investing heavily in research and development (R&D) and marketing budgets to secure market share and promote further growth and development. In most cases, the R&D is aimed at enhancing secondary metabolites with pharmanutraceutical potentials.

Gene manipulation has achieved significant progress in understanding and enhancing the biosynthesis of a number of pharmacologically useful secondary metabolites. The mostly studied and often used as an example of successful gene manipulation to produce transgenic plants with enhanced biosynthetic pathway and gene regulation research is that of *Catharanthus roseus* (L.) G.Don (Apocynaceae) production of anticancer compounds, vinblastine and vincristine (Fig. 1). Due to the low contents of these two compounds in naturally growing plants, it becomes

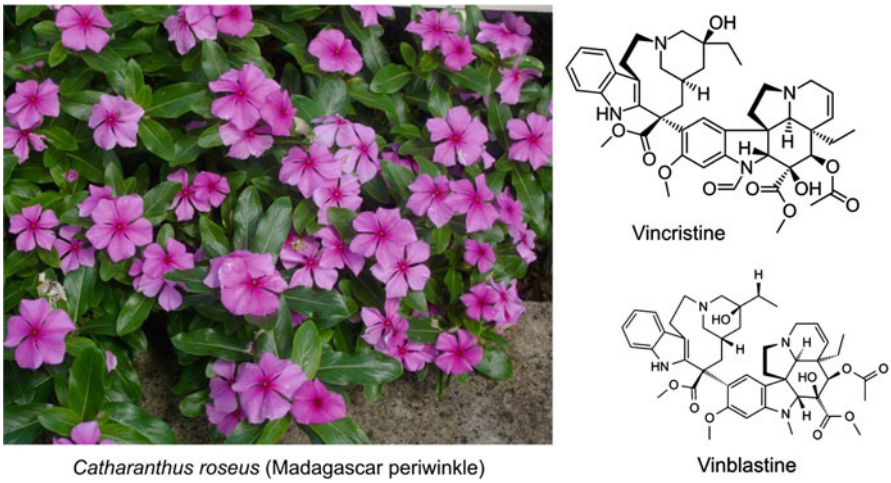

Fig. 1 *Catharanthus roseus* (L.) G.Don (Apocynaceae) and the two dimeric alkaloids, vinblastine and vincristine. The two compounds are used in the treatment of acute leukemia since the 1960s. The recent advances in the "omics" have enabled researchers to characterize the key genes and their resulting enzymes in the production of vinblastine and vincristine

important to clone and regulate expression of key genes (DXR, SLS, G10H, STR) involved in the pathway, in order to achieve high yields in in vitro culture systems. This was made possible because of the in-depth understanding of the biosynthetic pathways of the terpenoid indole alkaloids in *C. roseus* [7].

3 Gene Regulatory Networks for Secondary Metabolites

Most of the plant secondary metabolites, including phenolic compounds, flavonoids, alkaloids and theanine, are important components of plant chemistry and are closely related to the health benefits exhibited by most plants. Secondary metabolite biosynthesis in plants is differentially regulated in different networks and tissues during growth and development. However, little is still known about the expression patterns of genes involved in secondary metabolic pathways or their regulatory mechanisms.

3.1 Broadening the Horizons of Cancer Therapy: A Case of Vinblastine and Vincristine from Transgenic *C. roseus*

Catharanthus roseus (Madagascar periwinkle), presented in Fig. 1, accumulates more than 100 indole alkaloids including the monomeric alkaloids such as ajmalicine, serpentine, vindoline, and catharanthine. Ajmalicine is used to treat circulatory diseases such as hypertension, while vindoline and catharanthine are precursors of dimeric alkaloids such as vinblastine and vincristine (Fig. 1) which are used in the treatment of acute leukemia since the 1960s. Vinblastine and vincristine accumulates in the areal parts of *C. roseus* in very low amounts, making them very costly for commercial use.

A whole panthera of information has been generated in the direction of understanding the growth of cell suspensions, regulation of indole alkaloid production and the signals that trigger their biosynthesis. Monomeric alkaloids are easily, though noneconomic, produced by suspension-cultured cells. Transgenic *C. roseus* presents a good procedure to improve the alkaloid production and even to create new pathways for the synthesis of new pharmaceuticals. Alkaloids are mainly derived from the decarboxylation of amino acids precursors like ornithine, lysine, tryptophan, and histidine to give respective amines. Some are derived from anthranilic acid or nicotinic acid. The remarkable ability of plants to piece together amines and different chemical partners presents a number of chemical backbones for bioactive pharmanutraceuticals [8]. Characterization of genes responsible for the generation of these backbone and central chemical structures and their development into bioactive components have revolutionized the manipulation of biosynthesis of exciting molecules.

As mentioned before, the current advances in the "omics" have enabled researchers to characterize the key genes and their resulting enzymes and in some cases clone them. *Strictosidine synthase1* (*Str1*), a key enzyme in the biosynthesis of terpenoid indole alkaloids by catalyzing the formation of strictosidine from

tryptamine and the monoterpenoid secologanin in *C. roseus* has now been cloned [8, 9]. Strictosidine and secologanin are central backbones in the synthesis of monoterpenoid indole alkaloids like isoquinoline (e.g., berberine – fungitoxic and antibacterial), acridine (e.g., rutacridone – fungitoxic and antibacterial), and pyrrolizidine (e.g., scenecionine – hepatotoxic, insect pheromone precursor and antileukemic) [8, 10].

Vinblastine and vincristine are derived from the piecing together, through dimerization of catharathine and vindoline with some late stages involving cytochrome p450 monooxygenase activity. Tabersonine 16-hydroxylase (T16H) is a cytochrome p450 that has now been cloned and has added to the much anticipated manipulation of the biosynthesis of vinblastine and vincristine [11].

Perhaps in the future, functional genomics and proteomic analysis of alkaloid biosynthesis using expression-based analyses and computational modeling systems will be used to accelerate the comprehensive understanding of specialized and compartmentalization in the production of vinblastine and vincristine. For example, a glimpse into the future suggests that cell-specific compartmentation of alkaloid biosynthesis in *C. roseus* occurs in the epidermal, idioblast, and laticifer cells [12]. This is valuable information which could be used to enrich these pathways and allow growth of specific cells, in this case epidermal, idioblast, and laticifer, in a laboratory for the manufacturing of specific alkaloid as factories.

3.2 Enhancing Production of Antioxidants Including Vitamin A, C, and E in Plants

Most organisms including animals, microbes, and plants derive their energy for use in other processes from the oxidation of foodstuffs in the cells. However, there is a need to strike a balance between the oxygen consumption and metabolism as this process can lead to the production of excess aggressive forms of oxygen, reactive oxygen species (ROS), which are capable of serious damage to cell constituents, including membranes and DNA in humans and plants. Plant cells have evolved mechanisms of mopping up ROS, preventing uncontrolled oxidation, regulation of electron transport processes, and control of enzymatic reactions through natural antioxidants including flavonoids, phenols, tannic acid, glutathione, ascorbic acid, carotenoids, and enzymatic antioxidants such as superoxide dismutase (SOD). The production of compounds in plants with antioxidative capacity has generated interest among human health researchers and has directed human nutrition towards the use of enriched and biofortified foods with potential to prevent and decrease incidence of several diseases. With recent advances in genetic engineering to enhance the production of useful secondary metabolites in crop plants, there has been a renewed interest among scientists especially human nutritionists to enhance well-known antioxidants such as vitamins (Fig. 2) required for metabolic functions, i.e., vitamin C, vitamin E, vitamin B, and phytochemicals such as phenolic compounds (Fig. 3) including catechins, carotenoids, β-carotene, lycopene, zeaxanthin, diterpene, curcumin, and anthocyanins [13].

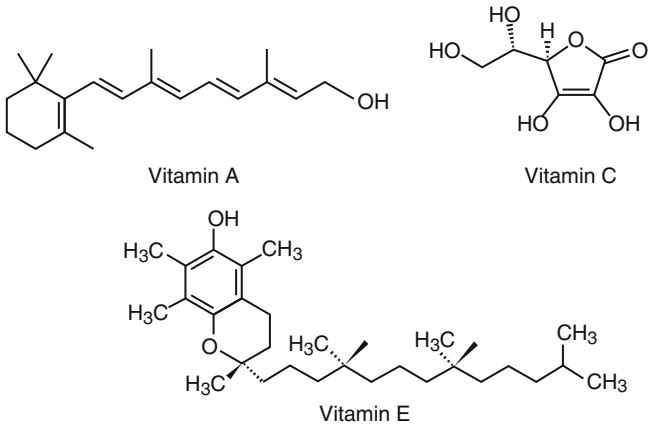

Fig. 2 Chemical structures of vitamins A, C, and E

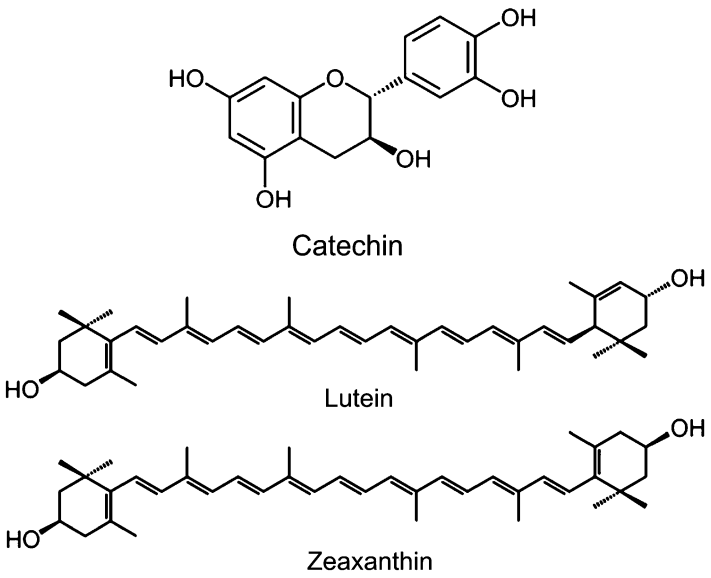

Fig. 3 Chemical structures of catechin, lutein, and zeaxanthin whose production can be enhanced through genetic manipulation of specific plants

3.2.1 Targeting Vitamin A

Dietary shortages of vitamin A (Vitamin A Deficiency – VAD) were estimated to cause fatalities to about 190 million children and 19 million pregnant women, in 122 countries in 2005 [14]. VAD is also responsible for more than 500,000 cases of irreversible blindness and millions of cases of xerophthalmia annually. The most vulnerable group is children under the age of five and pregnant women. Perhaps

genetic engineering of plants could offer the antidote to VAD among children and pregnant women [15]. In many communities where the diet is deficient in vitamin A, the much needed vitamin is supplemented orally and by injection. Be that as it may, several agricultural strategies coupled with genetic engineering have been employed across the world to combat undernutrition and most particularly VAD. In a classical example, a nutritious orange fleshed sweet potato variety is growing in popularity and becoming an important strategy to improve VAD across Africa.

In South Africa, the Agricultural Research Council, Institute of Vegetable and Ornamental Plants (ARC-VOP) is at the helm of promoting the vitamin A rich orange-fleshed sweet potato (OFSP) [*Ipomoea batatas* (L.) Lam – Convolvulaceae]. Funded by the South African Government's Department of Rural Development and Land Reform (DRDLR), agricultural extension researchers work closely with farmers, groups, and other parties to ensure widespread OFSP availability and sustainability. Elsewhere in Africa, OFSP is being disseminated with support from USAID under Feed the Future, the US Government's global hunger and food security initiative.

The R2R3-type protein IbMYB1 is a key regulator of some secondary metabolite biosynthesis in the storage roots of OFSP. Future transgenic work will likely look into introducing other necessary pharmaceutical ingredients into OFSP (Fig. 4). Recent studies on R2R3-type protein IbMYB1 have provided insight into further biofortification of OFSP by introducing genes that will overexpress anthocyanin pigmentation together with a provitamin A molecule, β-carotene (Park et al. [16]). Consequently, all IbMYB1 transgenic plants will have much higher antioxidant activities compared to the normal OFSP [16].

In another initiative, the ARC-VOP in collaboration with the Council for Scientific and Industrial Research (CSIR) which is South Africa's central and premier scientific research and development organization and Nestle (South Africa) is involved in the production of a biofortified household product noodles with a provitamin A rich *Amarathus* sp., dubbed *morogo* (indigenous vegetables) 2 min noodles (Fig. 4). Presently, the *Amarathus* sp. used is not transgenic, but elicitation

Fig. 4 β-Carotene biofortified foods: *Golden* rice with the potential of producing 23-fold increased β-carotene. *Orange*-fleshed sweet potato with the R2R3-type protein IbMYB1 set to further increase β-carotene in the edible parts of the crop, *Morogo* (indigenous vegetables) 2 min noodles biofortified with a provitamin A rich *Amarathus* sp.

methods of boosting the production of provitamin A β-carotene are being used to maximize the vitamin A benefit. However, future trend will focus on transgenic *Amarathus* sp. for the purpose.

In another classical example, genetic engineering has given rise to golden rice, a variety of rice (*Oryza sativa* L. – Poaceae) produced to biosynthesize large amounts of β-carotene, a provitamin A molecule, in the edible parts of rice. The transgenic golden rice differs from its parental strain by the addition of three β-carotene biosynthesis genes. The parental rice plant can naturally produce β-carotene in its leaves, where it is utilized during the photosynthesis processes. The plant does not normally produce the pigment in the endosperm, where photosynthesis does not occur. With the evolution of transgenic tools, a second generation variety of golden rice, 2, was announced, which produces elevated β-carotene up to 23-fold to the original golden rice. Issues on bioavailability of the β-carotene from golden rice have been tested and confirmed. This will make both the OFSP and golden rice effective sources of vitamin A for humans and will offer solutions for a range of natural-source β-carotene formulations that offer ease of use, superior color stability, and guaranteed minimum color intensity for the pharmaceutical industry.

3.2.2 Enhancing Vitamin C

Vitamin C or L-ascorbic acid has been classified as an essential nutrient for humans and many other animal species. It is also one of the well-researched plant secondary metabolites, possessing important pharmacological properties including being an antioxidant, enzyme cofactor, electron donor, and acceptor in the electron transport system. Apart from these, Vitamin C also participates in several physiological processes, among others, immune stimulation, synthesis of collagen, hormones, neurotransmitters, and iron absorption [17]. Its official IUPAC name is 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol. Deficiencies in vitamin C are widespread, affecting both developing and developed countries, with the latter actually trying to overcome this lack through dietary supplements and food fortification. It is therefore apparent that new strategies aimed to increase vitamin C production in plants would be of interest to benefit human health absorption [17].

All higher plants, some higher animals and a number of yeasts are able to synthesize ascorbic acid. However, some animals, including humans, are unable to synthesize the molecules due to the nonfunctional L-gulono-1,4-lactone oxidase gene which acts in the last step of the molecule's synthesis. The major pathway of ascorbic acid synthesis is the Smirnoff-Wheeler pathway where the molecule is synthesized from D-glucose via a complex 10-step pathway involving phosphorylated sugar intermediates and sugar nucleotides [18]. Interestingly, the pathway can be enhanced to provide a living bioreactor for vitamin C production in optimal growing conditions. The enhancement can be done through genotype selection by genetic engineering, classical breeding, and changes of the agronomic conditions, on the basis of the emerging concepts that plants can enhance vitamin C synthesis as part of defense responses [17].

As mentioned earlier, in developed countries vitamin C supplementation is largely adopted especially for preventing/reducing cold-related diseases. It has

been confirmed that the industrial production of vitamin C for the pharmaceutical industry represents a low efficient and expensive technology [17, 19]. In the last decade, an interesting phenomenon has also been demonstrated which shows that vitamin C from plant-derived food is more bioavailable than the chemically synthesized or purified molecule used in supplementation in the pharmaceutical scenario [17, 20]. This is because the molecule works in cohort with other molecules like vitamin E, polyphenols, and flavonoids in their pharmacological actions.

3.2.3 Enhancing Vitamin E

The term vitamin E is generic to a group of lipid-soluble antioxidant compounds, the tocochromanols, more specifically tocopherols and tocotrienols. Tocotrienols form the primary form of vitamin E in most plants. They contribute to the nutritive and pharmaceutical value of most plants as potent antioxidants in human diets, protecting polyunsaturated fatty acids against lipid peroxidation [21]. The initial stages of vitamin E synthesis involve the production of homogentisate from hydroxyphenylpyruvate (HPP) in a complex enzymatic reaction involving hydroxyphenylpyruvate dioxygenase (HPPD). The dioxygenase enzyme, HPPD has a key location in the tocochromanol pathway and is an important activity regulating tocochromanol fluxes in plants. However, overexpression of HPPD in Arabidopsis [*Arabidopsis thaliana* (L.) Heynh – Brassicaceae] and tobacco (*Nicotiana tabacum* L. – Solanaceae) resulted in the increase of tocopherols [22]. This is due to the fact HPP production is regulated by feedback inhibition of arogenate dehydrogenase by its product Tyr [22]. In the efforts to enhance the production of vitamin E, researchers succeeded in bypassing this feedback inhibition by expressing in tobacco a yeast (*Saccharomyces cerevisiae*) prephenate dehydrogenase (PDH) that catalyzes HPP production directly from prephenate [23]. This was even enhanced by the coexpression of PDH and Arabidopsis HPPD in tobacco which resulted in up to eightfold increase in vitamin E production in the tobacco leaves. Surprising this resultant eightfold increase in vitamin E production was mainly due to the increase in tocotrienols, which are normally produced in tobacco seeds but not in tobacco leaves. This presents a classical breakthrough in enhancing the production of vitamin E using transgenic plants.

3.3 Modulating Production of Functional Phenolic Compounds: A Case of Flavonoids

Among the major groups of bioactive compounds in plants, phenolic acids are the most abundant and provide the most beneficiary properties for human health. These are molecules containing a phenolic ring and an organic carboxylic acid function that function predominantly in electron exchange system by donating or accepting electrons which can delay or inhibit the oxidation of biomolecules such as DNA, proteins, and lipids [24]. Apart from this, the bioactive properties of phenolic acids are numerous. This has resulted in an increasing interest in production of plants with enhanced content of phenolic acids. Increasing the content in phenolic acids of

plants can be achieved by a variety of means, including development of improved cultivars, use of specific cultivation conditions, and application of postharvest treatments as well as genetic engineering to produce new cultivars [24].

Flavonoids are a ubiquitous group of plant secondary metabolites with numerous pharmacological functions ranging from antioxidants, anti-inflammatory, anticancer, antimicrobial and inhibition of coagulation, thrombus formation or platelet aggregation in blood, reducing the risks of cardiovascular diseases. They consist of a family of phenolic molecules with variable structures naturally occurring in vegetables, fruits, grains, flowers, and beverages. In the last two decades, the flavonoid biosynthetic pathway in plants together with the numerous enzymes required for the production of different structures and their regulation have been well studied and have been characterized [25]. Using this knowledge, enhancing flavonoid biosynthesis in plants may provide unlimited ingredients that have the potential for use in novel pharmanutraceuticals designed to benefit human health. Several plant models have been used including maize, petunia tomato, and Arabidopsis. With understanding of the biosynthesis pathways, genetic manipulation has made it possible to generate several tomato lines with altered flavonoid content. Of most notable success was the ectopic expression of a biosynthetic enzyme, chalcone isomerase which resulted in up to 78-fold increase in total fruit flavonols [25].

4 Potential of the Hairy Root for Enhancing Active Metabolite Production

During the last two decades, there has been much excitement about the ability to genetically engineer plants using gene isolation and insertion techniques. These techniques allow for the construction of transgenic plants that contain and express a single, well-defined gene from any source including microbes, animals, or other plant species. Hairy root cultures are obtained from the root transformation with *Agrobacterium rhizogenes* that inserts transfer-DNA into the genomes of the infected root cells, unbalancing their hormone physiology. The genetically transformed root cultures can produce and accumulate high levels of secondary metabolites comparable to that of intact plants. Hairy root cultures are currently used for high throughput production and enhanced bioactivity of some secondary metabolites used as pharmanutraceuticals, pigments, and flavors from many plants.

Rosmarinic acid has been found to accumulate in the hairy root cultures of *Salvia miltiorrhiza* Bunge (Lamiaceae, Danshen). Rosmarinic acid is a potent antioxidant with potential for Alzheimer's disease treatment [26]. *Arachis hypogaea* L. (Fabaceae, peanuts) hairy root cultures were reported to secrete pharmacologically important stilbenoids including 30-fold increase in resveratrol, arachidin-1,m and arachidin-3 [27]. Enhancement of many other pharmacologically important compounds has been reported in the literature, these include the anti-inflammatory compound phenylethanoid verbascoside from hairy root cultures of *Harpagophytum procumbens* (Burch.) DC. ex Meisn. (Pedaliaceae, Devils claw) [28]; antioxidant compounds caftaric, caffeic, and chlorogenic acids in *Echinacea purpurea* (L.)

Moench (Asteraceae, coneflower) hairy root cultures [29]; and the antimalarial drug artemisinin in hairy root cultures of *Artemisia annua* L (Asteraceae) [30].

5 Finding Nemo: Exploring Transgenic Organisms of the Aqua World for Important Secondary Metabolites

Recent trends in drug research from natural sources have shown that organisms from the aqua world, more especially algae, are promising organisms to furnish novel biochemically active compounds [31]. Just like plants, freshwater and marine algae have developed defense strategies for competitive survival, resulting in production of numerous structural and chemical diverse secondary metabolites from different metabolic pathways [32]. Currently, around 18 million tonnes (wet weight) of aquatic plants and organisms are cultivated/harvested annually with an estimated value of 4.4 billion US dollars [33]. Exploration of algae for pharmaceutical purposes has resulted in numerous prototypes for the discovery of new agents. This stimulates the use of sophisticated physical techniques and new syntheses of compounds with biomedical application. One such technique will be transgenesis for the enhancement of specific secondary metabolites.

However, transgenesis in algae is complex requiring selectable transformation techniques, and other genetic tools [34]. Be that as it may, transgenesis methods for some algae are already available with about 25 species already accessible to genetic transformation [34]. Recently, sequencing projects have been completed for several of the important species, providing the vast amount of genomic data on a number of algae, dramatically enlarging the algae's molecular toolbox [34]. Already, genetically modified algae are being used as bioreactors for the production of biofuel, recombinant antibodies, vaccines, and insecticidal proteins [35]. This unlocks promises and potentials on a much broader field of application, metabolic pharming, with emphasis on production of proteins or metabolites that are valuable to medicine. Perhaps the genetic modifications aimed at enhancing the physiological properties of algal strains and optimization of algal production systems will further improve the potential of this auspicious technology in the future.

6 The Curse of the Transgenic Plant: Biosafety Issues Surrounding Transgenic Plants

Because the metabolic pathways in plants and other organisms are intertwined to one another to form a complex system, it is expected that perturbation of a single step in the network usually have extensive effects on metabolic flux [36]. Metabolic engineering usually focuses on production of only one metabolite or a single metabolic gene and normally generates unexpected metabolic consequences [37]. In other scenarios, can promiscuous transgenic plants spread genes to other plants? Transgenic plants, like wild plants, are expected to breed with closely related species to produce hybrids, a phenomenon called out-crossing. Similarly, the possibility of

transgenic algae cultured near natural surface waters raises questions as raised above about transgenic plants and their potential to become invasive [35]. In worst-case scenarios, escaped transgenic algae might persist and produce toxins or might become so abundant that they create harmful algal blooms. If it is possible for free-living GE algae to become more invasive, more toxic, or more tolerant of extreme abiotic conditions than their wild counterparts are, this would be cause for concern [35]. So far, too little attention has been paid to these ecological questions. Research to support environmental risk assessment of novel transgenic plants and algae should be prioritized.

7 The Next-Generation Metabolic Engineering

One possible approach for improving metabolic engineering lies in the ability to predict variant effects on the product function and stability of the products of transgenic plants [38]. With the emergence of next-generation sequencing technologies, genetic variation in transgenic plants can now be determined on an unprecedented scale and resolution by resequencing thousands of plants systematically. To complement this, constraint-based reconstruction and analysis (COBRA) methods have gained popularity, tools for next-generation metabolic engineering [39]. The technique uses a genome-scale *in silico* representation of the metabolic network of a host organism to predict optimal genetic modifications that improve the rate and yield of secondary metabolite synthesis and production. A new generation of COBRA models and methods is under review to encompass a host of biological processes and simulation strategies to enable new types of predictions [39]. This will result in more efficient and precise production of desired secondary metabolites from transgenic plants. COBRA methods can also be used to predict some biosafety issues associated with transgenic plants. However, these methods are highly dependent on technology and database tools to predict variant metabolites.

8 Conclusions

Advanced genomics and biotechnology has resulted in the production of transgenic plants with the ability to synthesize desired plant secondary metabolites for the pharmaceutical and nutraceutical industry, in a phenomenon called pharming. A classic example is the production of golden rice and OFSP, with the ability to produce 23-fold β-carotene, a provitamin A molecule. Similarly, enhanced levels of alkaloids with anticancer activities in *C. roseus*, vinblastine and vincristine, have been achieved. Various natural antioxidants in the form of phenolic compounds including flavonoids and proantocyanidins have not been spared. Several techniques including hairy root cultures have been employed to enhance production of these. However, the unknown and unexpected results in metabolic pathway engineering still need to be researched and understood. The behavior of these transgenes in the environment still leaves a lot to be desired. While a decade ago production of

transgenic plants was expensive, time consuming, and had limitations on the host plants, next generation technologies are bringing efficient and precise methods enabling researchers to quickly edit genomes in a precise and accurate fashion at the single base-pair resolution level at multiple loci simultaneously. Perhaps it is what now lies in the future of multi-omics which could bring a concerted action between modelers, geneticists, microbiologists, and bioinformaticians to allow the full achievement of the predicted changes and novel metabolic capabilities.

Acknowledgments The work was partially supported by the National Research Foundation, Pretoria (Grants Nos. 93185 and 95662).

References

1. Teli NP, Timko MP (2004) Recent developments in the use of transgenic plants for the production of human therapeutics and biopharmaceuticals. Plant Cell Tiss Org Cult 79:125–145
2. Wilson SA, Roberts SC (2014) Metabolic engineering approaches for production of biochemicals in food and medicinal plants. Curr Opin Biotechnol 26:174–182
3. Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333
4. Lv Q, Cheng R, Shi T (2014) Regulatory network rewiring for secondary metabolism in *Arabidopsis* thaliana under various conditions. BMC Plant Biol 14:180
5. Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326
6. Gelvin SB (2010) Manipulating plant histone genes to improve *Agrobacterium*- and direct DNA-mediated plant genetic transformation. ISB News report 1–4
7. Zárate R, Verpoorte R (2007) Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 6:475–491
8. De Luca V, Laflamme P (2001) The expanding universe of alkaloid biosynthesis. Curr Opin Plant Biol 4:225–233
9. Pasquali G, Erven AS, Ouwerkerk PB, Menke FL, Memelink J (1999) The promoter of the strictosidine synthase gene from periwinkle confers elicitor-inducible expression in transgenic tobacco and binds nuclear factors GT-1 and GBF. Plant Mol Biol 39:1299–1310
10. St-Pierre B, Vázquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11:887–900
11. De Luca V, St-Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5:168–173
12. Qu Y, Easson MALE, Froese J, Simionescu R, Hudlicky T, De Luca V (2015) Completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline and its assembly in yeast. Proc Natl Acad Sci U S A 112:6224–6229
13. Sanghera GS, Malhotra PK, Sidhu GS, Sharma VK, Sharma BB, Karan R (2013) Genetic engineering of crop plants for enhanced antioxidants activity. Int J Adv Res Technol 2:428–458
14. WHO (2009) Global prevalence of vitamin A deficiency in populations at risk 1995–2005. WHO global database on vitamin a deficiency. World Health Organization, Geneva, Retrieved 12 Mar 2016
15. Humphrey JH, West KP Jr, Sommer A (1992) Vitamin a deficiency and attributable mortality in under-5-year-olds. Bull WHO 70:225–232
16. Park SC, Kim YH, Kim SH, Jeong YJ, Kim CY, Lee JS, Bae JY, Ahn MJ, Jeong JC, Lee HS, Kwak SS (2015) Overexpression of the IbMYB1 gene in an orange-fleshed sweet potato

cultivar produces a dual-pigmented transgenic sweet potato with improved antioxidant activity. Plant Physiol 153:525–537
17. Locato V, Cimini S, De Gara L (2013) Strategies to increase vitamin C in plants: from plant defense perspective to food biofortification. Front Plant Sci 4:1–12
18. Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Biochem Mol Biol 35:291–314
19. Hancock RD, Viola R (2002) Biotechnological approaches for L-ascorbic acid production. Trends Biotechnol 20:299–305
20. Vissers MC, Bozonet SM, Pearson JF, Braithwaite LJ (2011) Dietary ascorbate intake affects steady state tissue concentrations in vitamin C-deficient mice: tissue deficiency after suboptimal intake and superior bioavailability from a food source (kiwifruit). Am J Clin Nutr 93:292–301
21. Cahoon EB, Hall SE, Ripp KG, Ganzke KE, Hitz WD, Coughlan SJ (2003) Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. Nat Biotechnol 21:1082–1087
22. Matringe M, Ksas B, Rey R, Havaux M (2008) Tocotrienols, the unsaturated forms of vitamin E, can function as antioxidants and lipid protectors in tobacco leaves. Plant Physiol 147:764–778
23. Rippert P, Scimemi C, Dubald M, Matringe M (2004) Engineering plant shikimate pathway for production of tocotrienol and improving herbicide resistance. Plant Physiol 134:92–100
24. Kaushik P, Andújar I, Vilanova S, Plazas M, Gramazio P, Herraiz FJ, Brar NS, Prohens J (2015) Breeding vegetables with increased content in bioactive phenolic acids. Molecules 20:18464–18481
25. Verhoeyen ME, Bovy A, Collins G, Muir S, Robinson S, de Vos CHR, Colliver S (2002) Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. J Exp Bot 53:2099–2106
26. Tan RH, Zhang JJ, Zhao SJ (2014) Optimization of induction and culture conditions for hairy roots of Salvia miltiorrhiza. China J Chin Mater Med 39:3048–3053
27. Abbott JA, Medina-Bolivar F, Martin EM, Engelberth AS, Villagarcia H, Clausen EC, Carrier DJ (2010) Purification of resveratrol, arachidin-1, and arachidin-3 from hairy root cultures of peanut (*Arachis hypogaea*) and determination of their antioxidant activity and cytotoxicity. Biotechnol Prog 26:1344–1351
28. Homova V, Weber J, Schulze J, Alipieva K, Bley T, Georgiev M (2010) Devil's claw hairy root culture in flasks and in a 3-L bioreactor: bioactive metabolite accumulation and flow cytometry. Z Naturforsch C 65:472–478
29. Liu CZ, Abbasi BH, Gao M, Murch SJ, Saxena PK (2006) Caffeic acid derivatives production by hairy root cultures of *Echinacea purpurea*. J Agric Food Chem 54:8456–8460
30. Liu C, Wang Y, Guo C, Ouyang F, Ye H, Li G (1998) Enhanced production of artemisinin by *Artemisia annua* L hairy root cultures in a modified inner-loop airlift bioreactor. Bioprocess Eng 19:389–392
31. Cardozo KHM, Guaratini T, Barros MP, Falcão VR, Tonon AP, Lopes NP, Campos S, Torres MA, Souza AO, Colepicolo P, Pinto C (2007) Metabolites from algae with economical impact. Comp Biochem Physiol C 146:60–78
32. Barros MP, Pinto E, Sigaud-Kutner TCS, Cardozo KHM, Colepicolo P (2005) Rhythmicity and oxidative/nitrosative stress in algae. Biol Rhythm Res 36:67–82
33. Kannan RRR, Aderogba MA, Ndhlala AR, Stirk WA, Van Staden J (2013) Acetylcholinesterase inhibitory activity of phlorotannins isolated from the brown alga, *Ecklonia maxima* (Osbeck) Papenfuss. Food Res Int 54:1250–1254
34. Hallmann A (2007) Algal transgenics and biotechnology. Transgenic Plant J 1:81–98
35. Snow AA, Smith VH (2012) Genetically engineered algae for biofuels: a key role for ecologists. BioScience 62:765–768
36. Nielsen J, Olsson L (2002) An expanded role for microbial physiology in metabolic engineering and functional genomics: moving towards systems biology. FEMS Yeast Res 2:175–181

37. Sweetlove LJ, Last RL, Fernie AR (2003) Predictive metabolic engineering: a goal for systems biology. Plant Physiol 132:420–425
38. Cardoso JGR, Andersen MR, Herrgård MJ, Sonnenschein N (2015) Analysis of genetic variation and potential applications in genome-scale metabolic modeling. Front Bioeng Biotechnol 3:1–12
39. King ZA, Lloyd CJ, Feist AM, Palsson BO (2015) Next-generation genome-scale models for metabolic engineering. Curr Opin Biotechnol 35:23–29